Trials for Preparation of Monovalent Inactivated Equine Influenza Vaccine

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Abstract

Equine influenza virus (A/equi-2/Cairo-2/2000, H3N8) with egg passage 6 (Ep6) was inactivated by 0.1 % formalin (40 % formaldehyde) and complete inactivation was achieved after 24 hours at 37°C. Two forms of monovalent equine influenza vaccine were prepared using different adjuvants, the first with DEAE-Dextran, while the other with Alhydragel and saponin. Both vaccine forms (freeze dried and liquid one) proved to be safe and potent for horses and G. Pigs. The mean HI antibody titres in G. Pigs (three weeks post inoculation) were 1408, 1344, while in horses (six weeks post inoculation) were 640, 480 respectively for both vaccines.The antibody titre in horses vaccinated with both vaccine forms were monitored and persisted at protective level for one year.The keeping quality of the locally prepared EI monovalent inactivated vaccine either freeze-dried, reconstituted in DEAE-Dextran or the liquid one were studied and the shelf validity for both vaccines were found to be stable at 4°C for one year, while the freeze dried one can be kept at -20°C for three years.

Introduction

Equine influenza (EI) is one of the most serious viral respiratory diseases affecting horses with world-wide distribution. It is an infectious, highly contagious acute febrile respiratory disease and spreads rapidly among equine population (**12**). The disease is characterized by high morbidity rate which may reach 90% (**8**). Mortality rates are usually low except for young foals where severe viral pneumonia developed leading to death within 48 hours (**3 and 16**).

EI is caused by virus belongs to the family Orthomyxoviridae, type A, which classified into two antigenically distinct subtypes represented by reference strains A/equi-1/Prague/56 (H7N7) and A/equi-2/Miami/63 (H3N8) (**23, 24 and 25**).

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In Egypt, the first outbreak of EI disease was recorded in October (1989) where EI virus subtypes 1 and 2 were isolated (**12 and 6**). The second outbreak was recorded in winter (1999-2000) where subtype 2 was isolated (**9 and 17**). The third outbreak was recorded in June (2008) where EI virus subtype 2 was isolated (**Soliman et al., 2008**).

Vaccination is the most effective means of controlling EI disease. It must contain at least one representative of each subtype (H7N7 and H3N8) specially the current circulating strain, to obtain a potent vaccine.

So, the present study was planned as a trial for preparation of monovalent EI inactivated vaccine using formalin as an inactivating agent and different adjuvants (DEAE-Dextran and low viscosity aluminium hydroxide gel with saponin).

Material and Methods

Materials:

Virus:

Locally identified isolates of EI virus (A/equi-2/Cairo-2/2000) EP6 with HA titre 2048 and infectivity titre 9.5 $\log_{10}$ EID_{50}/0.1ml was used for vaccine preparation (**17**).

Antisera:

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

Animals:

Four groups of apparently healthy susceptible horses two to four years old (2/group) and groups of Guinea pigs 250-300 gm / weight (5/group) were used to determine the potency, safety and the stability of the prepared vaccines.

Embryonated chicken eggs (ECE):

Groups of specific pathogen free (SPF) 9-11 day old ECE were used for virus propagation, egg infectivity titration and to detect the residual infective virus in the inactivated viral fluid.

Formalin:

40 % formaldehyde was used as a virus inactivator.

DEAE-Dextran solution:

Solution of diethylaminoethyl (DEAE) Dextran, chloride form was obtained from MP Biomedicals LLC and used as diluents of the freeze-dried vaccine (2).

Alhydragel Low Viscosity:

Aluminium hydroxide gel low viscosity. It was used as an adjuvant and stored at 4°C (1 and 7).

Saponin:

It was obtained from Ubichem Plc. and used with alhydragel.

Methods:

Identity test:

The identity of EI virus fluid (A/equi-2/Cairo-2/2000) EP6, was confirmed by HI test with reference antisera against influenza virus subtype-1 and subtype-2.

Virus inactivation:

Vaccine virus fluid of EI subtype-2 (EP6) with HA titre 2048 and infectivity titre 9.5 log10 EID50/0.1 ml was incubated with two different concentrations of formalin (0.05 and 0.1 %) at 37°C for 36 hours. Samples were collected at different intervals and assayed for virus infectivity and haemagglutinating activity (HA).

Residual infective virus activity in ECE:

It was performed according to the method described by (19).

Sterility Test:

Samples from the inactivated virus fluid were cultured on different media to insure its freedom from bacterial or fungal contamination.

Preparation of freeze dried vaccine:

It was performed according to the method described by (10) and (18). Each vial contains 3 ml of vaccine virus fluid.

Addition of Adjuvant:

Each vial of the inactivated freeze-dried vaccine was reconstituted in 3 ml DEAE-Dextran solution (representing one horse dose). The dose must have a HA titre not less than 2^8 expressed in $\log_2$.

Preparation of liquid vaccine:

The inactivated virus fluid of (EI) was mixed with alhydragel and saponin. It was given as 5 ml/horse.

Safety test of the locally prepared EI inactivated vaccine in horses: According to **(19).**

Immunogenic potency of the locally prepared EI inactivated adjuvant vaccines in Guinea pigs: According to **(19)**

Fifteen seronegative Guinea pigs were divided into 3 groups (5 Guinea pigs/group). Group (A) was inoculated subcutaneously (S/C) with the horse dose (5ml) of the prepared liquid vaccine. Group (B) was inoculated S/C with the horse dose (3 ml) of the reconstituted freeze dried vaccine and Group (C) was kept as a control at the same conditions of the experiment. Twenty one days post inoculation, serum samples were collected from all groups.

Stability of the prepared EI vaccines (liquid and freeze dried vaccines:

* Random bottles of the liquid vaccine were divided into two groups:
- First group was kept at 4°C for 24 months.
- Second group was kept at room temperature (25-28°C) for one year.
* Random vials of the freeze dried vaccine were divided into 3 groups:
- First group was kept at 4°C for 24 months.
- Second group was kept at room temperature (25-28°C) for one year.
- Third group was kept at -20°C for 24 months.

Samples from each group from each vaccine was taken separately at different times and tested for potency in Guinea pigs.

Immune response of horses inoculated with inactivated freeze dried and liquid vaccines of EI virus:

Six seronegative horses to EI antibodies were divided into 3 groups (2horses/group). The first group was inoculated I/M with the prepared liquid vaccine (5ml/dose/horse), the second group was inoculated with the reconstituted freeze dried vaccine (one vial/3ml/dose/horse).

The third group was kept in the same conditions as a control.

Blood serum samples were collected at different intervals and screened for the immune response using HI test.

Results and Discussion

EI disease is characterized by high morbidity, high severity of clinical signs, moreover, secondary bacterial complications may affect the course of the disease especially unvaccinated, stressed and neglected animals or those under poor environmental conditions. In recent years, a number of EI vaccines specially inactivated one became available for use in horses (**7 and 26**).

The results of titration of EI virus (A/equi-2) in ECE 9-11 days old was represented in Table (1) where the infectivity titre was 9.5 $\log_{10}$ EID_{50}/0.1 ml and haemagglutinating titre was 2048 HA units at the 6^{th} passage in ECE. This result agreed with **(4) and (15)** who prepared EI inactivated vaccine from the field strain at 10^{th} passage in ECE with 512 to 1024 haemagglutinating units of A/equi-2.

Concerning with the inactivation of EI virus (A/equi-2) using different concentration of formalin 0.05 % and 0.1 % as shown in Table (2) and Fig. (1). The virus was not completely inactivated at concentration 0.05% formalin up to 36 hours while it was completely inactivated at concentration 0.1 % within 24 hours incubation at 37°C (**19**).

The HA titre of virus after inactivation was (512 HA units).

From the data obtained in Tables (3 and 5) and Fig. (2), G. pig and horse serum samples of group (A) which inoculated with alhydragel liquid EI vaccine and group (B) which inoculated with EI DEAE-Dextran adjuvant vaccine, showed that the mean HI antibody titre at three weeks post inoculation were (1344) and (1408), while in horses at six weeks post inoculation were (480) and (640) respectively for both vaccines. These results agreed with **(14) and (19)** who reported that the protective HI antibody titre should not less than (64).

Concerning with the effect of storage on the locally prepared EI (A/equi-2) monovalent inactivated vaccine revealed that the liquid vaccine is stable at 4°C for one year; while the freeze dried vaccine can be kept at 4°C for 18 months and at -20°C for three years. These results agreed with **(10), (20) and** (5) who recorded that freeze dried antigens were stable for at least 25 months when stored at 4°C and can be preserved for years at – 20°C.

From the obtained data as presented in Table (5) and Fig. (2), it is clear that the first dose of both vaccines (liquid and freeze dried vaccines) of inactivated influenza virus were able to stimulate HI antibodies in horses at 2 weeks post vaccination with mean value (80) for both vaccines. By boostering at 4th week post inoculation, much higher level of HI antibody titre were obtained at 6th week (480 and 640) for liquid and freeze dried vaccine, respectively. HI antibodies reached their maximum titre at 8th and 10th week where the mean HI antibody titre (2560) for both vaccines. Then the titre begin to decline gradually till the 12th month post inoculation with a considerable protective HI antibody level (80) for both vaccines, where the recorded protective HI antibody titre is 64 (**19**).

These obtained results come parallel to those mentioned by (**22, 21, 13, 11 and 19**).

From the obtained results, it could be concluded that EI virus was completely inactivated by 0.1 % formalin at 37°C for 24 hours. The use of DEAE-Dextran and alhydragel with saponin as adjuvants gave nearly the same efficient results and both vaccines proved to be safe and potent. We can deduce that it is important to monitor foreign epidemics and the appearance of variant strains then include these variants in the vaccine, also a suitable adjuvant should be added to enhance the antibody response and prolong the duration of immunity.

Tables

Table (1): Titration of EI virus fluid

	HA titre *	Infectivity titre **
Influenza A/Equi/2	2048	9.5

* HA titre expressed as the reciprocal of virus dilution.

** Infectivity titre expressed as $\log_{10}$ EID_{50}/0.1 ml

Table (2): Inactivation of locally isolated EI virus (A/equi-2/Cairo-2/2000) using 0.05 % and 0.1 % formalin at 37°C

Time of incubation	Virus fluid treated with 0.05 % formalin		Virus fluid treated with 0.1 % formalin		Untreated virus (Control)	
	Infectivity titre *	HA titre **	Infectivity titre *	HA titre **	Infectivity titre *	HA titre **
0	9.5	2048	9.5	2048	9.5	2048
6 hours	8.0	2048	7.0	2048	9.5	2048
12 hours	6.4	2048	5.0	2048	9.2	2048
14 hours	5.5	2048	3.0	2048	9.2	2048
16 hours	4.5	2048	2.0	2048	9.0	2048
18 hours	4.0	2048	1.0	1024	8.6	2048
24 hours	3.5	1024	0.0	512	8.3	2048
30 hours	3.0	1024			8.3	2048
36 hours	3.0	1024			8.3	2048

* HA titre expressed as the reciprocal of virus dilution.
** Infectivity titre expressed as $\log_{10}$ EID_{50}/0.1 ml

Table (3): HI antibody titres in sera of Guinea pigs inoculated with the prepared monovalent inactivated equine influenza vaccine

Animal* number	HI antibody titre					
	Group (A)		Group (B)		Group (C)	
	Pre-inoculation	21 days post inocula-tion	Pre-inocula-tion	21 days post inocula-tion	Preino-culation	21 days post inocula-tion
1	-	1280	-	2560	-	-
2	-	1280	-	640	-	-
3	-	2560	-	640	-	-
4	-	640	-	2560	-	-
5	-	1280	-	640	-	-
Mean	-	1344	-	1408	-	-

Group (A) : G. pigs inoculated with liquid EI vaccine
Group (B) : G. pigs inoculated with freeze dried EI vaccine
Group (C): Control group.
(-) : Negative result
* Number of animals in each group

Table (4): Stability of the prepared EI monovalent inactivated vaccines

Temperature	Time of stage	Mean HI antibody titre in sera of G. pigs *		
		Group (A) **	Group (B) ***	Group (C) Control group
4°C	0 time	1344	1408	- ve
	3 months	1344	1408	
	6 months	1280	1408	
	12 months	288	1408	
	18 months	55	160	
	24 months	-	-	
Room temp. (25-28°C)	0 time	1280	1408	- ve
	3 months	288	640	
	6 months	-ve	160	
	12 months	-ve	- ve	
- 20°C (for freeze dried vaccine only)	0 time		1408	- ve
	3 months		1408	
	6 months		1408	
	12 months		1408	
	18 months		1048	
	24 months		1048	
	30 months		1344	
	36 months		1280	

*Mean dilution giving the complete haemagglutination inhibition antibody titre expressed as the reciprocal of serum inhibition of haemagglutination.

**Group (A): G. pigs inoculated with EI liquid vaccine.

***Group (B): G. pigs inoculated with EI freeze dried vaccine.

Table (5): Seroconversion of horses inoculated with monovalent inactivated vaccine of EI virus tested by HI test

Time of sampling	HI titre in sera of vaccinated horses							
	Group (A)			Group (B)			Group (C)	
	H1	H2	Mean	H3	H4	Mean	H5	H6
Prevacc.	-	-	-	-	-	-	-	-
2 weeks	80	80	80	80	80	80	-	-
3 weeks	320	160	240	320	160	240	-	-
(b) 4 weeks	320	320	320	320	320	320	-	-
6 weeks	640	320	480	640	640	640	-	-
8 weeks	2560	2560	2560	2560	1280	1920	-	-
10 weeks	2560	1280	1920	2560	2560	2560	-	-
12 weeks	1280	1280	1280	1280	1280	1280	-	-
3.5 months	1280	640	960	1280	1280	1280	-	-
4 months	640	640	640	640	640	640	-	-
4.5 months	640	320	480	640	320	480	-	-
5 months	320	320	320	320	320	320	-	-
6.0 months	320	160	240	320	320	320	-	-
7 months	320	160	240	320	160	240	-	-
8 months	160	160	160	160	160	160	-	-
9 months	160	160	160	160	160	160	-	-
10 months	160	160	160	160	160	160	-	-
11 months	160	80	120	80	80	80	-	-
12 months	80	80	80	80	80	80	-	-

Group (A): Horses inoculated with inactivated EI liquid vaccine
Group (B): Horses inoculated with inactivated EI freeze dried vaccines reconstituted in DEAE-Dextran solution
Group (C): Control group
(-) : Negative result
H : Horse
(b) : Boostering

Fig. (1): Inactivation of locally isolated EI virus (A/equi-2/Cairo-2/2000) using 0.05 % and 0.1 % formalin at 37°C

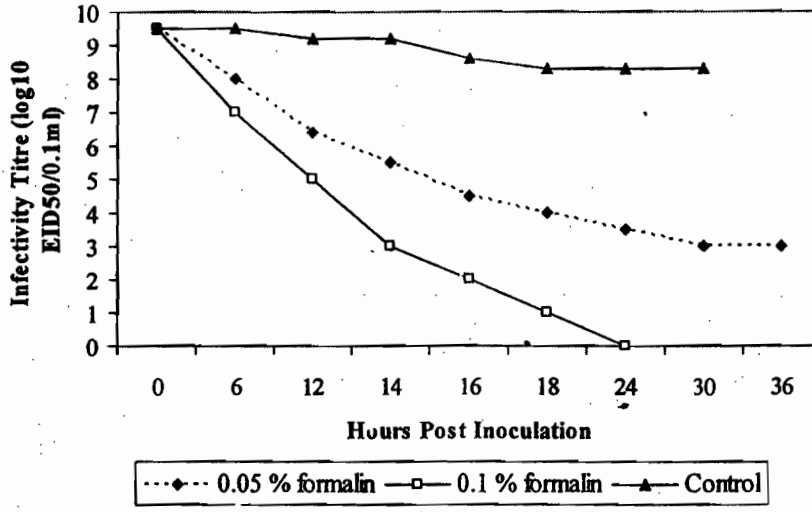
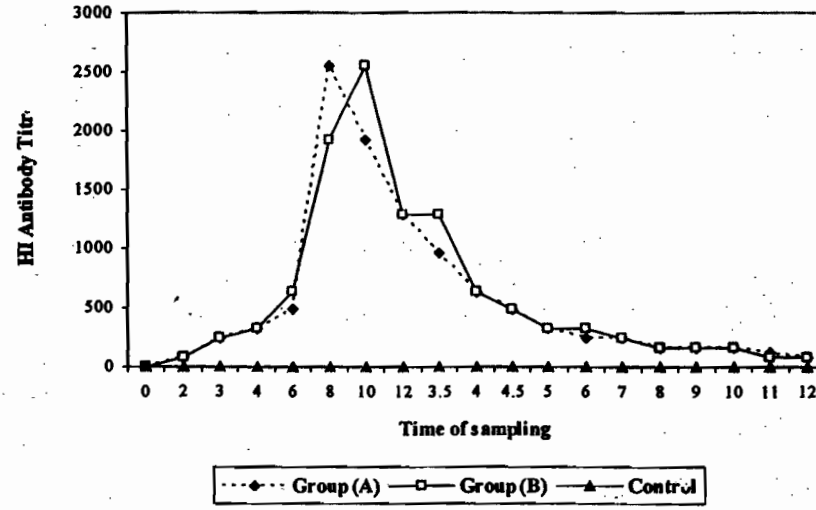


Fig. (2): Seroconversion of horses inoculated with monovalent inactivated vaccine of EI virus tested by HI test



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محاولات لتحضير إللقاح الأحادى المثبط لإنفلونزا الخيول

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الملخص العربى

الهدف من هذا العمل هو تحضير وتقييم لقاح أنفلونزا الخيول المحضر من العترة المعزولة محلياً لفيروس أنفلونزا الخيول النوع رقم ٢ وقد تم تثبيط الفيروس باستخدام الفورمالين ٠,١ % (٤٠ % فورمالدهيد) عند درجة حرارة ٣٧° م لمدة ٢٤ ساعة ثم إضافته الى نوعين من المحفزات إحداهما الديادكستران والأخر الهيدراجل مع الصابونين. وقد ثبتت كفاءة كل من اللقاحين عند حقنهم فى الخيول والأرانب الهندية وذلك بقدرتهم على إنتاج مستويات عالية من الأجسام المناعية. استمرت فى أمصال الخيول المحقونة بمستوى وقائى حتى عام كامل عند قياسها باختبار التلازن الدموى المثبط. تم تقييم كفاءة اللقاحين بعد حفظهما عند درجات حرارة مختلفة. ولقد وجد أن اللقاح السائل احتفظ بكفائته المناعية عند درجة ٤° م لمدة عام. أم بالنسبة للقاح المجفد فقد احتفظ بكفائته المناعية لمدة ١٨ شهر فى درجة حرارة ٤° م ولمدة ثلاث سنوات عند حفظه فى – ٢٠° م.