Study the effect of different adjuvants on inactivated Equine herpesvirus-1 vaccine

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ABSTRACT

Equine herpesvirus-1 is a highly prevalent and frequently pathogenic infection of equines. The most serious clinical signs of disease ranging in severity, from mild respiratory distress to abortion in pregnant mares, neonatal foal death and neuropathogenic disorders. Inactivated EHV-1 adjuvanted vaccines consider the best way for controlling infectious disease. In this research, the prepared EHV-1 adjuvanted vaccines was completely inactivated by binary ethyleneimine (BEI) with (0.008M) and proved to be, sterile, pure, safe and potent when inoculated on VERO cells, mice and horses. Challenge was applied only in inoculated mice to detect the role of all prepared vaccines in the reduction of virus clearance and excretion. There was no any undesirable post vaccinal reaction in horses vaccinated with the prepared vaccine reconistitued in saponin, DEAE-Dextran (100mg/dose) and adjuvanted with Montanide^{MT}Gel-01. The immunogenicity of vaccinated mares adjuvented with different three types of vaccine was assayed by different serological tests revealed that maximum antibodies titre was achieved at 3-4 months' post vaccination and EHV-1 inactivated vaccine reconstituted in DEAE-Dextran (100 mg/dose) is the best one of them followed by the vaccine reconstituted in saponin and vaccine adjuvanted with Montanide^{MT}Gel-01.

Keywords: Equine herpesvirus, inactivated vaccine, saponin, Montanide

(http://www.bvmj.bu.edu.eg) **(BVMJ-31(2): 8-15, 2016)**

1. INTRODUCTION

Equine herpesvirus-1 (EHV-1) infection is ubiquitous in most horse populations throughout the world causing a disease in horses with extensive economic losses (Slater, 2007). The relevant effects of this virus on the equine population are 3-fold. Firstly, sporadic occurrence of mild respiratory disease associated with pyrexia, principally affecting horses under 2 years of age. Secondly, abortion occurring during the 3rd trimester of pregnancy and perinatal mortalities results in important economic losses in foal production. Thirdly, neurological disease (equine herpes myeloencephalopathy (EHM) cause loss of life and disrupt breeding (Kydd et al., 2006). In Egypt, virus isolation and identification from several outbreaks were recorded in 1999, 2007 and 2009 (Abd El-Hafeiz et al., 2010; Hassanein et al., 2002 ; Nehal et al., 2009). Therefore, the goal of any vaccination program aimed to control the disease to prevent EHV-1 abortion or neurologic syndrome. Stimulation of immune responses can reduce or eliminate cell-associated virus, increase and/or modulate the intrinsic immunogenicity of an antigen and elicit strong and long lasting immune responses occurs by the addition of an adjuvant substance to the prepared vaccine (Tritto et al., 2009). Saponins are complex chemical adjuvants extracted from plants, often the tree Quillaia saponaria. Saponins are immunomodulators and can induce strong type 1 T helper (Th1) and type 2 T helper (Th2) responses as well as cytotoxic T lymphocyte (CTLs) (21). Montanide™Gel-01 was reported to be able to trigger a stronger TH1 response (Seppic). The mechanism of action of DEAE-Dextran is not clear; it may cause a stimulation of the helper T-cell function in antibody synthesis process (Houston et al., 1976).

The present work was designed to prepare tissue culture inactivated EHV-1 vaccine with three different adjuvants aiming to determine the best vaccine formula that induces the highest immune response in vaccinated mares with prolonged duration.

2. MATERIALS AND METHODS

2.1. Virus:

Freeze-dried locally isolated EHV-1 (Nehal et al., 2009; Safaa, 2003), supplied by Equine Vaccine Researches Department, Veterinary Serum and Vaccine Research Institute (VSRI), Abbasia, Cairo and used in antigen and vaccine preparation.

2.2. Animals

Fifteen adult apparently healthy mares, 3-4 years old (3 mares/ group) and SPF (Specific Pathogen Free) pregnant mice (10 mice/ group), were used to evaluate the safety, potency and immunogenicity of the prepared vaccine (OIE, 2015).

2.3. Embryonated chicken eggs:

Specific pathogen free (SPF) embryonated chicken eggs, 11-13 days of incubation were obtained from SPF Koum Osheim Farm, Fayoum. It used for viral re-isolation and detection of infective residual virus activity.

2.4. Cell Culture:

African green monkey kidney cells (VERO) was grown and maintained in Eagl's minimum essential media supplemented with 100 IU/ml. penicillin sodium, and 100mg/ml. It was used for virus propagation and vaccine preparation.

2.5. Antigen

Purified antigen of EHV-1 local isolate propagated on VERO cell cultures (Azmi and Field, 1993), used in serological tests.

2.6. Antisera

Reference freeze dried rabbit anti EHV-1 Serum was kindly supplied by Dr. Jennet Wellington and Local Rabbit anti EHV-1 hyper immune serum (Safaa et al., 2005), was used as positive serum control in serological test.

2.7. Binary Ethyleneimine (BEI):

Binary ethyleneimine 0.008 M (Aldrich chemical Co. LTD) was used as virus inactivator (Mark, 2004).

2.8. Adjuvants

2.8.1. Saponin:

Solution of 0.6 mg/dose of purified saponin was prepared in distilled water with pH 7.5. The solution was autoclaved at 108ºC for 45 minutes then stored at 4ºC. The vials of freeze dried vaccine reconstituted by saponin 2ml/ vial/dose (Nehal et al., 2013).

2.8.2. MontanideTMGel-01:

Montanide^{TM}Gel-01 is a white, opaque and flowable gel with characteristic viscosity, that able to work under all pH conditions. It is available in preserved (PR) or sterile autoclaved (ST) grades (Seppic).

2.8.3. DEAE-Dextran:

A solution of 0.1% Di-ethyl amino-ethyl Dextran MW: 500,000 Pharmacia Fine Chemicals, Sweden, was prepared at 0.25 M Tris-HCl buffer of pH 8.2. This was autoclaved and the pH was adjusted to 7.6-7.8. It was kept at room temperature until used as adjuvant of the vaccine (Nehal, 2006).

2.9. Preparation of locally prepared inactivated EHV-1 vaccine:

2.9.1. Virus identity test:

The freeze dried local isolate of EHV-1 was identified by serum neutralization test using reference freeze dried rabbit anti EHV-1 and EHV-4 Sera (OIE, 2015).

2.9.2. Titration of EHV-1:

Titration of EHV-1 was carried out on Vero cells using the microtiter technique and the titer was expressed in $\log_{10}TCID_{50}$/ml (Reed and Muench, 1938).

2.9.3. Virus Inactivation:

The prepared viral fluid was inactivated by Binary Ethyleneimine [BEI] in two concentrations, 0.008M and 0.01 M separately (Safaa and Hussien, 2012). The control virus and the inactivated one were incubated at 37ºC for 24 hours.

2.9.4. Residual infective virus activity:

Was established through inoculation of: 1) Chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE) 11-13 days incubated at 37ºC for five days with daily examination (Mayr et al., 1978). 2) VERO cells, incubated at 37ºC for seven days with daily observation, blind passage were done.

2.9.5. Preparation of freeze-dried inactivated EHV-1 vaccine:

Addition of equal volumes of Binary Ethyleneimine (BEI)-inactivated virus fluid and freeze drying solution was mixed, followed by freeze drying in Edward's apparatus for 24 hours. EHV-1 inactivated freeze dried vaccine was reconstituted with 2 ml of both Saponin, DEAE-Dextran (was reconstituted in two different concentration (100mg/dose & 200mg/dose)) while

Montanide™Gel-01was mixed v/v was mixed with inactivated virus fluid.

2.10. Evaluation of the prepared tissue culture inactivated EHV-1 vaccines:

2.10.1. Purity test:

Sample from the final prepared vaccines is tested for bacterial, fungal and mycoplasma contamination (OIE, 2015).

2.10.2. Safety test:

Was performed to insure complete virus inactivation in each inactivated vaccine batch of EHV-1 (OIE, 2015)

2.10.3. Safety in mice:

This test was performed on inactivated virus just after inactivation process as well as on the final product of EHV-1 vaccine. Intra-Peritoneal (I/P) and Sub-Cutaneous (S/C) inoculation of mice groups with EHV-1 adjuvanted vaccine with different types of adjuvant respectively.

2.10.4. Safety in horses:

Pregnant mares were inoculated Intra-Muscular (I/M) with two doses with 1 month interval from each vaccines (2 ml/dose/horse), any post vaccinal reaction observed for 2 weeks.

2.11. The potency of the locally prepared tissue culture inactivated EHV-1 vaccine in mice:

Five groups of mice, 4-6 weeks old (10 mice/group) were used for testing the potency of the prepared vaccine. Group 1, 2 & 3 inoculated with the prepared tissue culture EHV-1 inactivated vaccine reconstituted in Saponin (0.6mg/dose), DEAE-Dextran (200 mg/ml) and DEAE-Dextran (100 mg/ml) respectively. Group 4 inoculated with the prepared vaccine adjuvanted with Montanide™Gel-01, While Group 5 kept unvaccinated as control. All mice groups inoculated subcutaneously with two doses of the prepared vaccine (0.2 ml/mouse/dose) with one week interval. Serum samples were collected after 7 days from the second dose from all groups and the antibodies titre against EHV-1were determined by ELISA and AGPT. All mice groups were challenged (7 days post inoculation) intranasal with 0.45 µl/mice of EHV-1 local isolate (10^8 $TCID_{50}$ /ml). Two mice from each group were sacrificed at different time intervals 3rd, 4th, 5th, 6th and 7th days post challenge. 10% of liver and lung suspension of sacrificed mice were inoculated onto CAM of ECE to detect the presence of characteristic EHV-1 pock lesion.

2.12. Immune response of mares vaccinated with the locally prepared tissue culture EHV-1 inactivated vaccine:

Twelve mares (3horse / group) with low antibodies titer (≤4 neutralizing antibodies titer) were I/M inoculated with inactivated vaccine (2 ml / horse/ dose). Group 1 & 2 vaccinated with EHV-1 inactivated freeze dried vaccine reconstituted in Saponin and DEAE-Dextran (100 mg/ ml). Group 3 vaccinated with EHV-1 inactivated vaccine adjuvanted with Montanide™Gel-01, While Group 4, kept un-vaccinated as control. Each vaccinated mare was boostered by the corresponding vaccine four weeks after the first dose. Serum samples were collected from each group every two weeks up to seven months post vaccination. Serum samples collected from all horses were investigated by serological tests (ELISA, SNT and AGPT). The sites of inoculation were observed daily for seven days post vaccination for detection any undesirable post vaccinal or thermal reaction and nasal discharge after each immunization was observed.

2.13. Evaluation of the keeping quality of the prepared inactivated EHV-1 vaccine in mice:

Seven groups of mice inoculated with EHV-1 inactivated vaccine using different adjuvants just after preparation time as well as being kept at different time intervals at various temperature degrees (4ºC, 20ºC & 37ºC) for detecting the effect of storage on the locally prepared inactivated EHV-1 vaccines, the antibodies titer against EHV-1 determined by ELISA and AGPT.

3. RESULTS

The local isolate of EHV-1was identified using reference anti- EHV-1. EHV-1 was titerated on VERO cells and the titer was 10^8 $TCID_{50}$/2ml/dose. EHV-1 was inactivated completely at a final concentration of 0.008 M of BEI within 24 hours and 0.01 M of BEI within 22 hours at 37ºC (table, 1). For residual virus infectivity, there is no pock lesion on CAM, also no CPE was observed by two successive blind passages on inoculated VERO cells. Concerning the purity of prepared vaccine viral fluid or in the final vaccine product, achieved no any bacterial, fungal and mycoplasma contaminations when tested on specific media according to vaccine requirement production. Concerning the vaccine safety, neither abortion nor untoward symptoms of inoculated pregnant mice or mares groups except in groups that inoculated with the vaccine reconstituted in DEAE-Dextran (200mg/dose) detecting an appearance of swelling

and deaths in some mice and a slight inflammation at the site of injection in mares following first and booster injections. The result in table (2), revealing the immunogenicity of tissue culture adapted EHV -1 inactivated vaccine with three different types of adjuvants (Saponin, DEAE-Dextran (100 and 200 mg/dose) and Montanide^{TM}Gel-01) with the mean ELISA antibody titer against EHV-1 of inoculated mice groups were 1034.4, 1050.8, 1146.7 and 935 respectively. Challenge of inoculated mice groups with virulent EHV-1, for detecting the duration of viral re-isolation (table, 3), the antibodies titer increases gradually recording highest level at 5-7 dpc in all groups of inoculated mice. The immunogenicity of mares vaccinated by locally prepared inactivated EHV-1 vaccine reconstituted in Saponin and DEAE-Dextran 100mg/dose, vaccine adjuvanted with Montanide^{MT} Gel-01 tested by different serological tests (ELISA, SNT) (figure 1 & 2) and AGPT. Correlation between ELISA and Neutralizing antibodies, reveling there is an increase in the mean value of ELISA and neutralizing antibodies titer after two weeks from vaccination (352, 404 and 310) (1.2, 1.3 and 1.1) for the three different adjuvants respectively then it is slightly decreased after one-month post vaccination. After boostering the immune response for ELISA and VN antibodies titer will gradually increasing up to 3-4-month post vaccination reaching values (1480, 1637 and 895) (3.3, 3.5 & 3.2) respectively, then decreased gradually till 7th month post vaccination in both tests (123, 238.6 & 139.6) (1, 1 & 1). Also in AGPT, the immune response was detected by the appearance of precipitin line in all samples in-between antigen and sera sample which indicated as positive result in all mares vaccinated with the three different types of vaccine. The keeping quality of the locally prepared vaccine in mice (table 4) illustrated of the locally prepared Tissue culture inactivated EHV-1vaccine reconstituted with saponin, DAEA-Dextran (100mg/dose) can be kept at -20^{o}C for a period of 1.5 Y and 1 Y at 4^{o}C but the vaccine viral antigen was affected at 37^{o}C hence the freeze dried vaccine kept lower than 6 M after preparation. The locally prepared tissue culture inactivated EHV-1 vaccine adjuvanted with Montanide^{MT}Gel-01 in mice (table, 5) was kept for 1.5 Y at 4^{o}C and 2 M at 37^{o}C from the time of preparation with high immunization rate.

Table (1) Inactivation of EHV-1 by Binary Ethyleneimine (BEI).

Hours post incubation	Virus Titer of inactivated EHV-1 by different concentration of BEI Treated virus		Control untreated virus
	0.008 M	0.01 M	
0	8	8	8
8	7.3	5.8	7.8
14	4.8	3	7.6
18	3.2	2.1	7.4
20	1.9	1	7.2
22	1	ND	7
24	ND	ND	6.5

ND: Not detected.

Figure (1) Immune response of mares vaccinated with tissue culture EHV-1 inactivated vaccine reconstituted with saponin, DEAE-Dextran (100mg/dose) and vaccine adjuvanted with Montanide^{MT}Gel-01using ELISA.

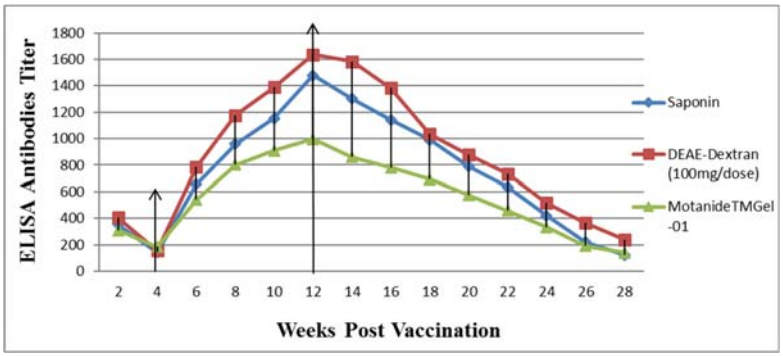

Table (2) I Immune response of mice inoculated with tissue culture EHV-1 inactivated vaccine using different adjuvants.

N0. of mice	Inoculated Mice Groups									
	Group 1		Group 2		Group 3		Group 4		Group 5	
	ELISA	AGPT	ELISA	AGPT	ELISA	AGPT	ELISA	AGPT	ELISA	AGPT
1	956	+	1067	+	1207	+	878	+	200	-
2	1113	+	955	+	1015	+	1011	+	110	-
3	995	+	1012	+	1147	+	959	+	270	-
4	1190	+	1028	+	1088	+	842	+	205	-
5	1022	+	997	+	1234	+	921	+	135	-
6	1053	+	1065	+	1304	+	1000	+	178	-
7	1001	+	1220	+	1003	+	807	+	119	-
8	908	+	1187	+	1251	+	1022	+	247	-
9	1064	+	972	+	1192	+	1009	+	211	-
10	1042	+	1005	+	1026	+	901	+	190	-
Mean	1034.4	+	1050.8	+	1146.7	+	935	+	186.5	-

Group (1): Mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in Saponin solution. Group (2): Mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in DEAE-Dextran solution (100 mg/dose). Group (3): Mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in DEAE-Dextran solution (200 mg/dose). Group (4): Mice inoculated with inactivated EHV-1 adjuvanted with Montanide™Gel-01. Group (5): Control un-inoculated mice. ELISA: Enzyme Linked Immunosorbent Assay. AGPT: Agar Gel Precipitation Test. (-) Negative result in AGPT. (+) Positive precipitin line in AGPT.

Table (3) Virus re-isolation from inoculated mice after EHV-1 challenge on CAM of embryonated chicken eggs (ECEs).

Group of mice	Days post challenge	Virus re-isolation on CAM of ECE*	% of virus re-isolation
Group 1	3rd	4/4	100%
	4th	3/4	75%
	5th	2/4	50%
	6th	2/4	50%
	7th	1/2	25%
Group 2	3rd	3/4	75%
	4th	2/4	50%
	5th	2/4	25%
	6th	1/4	25%
	7th	0/4	0%
Group 3	3rd	2/4	50%
	4th	1/4	25%
	5th	1/4	25%
	6th	0/4	0%
	7th	0/4	0%
Group 4	3rd	3/4	75%
	4th	3/4	75%
	5th	3/4	75%
	6th	2/4	50%
	7th	2/4	50%
Group 5	3rd	4/4	100%
	4th	4/4	100%
	5th	4/4	100%
	6th	3/4	75%
	7th	3/4	75%
	9th	1/4	25%

Group (1): mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in Saponin solution. Group (2): mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in DEAE-Dextran (100 mg/dose) solution. Group (3): mice inoculated with inactivated EHV-1 freeze dried vaccine, reconstituted in DEAE-Dextran (200 mg/dose)

solution. Group (4): mice inoculated with inactivated EHV-1 adjuvanted with Montanide™ Gel-01. Group (5): Control non inoculated mice but they were challenged at the same time of groups (1, 2, 3 & 4). CAMs of ECE: Chorioallantoic membranes of Embryonated chicken eggs. No. of eggs showed pock lesson/ total eggs No.

Figure (2) Immune response of vaccinated mares with tissue culture EHV-1 inactivated vaccine reconstituted with saponin, DEAE-Dextran (100mg/dose) and vaccine adjuvanted with Montanide^{MT}Gel-01 using serum neutralization test (SNT).

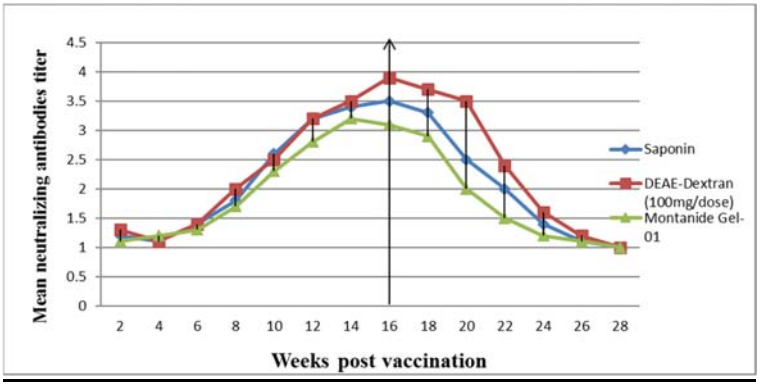

Table (4) Keeping quality of tissue culture EHV-1 inactivated vaccine reconstituted in saponin, DAEA-Dextran (100mg/dose) in mice.

Batch of vaccine	Storage temp.	Adjuvant	Group of mice	Storage time	ELISA	AGPT
Freeze Dried Vaccine	-	Saponin	1	At the time of preparation	1090	+
		DEAE-Dxtran (100 mg/dose)	1		1400	+
	-20° C	Saponin	1	6 M	1060	+
			2	1 Y	999	+
			3	1.5 Y	806	+
		DEAE-Dxtran (100 mg/dose)	1	6 M	1100	+
			2	1 Y	1020	+
			3	1.5 Y	840	+
	4° C	Saponin	1	6 M	980	+
			2	9 M	745	+
			3	1 Y	620	-
		DEAE-Dxtran (100 mg/dose)	1	6 M	1002	+
			2	9 M	901	+
			3	1 Y	658	+
	37° C	Saponin	1	3 M	960	+
			2	6 M	777	+
			3	9 M	510	-
		DEAE-Dxtran (100 mg/dose)	1	3 M	994	+
			2	6 M	801	+
			3	9 M	558	-

4. DISCUSSION

Inactivated vaccines are often ineffective and require co-administration with adjuvants to maximize the vaccine potency specially in valuable and sensitive Arabian horses. Adjuvant must not only enhance the immune response but also produce no undesirable either locally or systemic post vaccinal reaction. Arabian horses are highly sensitive animals constitute national economic value and special powerful adjuvants must be used to maximize the immune response, so this thesis is trails to study the effect of different of adjuvants in EHV-1 vaccine to choose the best effective vaccine for controlling the EHV-1 infection and the reduction of virus shedding during outbreaks.

Table (5) Keeping quality of tissue culture EHV- 1 inactivated vaccine adjuvanted with Montanide^{TM}Gel-01 in mice.

Batche of vaccine	Storage temp.	Adjuvant	Group of mice	Storage time	ELISA	AGPT
EHV-1 vaccine adjuvanted with Montanid^{MT}Gel-01	-	Montanid ^{MT} Gel-01	1	At the time of preparation	934	+
	4ºC		1	6 M	920	+
			2	9 M	875	+
			3	1 Y	798	+
			4	1.5 Y	720	+
			5	2 Y	503	-
	37ºC		1	1 M	790	+
			2	2 M	690	+
			3	3 M	490	-

The local isolate of EHV-1(vaccine seed virus) was identified by serum neutralization test (SNT) using reference anti- EHV-1 and EHV-4 sera (OIE, 2015). The prepared vaccine virus fluid was titrated, the titer was 10^8TCID$_{50}$/2ml/dose which is the recommended titer for preparation of inactivated EHV-1 vaccine (vaccinal dose for inactivated EHV-1 vaccine must not less than10^7TCID$_{50}$/2ml/dose (Mayr et al., 1978). The complete inactivation of EHV-1 is critical requirement in the production steps of inactivated EHV-1 vaccine to ensure the safety of the product. Vaccine virus fluid established completely virus inactivation at a final concentration of 0.008 M of BEI within 24 hours and 0.01 M of BEI within 22 hours at 37ºC (table, 1) (Safaa and Hussien, 2012). To decrease the harmful effect of inactivator, it is recommended to use 0.008M of BEI for virus inactivation.

The result of residual virus infectivity of the inactivated virus fluid revealed the absence of pock lesion on CAM and no CPE was observed on inoculated cell line respectively (OIE, 2015). Safety estimation of locally prepared inactivated EHV-1 vaccine reconstituted in saponin and DEAE-Dextran (100mg/dose), DEAE-Dextran (200 mg/dose) and vaccine adjuvanted with Montanide^{MT}Gel-01 was applied. Pregnant mice and mares inoculated two doses with one and four weeks interval for mice and mares respectively. No abortion or untoward symptoms of inoculated pregnant mice or mares with EHV-1 inactivated vaccine except in mice groups those inoculated with the vaccine reconstituted in DEAE-Dextran (200mg/dose) detecting an appearance of swelling and deaths in some mice and a slight inflammation at the site of injection in mares which disappeared after 72 hours from injection.

Evaluations of immune response of prepared vaccines were applied in both mice and horses. The immune response of inoculated mice with DEAE-Dextran (200 mg/dose) is the best one of them to induce highest immunogenicity against EHV-1 followed by DEAE-Dextran (100mg/dose), Saponin and Montanide^{MT}Gel-01, with the mean ELISA antibodies titer 1146.7, 1050.8, 1034.4 and 935 and positive result in AGPT were correlated with ELISA antibodies titer (table, 2). ELISA titer 300-600 in serum samples of inoculated mice resist challenge with the virulent virus (Kirisawa et al., 1995). After challenge there is an increase in immune response in all groups of inoculated mice from 5 to 7 (days post inoculation) dpc. The duration of viral re-isolation from challenged mice (table, 3), the vaccines has shortening the period of EHV-1 replication inside the vaccinated animals. The viral re-isolation in the inoculated mice groups with the saponin, DEAE-Dextran (100gm/dose), DEAE-Dextran (200gm/dose) and Montanide^{TM}Gel-01 were 50%, 75%, 25% and 75%, respectively at the 5th day post challenge (dpc) whereas 50%, 25%, 0% and 50% was registered at the 6th day post challenge (dpc). The virus re-isolations present till the 9 days post challenge in the control unvaccinated mice (Slater et al., 1993). This indicated that DEAE-Dextran adjuvanted vaccine (200 gm/dose) has the best lowest days for viral clearance achieved at the 6th (dpc).

The immunogenicity of mares vaccinated by locally prepared inactivated EHV-1 vaccine reconstituted in Saponin and DEAE-Dextran 100mg/dose, vaccine adjuvanted with Montanide^{MT}Gel-01 tested by different serological tests (ELISA, SNT) and AGPT (figure, 1 & 2), Booster immunization showed significant increase of ELISA and VN antibodies and development of higher Protective antibody titer achieved at 3-4 months post vaccination. Freeze dried inactivated vaccine could be stored at longer period than Montanide^{MT}Gel-01.

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