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Preparation of Combined Equine Influenza and Tetanus Toxoid Vaccine from local stains

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SUMMARY

Inactivated equine influenza from local isolate and tetanus toxoid (EITT) vaccine adjuvanted with saponin was prepared. It was inoculated into Guinea pigs as a preliminary evaluation for its potency, which exceeds the permissible protective level allowed to be used for vaccination of horses. The vaccine proved to be safe and potent for horses and Guinea pigs. The mean haemagglutination inhibition (HI) antibody titres (3 weeks post vaccination) were 704 and 200 and the mean toxin neutralizing (TN) antibodies (2 weeks after boostering) were 37.9 and 45 IU/ml respectively. The antibody titre in vaccinated horses was monitored up to 6 months post re-vaccination. The keeping quality of the prepared vaccine was studied for one year. It was found to be stable when stored at 4°C.

INTRODUCTION

Influenza and tetanus are important infectious diseases of horses that commonly targeted in equine immunization programmes (Wilson *et al.*, 1995).

Equine influenza (EI) is one of the most serious viral respiratory disease affecting horses world-wide. It is an acute highly contagious febrile respiratory disease which spread rapidly among equine population, involving all ages and both sexes (OIE, 2008). It is caused by virus belong to Family Orthomyxoviridae, Type A, which comprises two antigenically distinct subtypes represented by reference strains A/equi-1/praque/56 (H7N7) and A/equi-2/Miami/63 (H3N8) (Sovinova *et al.*, 1958, Waddell *et al.*, 1963 and WHO, 1979).

The disease is characterized by high morbidity rate which may reach 90% with low mortality except for young foals where sever viral pneumonia developed leading to death within 48 hours (Bryans, 1964 and Miller, 1965).

In Egypt, the disease was recorded for the first time in October (1989) where subtype 1 and 2 were isolated (Ismail *et al.*, 1990, Esmat *et al.*, 1992), the second outbreak was

recorded in Winter (1999-2000) where subtype 2 was isolated (Hamoda et al., 2001, Nashwa, 2004). The third outbreak was recorded in June (2008) where subtype 2 was isolated (Soliman *et al.*, 2008).

A/equi-2 virus was firstly isolated in Miami (1963) and continued to cause outbreaks and important economic losses to horse industry. Prevention and control of equine influenza largely depends on vaccination and the application of management regimes. The influenza H3N8 virus is highly prone to small changes (antigenic drift), so it is necessary to periodically update the vaccinal strains to reflect the latest epidemiological situation (Yates and Mumford, 2000, Minke *et al.*, 2004).

Tetanus is a highly fatal, infectious disease of all species of domestic animals caused by the toxin of *Clostridium tetani*. The neurotoxin of *C. tetani* is exceedingly potent, but there is considerable variation in susceptibility between the animal species, the horse being the most susceptible (Jansen and Knoeta, 1979).

Tetanus can never be eradicated because the spores of *C. tetani* are widely spread in the environment and found in the intestinal flora of domestic animals, horses, chickens and humans (Farrar and Newton, 2000).

C. tetani form resilient spores capable of surviving house hold disinfectants and boiling water for several minutes. In conditions of low oxygen tension (for example, deep and penetrating wounds). The spores germinate and the resultant bacteria multiply and produce a neurotoxin responsible for the clinical features of tetanus. The mortality rate may be as high as 90%.

The disease may be controlled by improved hygiene and vaccination. Active immunization with tetanus toxoid provides long term humoral and cellular immunity add to the short term immunity provided by antitetanus immunoglobulin (Goonetilleke and Harris, 2004).

So, the present study was planned for preparation of more effective and long lasting equine influenza and tetanus toxoid combined vaccine (EITT).

MATERIALS AND METHODS

Equine influenza (EI):

Virus: The locally identified freeze dried influenza virus A/equi-2/Alex-1/08 egg passage three (EP3) with infectivity titre 10 $\log_{10}$ EID50/0.1ml and HA titre 11 $\log_2$ was used for vaccine preparation (Soliman *et al.*, 2008).

Antisera: Reference antisera against A/equi-1/paragues/56 (H7N7) and A/equi-2/Miami/63 (H3N8) were obtained from National Veterinary Laboratories, United States Department of Agriculture, Veterinary Services (NVSL, USDA, VS).

Identity test: The selected vaccine seed virus EI (A/equi-2/Alex-1/08) EP3 was confirmed by HI test using reference antisera against EI virus subtype 1 & 2 and PCR (OIE, 2008).Molecular method using the matrix gene specific real time PCR. This was followed by amplification of the HA, NA genes and sequencing (Magda, et al,2011).

Preparation of vaccinal viral fluid: EI master seed virus was propagated for one or two successive passages (Ep4, Ep5) in specific pathogen free embryonated chicken egg (SPF-ECE) 9-11 days old (OIE, 2008), working seed virus was propagated to another passage in SPF-ECE. The virus fluid was harvested, tested for sterility, haemagglutinating activity expressed in log_2 (HA unit/0.05 ml) by using HA test (OIE, 2008) and infectivity titre expressed in log10 EID50/0.1 ml which calculated by the method of Reed and Muench (1938).

Virus inactivation: Vaccine virus fluid of EI-Ep6 with HA titre 11 log_2 and infectivity titre 10 log_{10} EID50/0.1 ml was inactivated with binary ethyleneimine (BEI) at a final concentration 0.003 M with continuous stirring at 37°C for 24 hours followed by immediate addition of sterile sodium thiosulphate at a final concentration 2% to stop the action of BEI on the virus and neutralize the toxic action of residual inactivator on target host (Eman, 2005). Inacticvated virus fluid was examined for residual infective virus activity in ECE and tested for sterility and safety (OIE, 2008).

Tetanus Toxoid:

Strain: Lyophilized *C. tetani* (Harvard strain, 49805) supplied from the Egyptian organization for Biological Products and Vaccines, Tatanus Department, Cairo, Egypt).

Preparation of tetanus toxoid:

Seed preparation: Lyophilized strain of *C. tetani* was reconstituted in seed medium according to Demain *et al.* (2005), and cultured for 3 successive subcultures. The last subculture was inoculated in fermentation medium for production of tetanus toxin as described by (El-Helw, 2007) and incubated at 35°C for 6 days.

The prepared toxin was assayed to determine its potency by flocculation test according to WHO (1978) and minimal lethal dose according to Lpsen (1941). Detoxification was carried out by adding 0.5 ml of 40% formaldehyde/100ml of the toxic culture fluid at pH 7 and continuing the incubation at 37°C until 1ml of the culture injected subcutaneously failed to kill a Guinea pig about 400 gm.

Addition of adjuvant: Inactivated fluids of EI and tetanus toxoid were mixed with saponin as an adjuvant, then dispensed in vials, each of them contain 1ml that representing one horse dose. Each 1 ml of inactivated EITT vaccine contain 40 Lf (limit of flocculation) of tetanus toxoid, 10 log_2 HA units and 1 mg saponin.

Safety test of the prepared vaccine: It was performed according to OIE (2008) and British Veterinary Pharmacopoeia (2009).

Potency test of the EITT vaccine in Guinea pigs: Fifteen seronegative Guinea pigs were divided into 3 groups (5 Guinea pigs/group). Group (A & B) were inoculated subcutaneously (S/C) with the horse dose (1ml) of the prepared vaccine. Group (C) was kept as a control at the same conditions of the experiments. Twenty one days post inoculation, sera samples were collected from groups A and C and tested for HI antibody titre using HI test (OIE, 2008).

After 28-30 days post inoculation, group B received the second dose of the vaccine then 14 days later, sera samples were collected from group B and C and tested for toxin neutralizing antibodies using (TN) test (British Veterinary Pharmacopeia, 2009).

Immunogenicity of the EITT vaccination in horses:

Six seronegative horses (two to four years old) were divided into 2 groups:

Group (A):

Four horses were inoculated with two doses of the prepared vaccine one month apart. Each dose (1ml) was injected (I/M) in the lower third of the neck and revaccinated at 6 months with the same dose and route (Wilson, 1999).

Group (B):

Two horses, was kept at the same conditions as a control.

Serum samples of horses in groups A&B were collected at different intervals weekly in the 1st month, every two weeks up to the 4th month, then monthly till 6 months post revaccination and screened for the immune response using HI and TN tests.

Stability of the prepared EITT vaccine: Random vials of the vaccine were divided into two groups. The first group was kept at 4°C for 12 months. The second group was kept at room temperature (25-28°C) for the same period. Samples from each group of the vaccine were taken separately at different times and tested for its potency in Guinea pigs (15 Guinea pigs for each time) and tested for HI and toxin neutralizing antibodies.

RESULTS AND DISCUSSION

According to the locally available static, the number of equine population in Arab Republic of Egypt in 1990 reach approximately one million and two hundred forty thousand of which are 6200 horses, 5500 mules, 123000 donkeys. From the economical point of view, the field of selecting rearing and trading pure Arabian horses constitute remarkable additional support to the national incomes. For this reason, it becomes a matter of importance to protect solipeds in Egypt against epidemic and infectious diseases, either viral or bacterial diseases specially those causing great economic losses.

Vaccination is the most effective means of control EI disease. Also the protection against tetanus either by tetanus antitoxin or by vaccination with tetanus toxoid is recommended and the later is highly effective and the only practical means of long term protection (Goonotilleke and Harris, 2004).

In this study, the results of titration of EI virus (A/equi-2/Alex-1/08) in SPF ECE 9-11 days old was 10 $\log_{10}$ EID_{50}/0.1 ml for infectivity and haemagglutinating titre was 11 $\log_2$ HA units / 0.05ml at the 6th passage in ECE. These results were in agreement with Kucera and Bechenhaur (1977) who prepared EI (A/equi-2) inactivated vaccine from the field strain at 10th passage in ECE with 9 $\log_2$ to 10 $\log_2$ haemaggluinating units.

The virus was inactivated with binary ethyleneimine (BEI) at a final concentration 0.003 M at 37°C for 24 hours (Eman, 2005) HA titre of the virus after inactivation was (11 $\log_2$ HA unit).

The modified medium which described by El-Helw (2007) gave high significant production of tetanus toxin. It was evaluated by minimal lethal dose (MLD) which was found to be 600,000 (MLD) and by flocculation showed 120 Lf. The toxin was completely detoxified after 21 days of incubation at 37°C.

From the obtained data in Tables (1 and 2), Guinea pigs serum samples of groups A and B which inoculated with EITT vaccine showed that the mean HI antibodies titre at 3 weeks post inoculation was 704 in group (A). The result was in agreement with OIE (2008) who reported that the protective HI antibody titre should not less than 64. The mean TN antibodie was 45 IU/ml after 2 weeks from boostering dose in group (B). This was in agreement with British Veterinary Codex (1970) and British Veterinary Pharmacopeia (2009) as they reported that toxin neutralizing antibody titre should not less than 30 IU for evaluating the potency of tetanus vaccine for equines.

Serum samples obtained from Guinea pigs inoculated with either Tetanus toxoid or inactivated EI vaccine in combination with saponin showed nearly the same results obtained from those inoculated with EITT combined vaccine.

Tables (3 & 4) and Fig. (1 & 2) clarify that the first dose of EITT vaccine was able to stimulate HI and TN antibodies in horses at 2 weeks post vaccination. By boostering at 4th week post inoculation much higher level of HI and TN antibodies were obtained. Antibodies reached their maximum titre at 3 and 3.5 months respectively, then the titre begin to decline gradually till 6 months post vaccination.

By revaccination at 6 months, the HI and TN antibodies titre increased till its maximum level at 9 months (1040 and 47) respectively, then decline with considerable protective titre till 12 months post vaccination. These obtained results come

parallel with those mentioned by Heldens *et al.* (2010) who reported that inactivated equine influenza and tetanus combination vaccine adjuvanted with ISCOM (Immuno stimulating complex) induce high antibody titre at two weeks after the second vaccination (4 weeks apart from the first vaccination), 3rd vaccination (6 months later) was recommended, it induced protection until time of annual revaccination.

Concerning with the effect of storage on the prepared vaccine (Table 5) revealed that the vaccine was more stable at 4°C for 1 year.

From the aforementioned results it is clear that the newly prepared vaccine which contains the locally isolated strain of EI and tetanus toxoid with the presence of a suitable adjuvant (saponin) induce protective antibody levels with prolonged duration of immunity.

So, the control of equine influenza and tetanus could be achieved by vaccination of unvaccinated horses 3 times with that prepared inactivated (EITT) vaccine. The first two dose 4 weeks apart and the 3rd (revaccination) 6 months later. Further study must be done on the vaccination program of pregnant mares and previously vaccinated horses.

Table (1): HI antibody titre in sera of Guinea pigs inoculated with EITT vaccine

Guinea pigs group A	HI antibody titre	
	Pre-inoculation	21 days post inoculation
1	-	320
2	-	640
3	-	640
4	-	640
5	-	320
Mean	-	704

Control group gave negative results

Table (2): Tetanus antitoxin titre in sera of Guinea pigs inoculated with EITT vaccine tested by TN test

Guinea pigs group B	Antitoxin Titre (IU/ml)	
	Pre-inoculation	14 days after 2nd dose
1	-	40
2	-	50
3	-	40
4	-	45
5	-	50
Mean	-	45

Control group give negative result

Table (3): Immune response of horses inoculated with EITT vaccine tested by HI test

Time of sampling	HI titre in sera of horses						
	Group (A)					Group (B)	
	H1	H2	H3	H4	Mean	H5	H6
Prevacc.	-	-	-	-	-	-	-
2 W	80	80	80	40	70	-	-
3 W	320	160	160	160	200	-	-
(b) 4 W	160	160	80	80	120	-	-
6 W	320	160	320	160	240	-	-
8 W	640	640	320	320	480	-	-
10 W	640	640	640	1280	800	-	-
12 W	1280	640	640	1280	960	-	-
3.5 M	1280	1280	320	640	880	-	-
4 M	640	640	160	320	440	-	-
5 M	160	160	160	160	160	-	-
(R) 6 M	80	80	160	160	120	-	-
7 M	160	160	320	160	200	-	-
8 M	320	320	640	320	400	-	-
9 M	1280	1280	1280	640	1040	-	-
10 M	1280	1280	640	640	880	-	-
11 M	640	640	320	320	480	-	-
12 M	160	320	80	40	150	-	-

Group (A): Horses inoculated with EITT vaccine
Group (B): Control group
(-): Negative result (H): Horse
(b): Boostering (R): Revaccination

Table (4): Antitoxin titre in serum of horses inoculated with EITT vaccine tested by toxin neutralization test

Time of sampling	Antitoxin titre in sera of horses (IU/ml)						
	Group (A)					Group (B)	
	H1	H2	H3	H4	Mean	H5	H6
Prevacc.	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2 W	12*	11	8.6	11	10.7	< 0.01	< 0.01
3 W	19.3	24	16.5	16	19	< 0.01	< 0.01
(b) 4 W	16	17	11	12	14	< 0.01	< 0.01
6 W	35	30	30	33	32	< 0.01	< 0.01
8 W	36	35	40	40.5	37.9	< 0.01	< 0.01
10 W	40.5	42.5	42	42	41.8	< 0.01	< 0.01
12 W	48	40	43.5	43	43.6	< 0.01	< 0.01
3.5 M	45.1	46	50	42.5	46	< 0.01	< 0.01
4 M	37.5	40	31.5	37	36.5	< 0.01	< 0.01
5 M	32.5	29.3	36	32	32.5	< 0.01	< 0.01
(R) 6 M	30	27.3	32.3	30	30	< 0.01	< 0.01
7 M	40	35.5	42	35	38	< 0.01	< 0.01
8 M	48	42	43.5	45	44.6	< 0.01	< 0.01
9 M	50	48	45	45	47	< 0.01	< 0.01
10 M	45	42.5	40	42	42.4	< 0.01	< 0.01
11 M	40.5	38.3	35	36	37.5	< 0.01	< 0.01
12 M	37.5	28.5	28	28.5	30.6	< 0.01	< 0.01

Group (A): Horses inoculated with EITT
Group (B): Control group H: Horse (w) : week
(b): Boostering (R): Revaccination (M) : month
• International antitoxin unit / ml of serum

FIG.(1): Immune response of horses inoculated with EITT vaccine tested by HI test

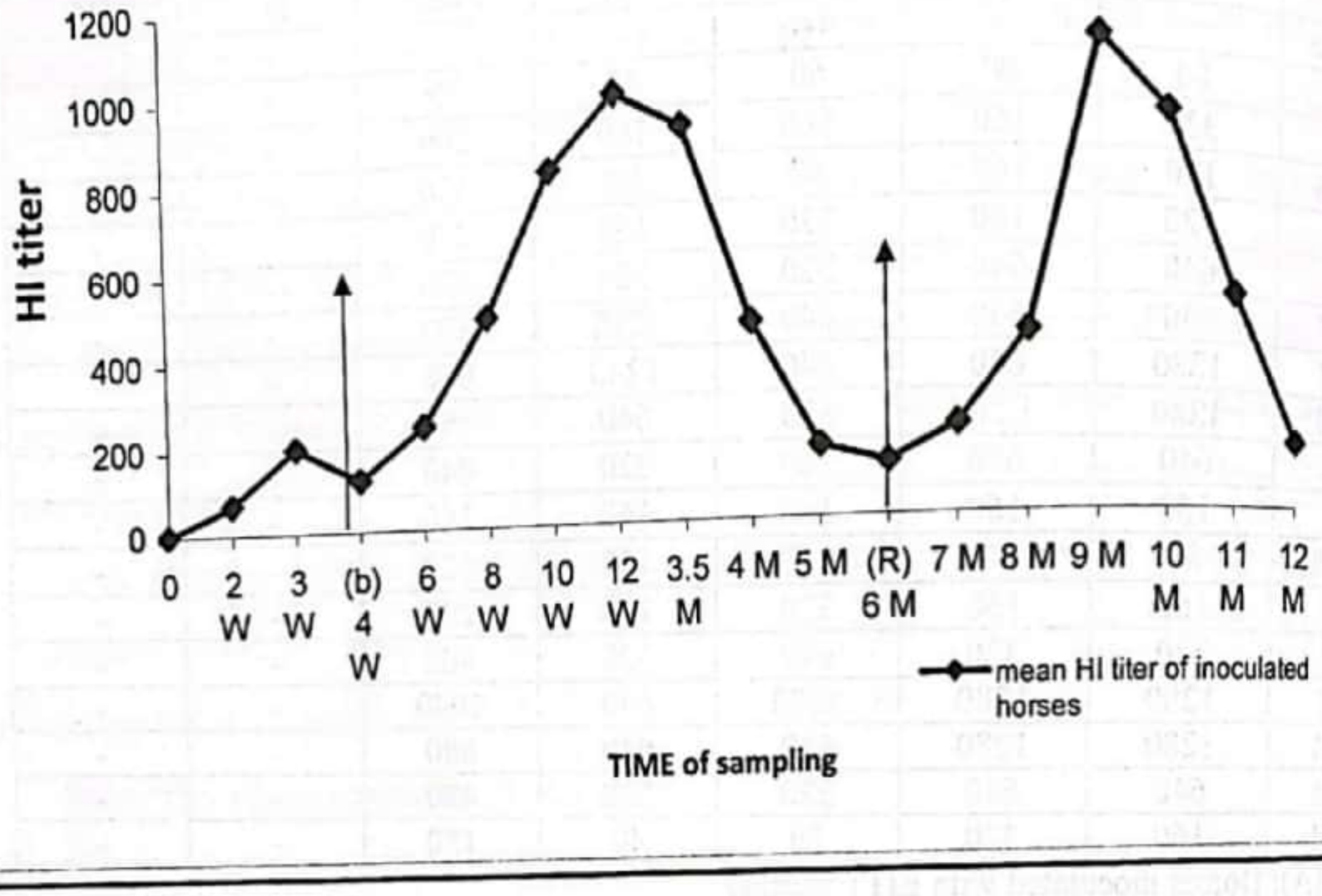
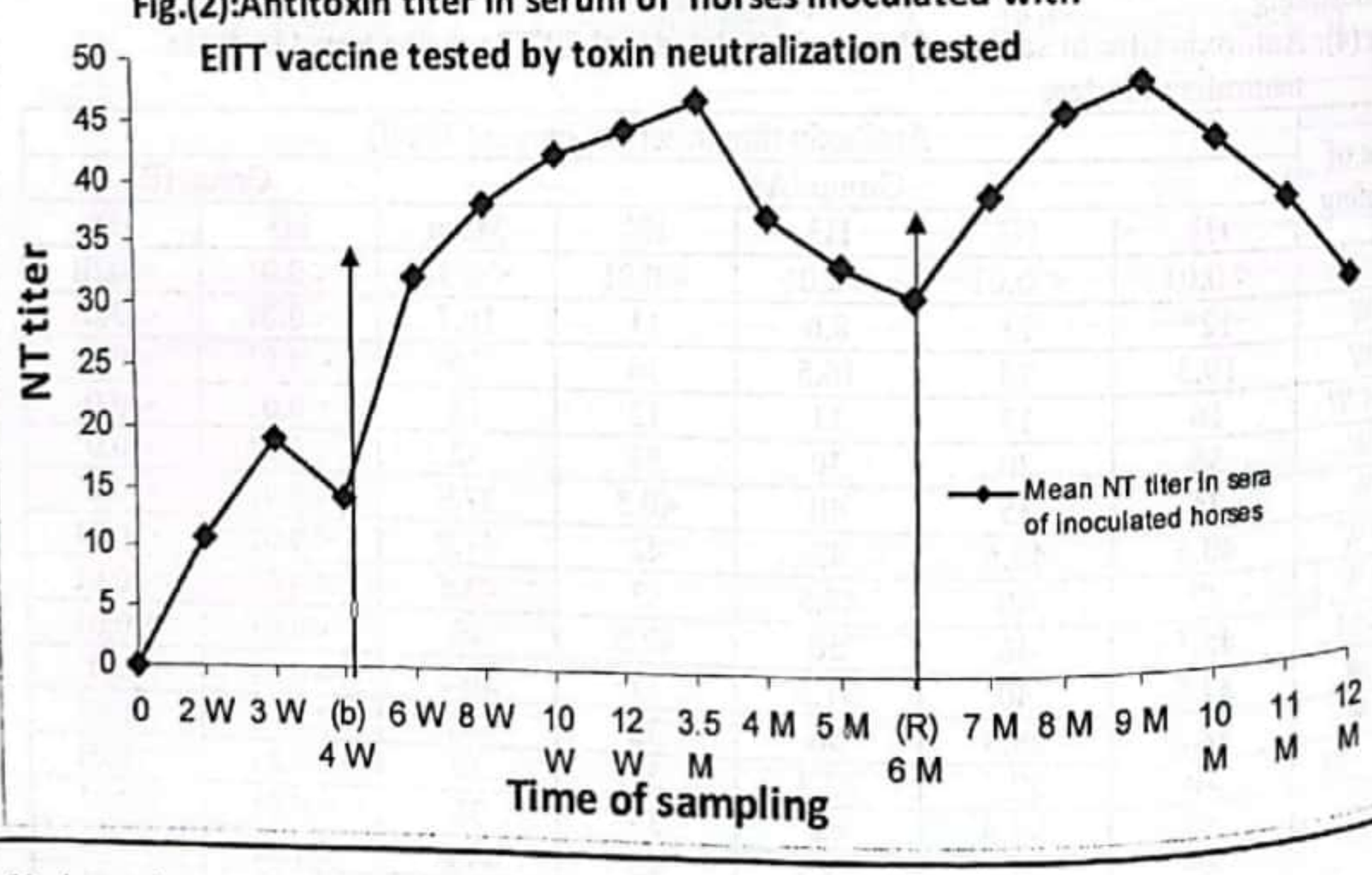


Fig.(2):Antitoxin titer in serum of horses inoculated with EITT vaccine tested by toxin neutralization tested



(b) : boostering. (R): Revaccination.

Table (5): Stability of the prepared EITT vaccine tested in groups of Guinea pigs

Temperature of storage (°C)	Time of storage	* The mean HI antibody titre in sera of group (A)	** The mean TN antibody titre in sera of group (B)	*** Group (C)
Vials of group (1) stored at 4°C	0	704	45	-
	3 Months	704	45	-
	6 Months	704	45	-
	9 Months	640	43	-
	12 Months	480	40	-
Vials of group (2) stored at Room Temperature (25°C-28°C)	0	704	45	-
	3 Months	480	36.5	-
	6 Months	93	30	-
	9 Months	-	-	-
	12 Months	-	-	-

* Sera samples collected from group (A) at 21 days post inoculation.

** Sera samples collected from group (B) at 14 days after the 2nd dose.

*** Group (C): Control group

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تحضير لقاح مركب من أنفلونزا الخيول وتوكسيد التيتانوس

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معهد بحوث الأمصال واللقاحات البيطرية – العباسية – القاهرة

الهدف من هذا العمل هو تحضير لقاح مركب من انفلونزا الخيول المحضر من عترة محلية وتوكسيد التيتانوس مع إضافة مادة الصابونين كمحفز. وقد ثبتت كفاءة اللقاح فى كل من الخيول وارانب غينيا وذلك بقدرته على إنتاج مستوى عالى من الاجسام المناعية حيث كان متوسط الاجسام المناعية باستخدام اختبار التلازن الدموى المثبط عند 3 أسابيع بعد التحصين 200، 704 وكان متوسط الاجسام المناعية للتيتانوس باستخدام اختبار السيرم التعادلى 379، 45 وحدة دولية لكل ملليمتر من الجرعة التعزيزية على التوالى وقد أستمر معدل الاجسام المناعية مرتفع فى سيرم الخيول المحصنة حتى 6 شهور من تكرار التحصين. تم تقييم كفاءة اللقاح عند حفظه فى درجات حرارة مختلفة لمدة عام. ولقد وجد أن اللقاح احتفظ بكفاءته المناعية على درجة 4^{0}م.