



Improvement Of Inactivated Equine Herpes Virus-1 Vaccine Using Carbomer

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ABSTRACT

An inactivated Equine herpes virus-1 vaccine was successfully improved using carbomer as adjuvant inducing high and long immunity in vaccinated mares in comparison with the convention one adjuvanted with Al-hydra gel and saponin. Such purpose was established by using 0.5% carbomer as adjuvant to the inactivated *EHV-1*. The applied quality control tests carried out on such vaccine revealed that it is free from foreign contaminants, safe in pregnant mares and mice and potent induced high levels of specific *EHV-1* antibodies in vaccinated Guinea pigs and mares as measure by ELISA and SNT. This immunity was sufficient to protect vaccinated horses up to 28 weeks (7months) post-vaccination.

Keywords: Carbomer, ELISA, SNT, Inactivated EHV-1, Equine Virus.

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INTRODUCTION

Equine herpesvirus type 1 (EAV-1) causes a broad range of manifestations in horses, including a central nervous system disease involving the spinal cord and brain (myeloid cephalopathy), respiratory disease, abortions and perinatal death (Meaquita *et al.*, 2017). Abortion may result from exogenous or endogenous infection, i.e., reactivation of latent virus (Allen *et al.*, 1998).

In recent years, increased incidence of Equine herpesvirus myeloid Cephalopathy (EHM) has been observed by infection with the same virulent strains of EHV-1 leading to inflammation of the blood vessels supply the brain and spinal cord (Henninger *et al.*, 2007). Control of EHV-1 infection is so tricky that the symptoms may be subclinical and may be reactivated after many months or years after primary infection under stress factors (Browning *et al.*, 1988). Vaccination is crucial to control EHV-1 infection using

either live-attenuated or inactivated vaccines with the inactive one's preferable use (Mayer *et al.*, 1978).

In inactivated vaccines, an ideal adjuvant should be safe, stable, bio gradable and ensure vaccine potency's reproducibility during manufacture (Cater and Reed, 2010). Carbomer has low reactivity, no virucidal nature and efficacy in one shot vaccination schedules; a lightly cross-linked polymer of acrylic has become widely used as an adjuvant in the veterinary field (Diamantstein *et al.*, 1971) enhance the strength and duration of antibody responses stimulated by inactivated equine influenza vaccines as compared with vaccines of equivalent antigenic content (Wood *et al.*, 1983). Production of GHPV antigen inactivated adjuvanted vaccine (Jac Queline *et al.*, 2010) vaccine induces a robust serological response in growing gosling and breeder. Carbomer promotes an early onset of cellular immunity by facilitating the cell differentiation towards effector phenotypes and efficiently inducing native to memory transition (Mair *et al.*, 2015). Using carbomer in animal models results

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in adjuvant system activity, including strong proinflammatory type-1 T cell (TH1) polarization (Schwabe *et al.*, 1977). Carbomer adjuvanted rabies vaccines are preferable to aluminum hydroxide gel vaccine providing a high protective level in vaccinated dogs.

Saponin is extracted from Saponaria Molina can stimulate a strong immune response against exogenous antigens when used as an adjuvant (Eman *et al.*, 2013). Tissue culture inactivated ISA-70 oil adjuvanted EHV-1 vaccine was successfully prepared from the locally isolated virus, which was entirely inactivated by 0.008 M of binary ethyleneimine (BEI) within 24 hours at 37°C (Safaa *et al.*, 2012). Vaccination was one of the best options to fight EHV-1 infection in combination with reasonable management measures. Successful vaccination against EHV-1 requires both humoral and cellular immune responses, which require the presence of a potent adjuvant to stimulate both immune responses (Paillot *et al.*, 2008).

However, the cellular immune response is also essential in pregnant mares, positive correlation between the frequency of EHV-1 CTL (cytotoxic T-lymphocyte) and the protection against the disease (Kydd *et al.*, 2003). This study was designed to prepare and evaluate the safety and potency of inactivated EHV-1 adjuvanted with carbomer and a mixture of saponin as vaccine adjuvants.

MATERIALS AND METHODS

1. Equine herpes virus-1 (EHV-1)

Locally isolated EHV-1 isolated by Hassanein *et al.* (2002) and adapted on Vero cell culture by Safaa (2007) identified by reference freeze-dried rabbit anti-EHV-1 (kindly supplied by Dr Jennet Wellington, Research Follow Department of Biological Science, Macquarie Univ., NSW Australia) was supplied by the Department of Equine Vaccine Research (DEVIR); Veterinary Serum and Vaccine Research Institute (VSVRI) and used for an inactivated vaccine.

2. EHV-1 antiserum

Locally prepared EHV-1 antiserum (Safaa *et al.*, 2005) was supplied by DEVIR and used as a positive control in the serological tests.

3. Experimental animals

3.1. Mares

Eleven health adult mares were used in the present study where 3 of them were used to test the safety, and six mares were used to test the potency of the prepared vaccine while two mares were kept without vaccinates as a test control. All mares were housed under hygienic measures receiving balanced ration and adequate water following animal ethics.

3.2. Mice

Three groups of pregnant Swiss Albino mice of 4-6 weeks old (4mice/ group) were used for safety testing of the prepared inactivated EHV-1 vaccine

3.3. Guinea pigs

Twelve seronegative Guinea pigs of about 300-400gm body weight were divided into three groups were the first two groups were used to assess the potency of the carbomer and Al-hydra gel with saponin adjuvanted vaccines respectively, keeping the third group without inoculation as test control and the second group was used

4. Cell culture

African green monkey kidney cell line (Vero) was maintained and propagated using Minimum Essential medium with Eagle's salts and used to prepare EHV-1 suspension, virus titration, and the residual viral testing inactivated virus suspension and serum neutralization test.

5. Embryonated chicken eggs

Specific pathogen-free Embryonated chicken eggs (SPF-ECE) of 11-13 days old were obtained from SPF eggs farm Koum Osheim, Fayoum Governorate, Egypt and used for assurance of complete virus inactivation

6. Saponin

Saponin was obtained from Ubichem. PLC as powder prepared as a solution of 2mg/ml of purified saponin in distilled water with PH 7.5 and sterilized by autoclaving at 108°C for 15 minutes according to Hamdy (2016)

7. Preparation of carbomer solution

Carbomer was supplied from Lubrizol Co. as a fluffy white powder. It was dissolved in hot water to prepare 0.5% aqueous stock solutions, sterilizes by autoclaving at 121°C under the pressure of 1.5lb/ inch for 20 min, then stored at 4°C until use (United States pharma Cofield Convention, 1990).

8. Preparation of EHV-1 suspension

The local EHV-1 was propagated in three successive passages in Vero cells. The virus suspension was collected and clarified by centrifugation at 3000 rpm for 15 minutes, then titrated where the virus titer was calculated according to Reed and Muench (1938). It was recommended that the virus titer should not be less than 7log₁₀ TCID₅₀/ ml for preparation of inactivated EHV-1 vaccine (OIE, 1990).

9. Virus inactivation

EHV-1 inactivation was carried out using 0.008M of Binary ethylenimine (BEI) for 24 hours according to Nehal (2006)

10. Detection of residual infective virus

This step was carried out through:

10.1. Inoculation of the chorioallantoic membrane of ECE incubated at 37°C for five days with daily examination for detection of pock lesions, which should not be detected as recommended by **Safaa and Hussein (2012)**.

10.2. Inoculation of Vero cells to ensure the absence of EHV-1 CPE through three blind passages according to **OIE (2017)**

11. Vaccine preparation

Two batches of inactivated EHV-1 vaccine were prepared where one of them was 20% of Al-hydra gel and the second one was mixed in equal volumes carbomer according to **Naglaa et al. (2020)**.

12. Quality control testing of the prepared vaccine

12.1. Sterility test

Random samples of the prepared inactivated EHV-1 vaccine with carbomer were cultured on different media to ensure the vaccine freedom of foreign contaminants (aerobic and anaerobic bacteria, fungi and mycoplasma) according to the recommendations of **OIE (2017)**

12.2. Safety test

12.2.1. In mice

This test was performed in 2 groups of pregnant mice (10 mice/group) where the first group was inoculated I/N with 45µl of the inactivated virus/mouse while the 2nd group was inoculated S/C with 0.3ml of the prepared vaccine/ mouse according to **Kirisawa et al. (1995)** and kept under a hygienic condition and daily observation for two weeks

12.2.2. In mares

Each of three mares was inoculated I/M with a dose of 2ml of one of the prepared vaccines/mares receiving a second dose one month later. These mares were kept under hygienic measures for two weeks.

12.2.3. Potency test

12.2.3.1. In Guinea pigs

This test included three groups of Guinea pigs of about 300-400 gm body weight (3 animals/group). The first group was inoculated S/C with a 0.2ml/animal of the inactivated EHV-1 vaccine with rehydrated gel and saponin. The second group was

inoculated in the same manner with the same dose of the prepared vaccine with carbomer. Each group received a second dose after one week. The third group was kept without vaccination as test control and serum samples were obtained from all groups on the 3rd and 5th-week post-vaccination.

12.2.3.3. In mares

Eight healthy mares with low EHV-1 antibody titer (≤ 4 NI) were divided into three groups as follow: Group-A of 3 mares were vaccinated I/M with a dose of 2ml of Al-hydra gel and saponin adjuvanted vaccine/ mare

Group-B of 3 mares were vaccinated I/M with a dose of 2ml of carbomer adjuvanted vaccine/ mare

Group-C of 2 mares was kept without vaccination as test control

Each of the vaccinated groups received a booster of the corresponding vaccine four weeks post the first vaccination.

Serum samples were obtained from all mares on 2-week interval up to 28 weeks post-vaccination to follow up the induced levels of EHV-1 antibodies in vaccinated mares using serum neutralization test and ELISA

13. Serological tests

13.1. Enzyme-linked immune sorbent assay (ELISA)

Solid-phase enzyme-linked immune sorbent assay (ELISA) was carried out on mares and Guinea pig sera as described by **Sugiura et al. (1997)**.

13.2. Serum neutralization test (SNT)

SNT was carried out on vaccinated mare sera and the antibody titer was expressed as serum neutralization index (NI) according to **Shanker et al. (1989)** and **Hamdy (2016)**.

RESULTS

The applied quality control testes on the prepared vaccine revealed that it is free from foreign contaminants (aerobic and anaerobic bacteria; fungi and mycoplasma); safe inducing no abnormal local or systemic post inoculation reactions in Guinea pigs and mares and potent providing vaccinated animals with good levels of specific EHV-1 antibodies as shown in table (1).

Table 1: Quality of the prepared inactivated EHV-1 vaccine adjuvanted with carbomer

Applied tests						Result
Virus titer before inactivation	Sterility	Safety		Potency		
		In mice	In mares	In Guinea pigs	In mares	
8.5log ₁₀ TCID ₅₀ /ml	Free from foreign contaminants	Safe inducing no abnormal signs in both animals		Potent inducing high specific EHV-1 antibodies		Valid

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Application of indirect ELISA on serum samples of vaccinated Guinea pigs showed that these animals exhibited antibody titers 400 and 490 by the 3rd week post vaccination in those vaccinated with the alhydra gel vaccine and those vaccinated with the carbomer vaccine reached to titers of 940 and 1019 by the 5th week post vaccination respectively (table 2).

Table 2: Mean EHV-1 ELISA titer in vaccinated Guinea pigs

Periods post-vaccination	Mean EHV-1 ELISA titer (log10) in Guinea pigs vaccinated with		
	EHV-1 with Al-hydra gel and saponin	EHV-1 with carbomer	Unvaccinated control
0 time	0	0	0
3weeks	400	490	0
5weeks	940	1019	0

Regarding vaccination of mares tables (3 and 4) showed that the inactivated EHV-1 vaccine adjuvanted with carbomer induced higher ELISA titer (1480) by the 6th week post vaccination while the gel vaccine showed ELISA titer (1360) on the same period with SNI 2.0 and 1.9 by the 2 vaccines respectively. Peak titers (1690 and 1595 by ELISA and 3.5 and 3.2 SNI respectively by the 10th week recording their lowest levels (803 and 770 by ELISA and 1.8 and 1.3 SNI respectively by the 28th week.

Table 3: Mean EHV-1 ELISA titer in vaccinated mares

Weeks post-vaccination	Mean EHV-1 ELISA titers (log10) induced by		
	Adjuvanted vaccine with carbomer	Adjuvanted vaccine with Al-hydra gel and saponin	Unvaccinated control mares
2	605	530	100
4 (Bootstring)	550	400	93
6	1480	1360	95
8	1605	1590	105
10	1690	1595	95
12	1620	1980	110
14	1508	1230	100
16	1390	1120	95
20	1120	1001	150
24	990	880	95
28	803	770	95

Table 4: Mean EHV-1 serum neutralizing indices in vaccinated mares

Weeks post-vaccination	Mean EHV-1 serum neutralizing indices induced by		
	Adjuvanted vaccine with carbomer	Adjuvanted vaccine with Al-hydra gel and saponin	Unvaccinated control mares
0 time	0.4	0.3	0.2
2	0.7	0.6	0.3
4 (Bootstring)	0.8	0.6	0.3
6	2.0	1.9	0.3
8	3.0	2.8	0.5
10	3.5	3.2	0.3
12	3.5	2.8	0.3
14	3.3	2.5	0.4
16	3.0	2.0	0.4
20	2.8	1.5	0.5
24	2.5	1.5	0.5
28	1.8	1.3	0.3

DISCUSSION

The present study was performed for the preparation and evaluation of inactivated EHV-1 vaccine adjuvanted with carbomer. The present obtained results showed that the prepared inactivated EHV-1 adjuvanted with carbomer is free from foreign contaminants; safe in mice and mares and potent in Guinea pigs and mares (table-1), coming in agreement with the directions of **OIE (2017)**. The obtained virus titer after three blind passages in Vero cells was found to be $8.5 \log_{10}$ TCID₅₀/ml recording a higher value than that recommended by **Hamdy (2016)** and **OIE (2019)**. They concluded that EHV-1 titer should not be less than $7 \log_{10}$ TCID₅₀/ml before virus inactivation. Such a high virus titer ensures the production of a potent inactivated vaccine.

Insurance of the safety of the presently prepared vaccine showed that all vaccinated horses showed average body temperature and no local or systemic postvaccinal reactions following neither the first of the second dose and there were no abortion in pregnant mares and mice, coming in agreement with the recommendations of **OIE (2019)**.

The preliminary study of the prepared vaccine immune response was conducted in Guinea pigs. Table (2) showed detectable EHV-1 antibodies with mean ELISA titer 490 and 400 on the third-week post-vaccination with carbomer rehydra gel vaccines, respectively. These titers increased to 1019 and 940 by the two vaccines respectively by the second-week post the booster dose (5 weeks post the first dose). These findings coincidence with those of **Guo et al. (1989)** and **Dalia (2017)**.

Table (3) demonstrates that the mean EHV-1 ELISA antibody titers were 605 and 530 induced in vaccinated horses with carbomer and rehydra gel with saponin adjuvanted vaccines respectively on the 2nd-week post administration of the 1st dose and increases to 1480 and 1360 respectively on the 2nd-week post bootstring recorded peak titers (1690 and 1595 by the two vaccines respectively) on the 10th-week post the first vaccination then declined till the 28th week with mean values 803 and 770 respectively. These results correlated with those of **Kydd et al. (2006)** and **Nashwa et al. (2016)**, who stated that vaccinated horses became protected against EHV-1 infection to 3-6 months with a decline in abortion.

Table (4) represented the results of SNT expressed as NI, which was detectable two weeks post-vaccination by mean values 0.7 and 0.6 induced by carbomer and rehydra gel with saponin vaccines, respectively. After boosting, there was a gradual increase significantly in NI with mean values 3.5 and

2.8 by the 12th-week post-vaccination, then began to decline to reach 1.8 and 1.3 by the 28th week. These results agree with those of **Senthil et al. (2014)**, **Shankar et al. (1989)** and **Hamdy et al. (2016)** who reported that NI of EHV-1 was ranged between 1.5-3.5 could resist the challenge of EHV-1 infection. Also, these results agree with **Bannai et al. (2014)** and **OIE (2019)**, who found that ELISA antibodies begin to increase by day 14 post-vaccination then reached their peak at two months with a 4-fold increase indicated an excellent immune response.

The obtained data indicate that carbomer has a potential effect eliciting higher levels of humoral antibodies with a longer duration of immunity than the conventional Al-hydra gel adjuvant.

CONCLUSION

Depending on the present obtained results, it could be concluded that the prepared EHV-1 inactivated vaccine adjuvanted with carbomer is safe and more potent than that prepared with Al-hydra gel.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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