



## **Trials For Preparation Of Combined Vaccine Of Equine Influenza, Equine Herpesvirus–1, Rabies Virus And Tetanus Toxoid**

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### **ABSTRACT**

A combined vaccine of Equine influenza (EI), equine herpesvirus–1 (EHV-1), rabies virus and tetanus toxoid adjuvanted with saponin and Alhydrogel was prepared. Quality control testing of such vaccine revealed that it was free from foreign contaminants, safe for Guinea-pigs, mice and mares. It was inoculated into Guinea -pigs as a preliminary evaluation for its potency and it was proven safe and potent. Also, it exceeded the permissible protective level allowed to be used for the vaccination of horses. Two groups of mares were used for evaluating the potency of the vaccine. The first one received two doses (5 ml) of the prepared vaccine with 4 weeks interval while the 2<sup>nd</sup> group was kept without vaccination as control. The mean haemagglutination inhibition (HI) influenza antibody titer reached its maximum at the 2<sup>nd</sup> month post-vaccination (MPV); 2048 while EHV-1 antibodies reached the peak at the 3<sup>rd</sup> MPV as recorded by ELISA and neutralizing indices; 1790 and 3.50. Rabies antibodies were detectable in vaccinated mares by the 2<sup>nd</sup> week post-vaccination showing titers of 16 and 1.0 by serum neutralization test (SNT) and ELISA, respectively recording their peaks (128 by SNT and 2.15 by ELISA) at the 2<sup>nd</sup> MPV. Tetanus antitoxic titer increased till reaching the peak at the 2<sup>nd</sup> MPV (40IU/ml) as determined by toxin neutralization test. Depending on these results, it could be concluded that the prepared inactivated EI, EHV-1, rabies and tetanus vaccine is safe and potent for mares providing them with good protective levels of specific antibodies against the 4 used antigens up to 6 months.

**Keywords:** Equine influenza, herpesvirus-1, Rabies, tetanus , Vaccine.

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### **INTRODUCTION**

Equine influenza (EI) is a highly contagious viral respiratory disease characterized by fever, depression, mucoid or mucopurulent discharge and coughing which develop after a short incubation period. The disease spreads quickly through direct aerosol transmission or indirect as a consequence of inadequate hygienic precautions and the co-transport of infected and uninfected horses (Laabassi *et al.*, 2015). The virus currently circulating in horses is H3N8 antigenic subtype (Cullinane and Newton, 2013) and (Gildea *et al.*, 2016)

Equine herpes viruses (EHVs) belong to the *Herpesviridae* family. The family is subdivided into three subfamilies ( $\alpha$ ,  $\beta$  and  $\gamma$ ) based on tissue tropism, pathogenicity and behavior in tissue culture (Davison *et al.*, 2002). Both EHV1 and 4 are economically important and affect the respiratory tracts of horses worldwide (Allen and Bryans, 1986). EHV-1 is an alpha herpes virus that causes a broad range of manifestations in horses, including a central nervous system disease that includes spinal cord and brain disorders (myeloencephalopathy), respiratory disease, abortions, perinatal death and chorioretinopathy

(Mesquita *et al.*, 2017). Some vaccines are available but their efficacies remain limited (Khusro *et al.*, 2020).

Rabies is a zoonotic disease that affects the Central Nervous System (CNS) and causes acute and fatal encephalitis in the affected hosts. The etiologic agent of the disease is rabies virus; a neurotropic, RNA virus of the order Mononegavirales, family Rhabdoviridae genus Lyssavirus. The rabies infection is mainly transmitted when infected saliva reaches nervous tissue through a bite wound or skin injuries, or breached mucous membranes. Once clinical symptoms appear, it is almost 100% fatal (WHO, 2018).

Today, rabies vaccines which are used for both humans and animals as recommended by the World Health Organization are made of inactivated viruses produced in continuous cell lines (WHO, 2018). Tetanus is a highly fatal bacterial infectious disease caused by an anaerobic spore-forming bacterium; *Clostridium tetani*. This disease is prevalent globally and affects all types of domestic animals (Brüggemann *et al.*, 2003). Horses are the most susceptible of all domestic animals, representing more than 80% of affected hosts. Infection occurs due to its spore as it enters the body via any wounds especially deep lacerated wounds caused by sharp objects or internal wounds. Vaccination is effective, practical and give long-term protection by using the tetanus toxoid which is the most effective prophylactic immunogene today. Regular vaccination of all horses against tetanus is essential (pailot *et al.*, 2017).

The protection induced by first generations of whole inactivated, aluminum hydroxide adjuvanted EI vaccine primarily relied on stimulation of high antibody levels. It is known that Aluminum hydroxide triggers a Th2, antibody -orientated immune response (Lindblad, 2004 and Dalia, 2017). Saponin based adjuvants can modulate the cell mediated immune system as well as enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (Rajput *et al.*, 2007 and Abdelhady, 2016). Vaccine formulation that enables a reduction in the number of different vaccinations required each year will provide protection from the most critical pathogens as a combined vaccine against Equine influenza and EHV-1 in horses is highly desirable (Nashwa *et al.*, 2016).

The combined inactivated vaccine (EIV, EHV-1 and Tetanus Toxoid (TT) adjuvanted with saponin and Alhydrogel was evaluated in guinea pigs and horses. The results obtained were proved it to be safe, potent and stable at 4°C for up to 12 months (Bayoumi *et al.*, 2018a)

Thus, the present study was planned to assess the immune response of horses to a combined inactivated EI, EHV-1, rabies and TT vaccine adjuvanted with saponin and Alhydrogel.

## MATERIALS AND METHODS

### 1. Strains used for vaccine preparations

- A locally identified freeze-dried equine influenza virus (A/equi-2/Egypt /6066 NAMRU3-VSVRI/2008) egg passage three (EP3) (Magda *et al.*, 2011) and locally isolated freeze dried EHV-1 (EHV-1/Egypt/ VSVRI/Zahraa /2014) at its VERO cell passage two (VEp<sub>2</sub>) were supplied by the Department of Equine Vaccine Research (DEV), Veterinary serum and vaccine Researches Institute (VSVRI), Abbasia, Cairo, Egypt
- Tissue culture adapted Evelyn Rokitnicki Abelson (ERA) rabies virus strain (with a titer of 10<sup>8.5</sup> TCID<sub>50</sub>/ml) was kindly supplied in a lyophilized form by WHO Collaborating Center for References and Researches in rabies Pasteur, Paris-France
- Lyophilized *C. tetani* strain (Harvard strain, 49805) obtained from Antigen and Sera Department, VSVRI, Abbasia, Cairo, Egypt.

These strains were used for preparation of an inactivated combined vaccine against equine influenza, equine herpes virus – 1, rabies virus and TT.

### 2. Vaccine preparation

The vaccine was prepared according to (Bayoumi *et al.*, 2018a). An equal volume of inactivated of EIV and EHV-1 fluids and TT were mixed with inactivated rabies virus (Edries *et al.*, 2017) then adjuvanted with 20% alhydrogel with saponin and dispensed in vials (five ml per vial). Each horse dose of inactivated combined vaccine contains 11 log<sub>2</sub> HA units of EIV and 8.5 log<sub>10</sub> TCID<sub>50</sub> /ml of EHV-1, inactivated rabies virus 7.5 - 8 log<sub>10</sub> TCID<sub>50</sub>/ml and tetanus toxoid 40 limits of flocculation (Lf/ml) (Heldens *et al.*, 2010 and Soliman *et al.*, 2011).

### 3. Experimental animals

#### 3.1. Mares

Ten apparently healthy adult mares (2-4 years old) were used in the present study with 4 pregnant mares to test the safety and 3 mares were used to assess the potency of the prepared vaccine while last 3 mares were kept unvaccinated as control animals. All mares were housed under hygienic measures receiving balanced ration and adequate water according to animal ethics.

#### 3.2. Mice

Four pregnant Swiss Albino mice of 4-6 weeks old were used for safety testing of inactivated EHV-1 as a component of the prepared vaccine. About 60 mice were used in the toxin neutralization test for evaluating the potency of tetanus antitoxin.

### **3.3. Guinea pigs**

Fifteen seronegative Guinea pigs of about 300-400 gm body weight were divided equally into three groups; the first group was used to assess the safety of the prepared TT, the second group was used for assessing the potency of the prepared vaccine and the 3<sup>rd</sup> group was kept without inoculation as control group.

## **4. Vaccine quality control**

### **4.1. Sterility**

This test was performed on each inactivated virus fluid before mixing as well as on the final product. Samples from them were cultured on different media to exclude bacterial, fungal, mycoplasma contaminations. (OIE, 2019a).

### **4.2. Safety**

#### **4.2.1. Residual infective virus activity**

This test was performed on the inactivated virus fluid immediately after inactivation process to ensure complete virus inactivation as follow:

a) Undiluted inactivated EIV was inoculated into the allantoic cavity of a group of specific pathogen free (SPF) Embryonated Chicken Egg (ECE) 9-11 day old and haemagglutinating activity should not be detected (OIE, 2019b).

b) Undiluted inactivated EHV-1 was inoculated on both VERO and Chorioallantoic Membrane (CAM) of embryonated chicken eggs 11-13 days (Safaa and Hussein, 2012) revealing no CPE in the cell culture and no lesions in CAM.

c) Undiluted inactivated ERA rabies virus was inoculated in both BHK cell culture and in weaned albino Swiss mice with no CPE in BHK cells and no symptoms in mice (OIE, 2018)

d) Five ml of TT were injected subcutaneously as two equally divided doses at separate sites into each of five Guinea-pigs, each weighing 350-400 g. No significant local or systemic reaction is produced. The Guinea-pigs were observed for 21 days. No Guinea-pig showed signs of tetanus or died from it (British pharmacopoeia, 2009)

#### **4.2.2. Animals safety test**

It was performed on the final vaccine product as follow:

a) Four pregnant mares in the last trimester of pregnancy were inoculated I/M with 5ml of the prepared vaccine (OIE, 2019b) and observed for two weeks.

b) Four pregnant mice were inoculated subcutaneously (S/C) with 0.2 ml of the prepared vaccine (Slater *et al.*, 1993).

All groups of both mares and mice were kept under observation for two weeks in good hygienic conditions.

c) Five Guinea pigs were injected with 5ml of the vaccine S/C as 2 equally divided doses, at separate sites and kept under observation in a good hygienic condition for 21 days to evaluate the safety of tetanus toxoid in the vaccine. (British Pharmacopoeia, 2009).

### **4.3. Potency of the prepared vaccine**

#### **In Guinea pigs**

Ten seronegative Guinea pigs (G. pigs) were divided into 2 groups (5 G. pigs/group). Group (A) was inoculated subcutaneously (S/C) with 5ml of prepared vaccine; group (B) was kept as a control under the same conditions of the experiments. Twenty one days post inoculation, serum samples were collected from both groups and tested for HI antibody titer using HI assay for EI virus (OIE, 2019b). Twenty one days post inoculation, group A received a booster dose of the vaccine then 14 days later, serum samples were collected and tested for EHV-1 antibodies using ELISA (Guo *et al.*, 1989), rabies antibodies using SNT and ELISA (OIE, 2018) and tetanus antitoxin titer using toxin neutralization test (TN) (British Pharmacopoeia, 2009)

#### **In horses**

Six local mares, two - four years old, seronegative for rabies and tetanus or with low antibodies titer against EIV&EHV-1 were divided into 2 groups. Group (A) was vaccinated with two doses of the prepared vaccines one month apart using a dose of 5ml and group (B) was kept as a control group under the same conditions of the experiments. Serum samples were collected from each group every two weeks for two months then monthly until the 7<sup>th</sup> months post vaccination for monitoring the immune response using HI test for EI virus, neutralization indices and ELISA for EHV-1, SNT and ELISA for rabies and TN test for tetanus antitoxin titer.

## **5. Serological examination**

### **5.1. Haemagglutination test (HA) and Haemagglutination Inhibition test (HI)**

They were performed according to the method described by (OIE, 2019b) for measuring the equine influenza virus titer and antibody response.

**5.2. Enzyme linked immunosorbent assay (Solid phase ELISA)** (Single-dilution) was carried out according to (SuGiura *et al.*, 1997) and neutralization indices according to (Shankar and Yadav, 1986) and (Abdelhady, 2016) for measuring the EHV-1 antibodies.

### **5.3. Serum neutralization test (SNT) and ELISA for monitoring of rabies antibodies**

SNT was carried out using the microtiter technique according to (Rossiter *et al.*, 1985) and the rabies serum neutralizing antibodies titer was calculated by the method of (Singh *et al.*, 1967).

Zhenrui Rabies virus Antibody Quantitative ELISA kit, version RBV1805003 was supplied by Shenzhen Zhenrui Biotech Co., Ltd. China and the rabies antibody titer was expressed as IU/ml according to the standard curve performed by the kit manufacturer.

**5.4.Toxin neutralization test for monitoring of tetanus antitoxin titer**

The antitoxin titer was measured by mice TN which was done according to (Martha and Craig, 1997).

Ethical approval: Institutional Animal Care and use committee at veterinary serum and vaccine research institute hereby acknowledge the research manuscript and it has been reviewed under its research authority.

**RESULTS**

Concerning the safety of the prepared vaccine, it was found that there are no residual virulent viruses in the inactivated viral fluids as proved by absence of haemagglutinating activity for EIV, pock lesion and CPE in both CAM and VERO cells for EHV-1 respectively. No CPE of rabies virus in BHK cells and no rabies signs in mice as recommended by OIE (2018 and 2019b).Also, there was no signs of tetanus in guinea pigs. Also, all inoculated horses showed normal body temperature and no undesirable local or systemic reaction observed following primary and booster injections. There is no abortion in pregnant mares.

Testing the potency of the prepared vaccine in G. pigs; the demonstrated data in table (1) showed that G. pigs serum samples of group (A) exhibited Equine Influenza antibodies with mean HI titer 1843.2 by the 3<sup>rd</sup> WPIwhile, EHV-1 elicited an obvious seroconversion in Guinea pigs; serum neutralizing antibodies were detectable (550) twenty one days after the primary inoculation and a marked increase in the titer of virus neutralizing antibodies was obtained by 2 weeks post second dose (about 2 fold increase; 1250). Tetanus antitoxin titer was 40 IU/ml by the 2<sup>nd</sup> week post second inoculation.

Table 1: Mean antibody titers in sera of Guinea pigs inoculated with the prepared vaccine.

Applied test	Group-A			Group-B
	Pre-vaccination	*3WPI	*2 WPB	
EI-HI	-	1843.2	-	0
HV-1 EHV-1	-	550	1250	0
TT(TN)	-	20	40	0

Group A: was vaccinated with (5ml) of prepared vaccine.  
Group B: was kept without vaccination.  
\*WPI: Weeks post-inoculation.  
\*WPB: Weeks post boosting.

Regarding the immunogenicity of the prepared vaccine in mares, the demonstrated data in table (2) show that EIV antibodies determined by HI test were detectable at 2 weeks post inoculation with a mean titer 256, which at 4 weeks reached 128. Such antibodies reached their maximum titer at 2 months post vaccination (MPV) to be 2048, then began to decline gradually till the 6<sup>th</sup> month.

Table 2: EI- HI antibodies titer in sera of mares inoculated with the prepared vaccine

Periods post-vaccination	EI-HI antibody titer/ WPV	
	Group-A	Group-B
0 time	8	≤ 4
2WPV	256	
4WPV	128	
Boostering		
6WPV	1024	≤ 4
2MPV**	2048	
3MPV	2048	
4MPV	1024	
5MPV	512	
6MPV	512	
7MPV	128	

Group A: was vaccinated with (5ml) of prepared vaccine.  
Group B: was kept without vaccination.  
\* WPV: Weeks post vaccination.  
\*\* MPV: Months post-vaccination.

Results in table (3) indicates that EHV-1 antibodies were detectable (656) at 2WPV with mean ELISA titer and neutralizing index (0.60) for group(A) and reached its maximum titer at 3<sup>rd</sup>MPVwith mean ELISA titer (1790) and neutralizing index (3.50).

Table 3: Mean ELISA and VNT titers of EHV-1 antibodies in horses vaccinated with the prepared vaccine.

Periods post-vaccination	Group-A		Group-B	
	ELISA	NI	ELISA	NI
0 time	95	0.20	98	0.20
2WPV*	656	0.60	90	0.20
4WPV	420	0.60	92	0.20
Boostring				
6WPV	1390	2.60	95	0.20
2MPV**	1530	3.50	92	0.20
3MPV	1790	3.50	92	0.20
4MPV	1680	3.50	92	0.20
5MPV	1430	2.80	92	0.20
6MPV	1100	1.60	92	0.20
7MPV	910	1.40	92	0.20

Group A: was vaccinated with (5ml) of prepared vaccine.  
Group B: was kept without vaccination.  
\*WPV: Weeks post vaccination.  
\*\*MPV: Months post- vaccination.

**DISCUSSION**

Horses are commonly vaccinated to be protected against pathogens which are endemic within the general horse population, such as EIV and EHV-1, and against a variety of diseases which are less common but lead to greater morbidity and mortality, such as tetanus (Ribeiro *et al.*, 2018) and rabies (Schwarz *et al.*, 2020). Recent equine vaccines trials have indicated that the efficacy of vaccines is unaffected by their administration as monovalent or polyvalent formulations (Heldens *et al.*, 2002). So, this study was developed to evaluate the immune response of equines to combined vaccine of inactivated equine influenza virus, equine herpes virus-1, rabies virus and tetanus toxoid.

The vaccine was inoculated in guinea pig as a preliminary evaluation for its potency. As seen in table (1), the Equine Influenza HI antibodies recorded exceeds the protective level (64) that reported by (OIE, 2019). As for Herpes virus-1 antibodies the results came in agreement with those of (Guo *et al.*, 1989) and (Nashwa *et al.*, 2016) who mentioned that there was more than 2 fold increase after the second dose. Tetanus antitoxic titer reached 40 IU/ml by the second week post second dose and this titer exceeds the requirements of **British Pharmacopoeia (2009)**; as it requires a minimum of 30 IU/ml in Guinea pig for tetanus toxoid to be adequate in vaccination of horses.

Regarding the immunogenicity of the prepared vaccine in mares, the elicited HI antibodies against equine influenza exceeds the requirements of OIE (OIE, 2019b) who reported that the protective EI-HI antibody titer should not be less than 64.

Table (3) showed the immune response against herpes virus-1, the results indicate more than fourfold increase in antibody titer against the virus and this agree with those of (Bannai *et al.*, 2014) and (Rusli *et al.*, 2014) who found that ELISA antibodies began to increase by day 14 post injection then reached its peak at 2 months with 4-fold increasing, which indicated good immune response.

As shown in table (4) rabies antibodies values indicated that the tested vaccine is potent for mares where similar results were obtained by Khodeir, (1999); Khodeir and Daoud, (2008); Albehar, (2009) and Eiaka, (2017) using cell culture inactivated rabies vaccine adjuvant with aluminum hydroxide gel and recommended that the protective level of rabies serum neutralizing antibodies should not be less than 16 by SNT and 1.0 by ELISA.

Regarding tetanus antitoxic titer, the titer reached 2 IU/ml at the 7<sup>th</sup> month, which could be

Table (4) demonstrates that rabies antibodies were detectable in vaccinated mares by the 2<sup>nd</sup> week post vaccination showing titers of 16 and 1.0 by SNT and ELISA respectively recording their peaks 128 by SNT and 2.15 by ELISA by the second month.

Table 4: Rabies serum ELISA and neutralizing antibody titer in vaccinated mares with the prepared vaccine

Periods post-vaccination	Group-A		Group-B	
	ELISA	SNT titer*	ELIS A	SNT titer
0 time	0.01	0	0.01	0
2WPV**	1.0	16	0.01	0
4WPV	1.2	32	0.10	0
Bootstring				
6WPV	1.35	64	0.01	0
2MPV***	2.15	128	0.01	0
3MPV	2.15	128	0.01	0
4MPV	2.13	128	0.01	0
5MPV	2.13	128	0.01	0
6MPV	2.11	128	0.01	0
7MPV	2.10	128	0.01	0

Group A: was vaccinated with (5ml) of prepared vaccine.

Group B: was kept without vaccination.

\*\*WPV: Weeks post-vaccination.

\*\*\*MPV: Months post-vaccination.

\*Rabies serum neutralizing antibody titer= the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID<sub>50</sub> of rabies virus.

ELISA results were expressed as IU/ml where the protective value should not be less than 0.5 IU/ml.

Regarding the tetanus toxoid component in the combined vaccine, table (5) showed that the antitoxins were detected in vaccinated animals at the 2<sup>nd</sup> week postvaccination (12 IU) reached their peak by the 2<sup>nd</sup> MPV(40 IU) then declined till the end of the experiment(2 IU).

Table 5: Tetanus antitoxin titer in sera of mares vaccinated with the prepared vaccine tested by TN test

Periods post-vaccination	Tetanus antitoxin (IU/ml)	
	Group-A	Group-B
0 time	< 0.01	< 0.01
2WPV*	12	
4WPV	25	
Bootstring		
6WPV	30	< 0.01
2MPV**	40	
3MPV	30	
4MPV	20	
5MPV	10	
6MPV	5	
7MPV	2	

Group (A): was vaccinated with 5ml of the prepared vaccine.

Group (B): was kept without vaccination as a control at the same conditions of the experiment.

\*WPV: Weeks post-vaccination

\*\*MPV: Months post-vaccination

accepted as protective level of tetanus antitoxin titer in animal sera, as measured by TN as it is recommended that it must be at least 0.01IU/ml as there are indications that this is the actual protective level (Gupta *et al.*, 1985).

These results agree with those obtained by (Heldens *et al.*, 2001) who indicated that a combined vaccine against EI, EHV-1 and tetanus is immunogenic and confer immunity for up to 6 months post vaccination. From the above result it is clear that after the 6<sup>th</sup> month revaccination with combined EIV, EHV-1 and tetanus vaccine is required as stated by (Bayuomi *et al.*, 2018b) who pointed out that the third dose developed high antibodies titer above the required level for clinical protection against influenza and tetanus until 12 months and 6 months for EHV-1. Thus, alternative additional inactivated EHV-1 should be used.

### CONCLUSION

The present obtained results indicate that there is no antagonizing effect of the used vaccine components on the immune response of vaccinated animals and accordingly it can be conclude that the combined vaccine is safe and immunogenic using a vaccination scheme consisting of 2 doses with one month apart followed by a third vaccination at 6<sup>th</sup> MPV to maintain a maximal protection against equine influenza, Herpesvirus-1, Rabies and tetanus infections.

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