Immune Response Against Inactivated Equine Herpes Virus Vaccine Prepared From Local Isolate In Horses And Donkeys In Egypt

Mohamed G. Abdelwahab[1], Hazem M. El Moghazy[2], Elsayed M. Ibrahim[1], Faiysal I. Hamouda[1] and Safaa M.A. Warda[3].

[1] Animal Medicine Department, Faculty of Veterinary Medicine, Benha University, Egypt
[2] Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Benha University, Egypt
[3] Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Abstract: This study was designed to evaluate the immune response of Equine Herpes Virus-1 (EHV-1) inactivated vaccine in horses and donkeys in Egypt. The practical part of this study was undertaken in the Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. Propagation of EHV-1 local isolate was subjected for further passages (Passage 4, 6 and 8) on VERO cells. The infectivity titer of the stock virus was 8 $\log_{10}$ TCID50/ ml. Six horses and six donkeys were used in this study, in addition four Mares and two horses were used for safety test of the prepared vaccine. Three of horses and three donkeys were vaccinated through intramuscular route by 2 doses of the vaccine with one Month interval, as well as, another three horses and three donkeys were kept as control. Serum samples were collected from all vaccinated and control animals every 2 weeks till to the 26th weeks post vaccination (WPV) which were monitored for EHV-1 antibodies using ELISA, AGPT and VNT. None of the vaccinated horses and donkeys showed nasal charge, swelling at the site of injection or increase in body temperature post vaccination. Vaccination of horses and donkeys revealed an increase in the antibodies response against EHV-1 at 2WPV with the mean ELISA antibodies titer 335.3 and 352 in horses and donkeys respectively. After boostering, antibodies titer is continuing to increase until reaching to its peak at the 12 WPV with mean antibodies titer 1269.7 and 1153 in horses and donkeys respectively, then the antibodies titer decreased gradually till to the 26th WPV with a considerable protective antibody level. Also, AGPT showed precipitin line in collected samples having high antibodies. The results also revealed an increase in the virus neutralizing antibody titer at 2WPV with the mean antibodies titer 1 and 1.26 in horses and donkeys respectively. After boostering, antibodies titer is continuing to increase until reaching to its peak at the 12 WPV) with mean antibodies titer 3.1 and 3.16 in horses and donkeys respectively, then the antibodies titer decreasing gradually till to the 26 WPV with a considerable protective antibody level.

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1. Introduction:

Equine herpes virus-1 (EHV-1) is an important equine viral pathogen that exerts its major impact by inducing abortion storms or sporadic abortions in pregnant mares, early neonatal death in foals, respiratory disease in young horses and myeloencephalopathy in adult horses **(van Maanen, 2002; Patel and Heldens, 2005).** The current vaccines can only reduce clinical symptoms and virus excretion in vaccinated animals, which is a desirable effect from an epidemiological point of view. The duration of this immunity can be boosted up to 6 months. Vaccination remains today an important strategy to fight EHV-1 infection, in combination with management measures. Whole inactivated EHV-1 vaccines which provide variable levels of protection against the disease through the induction of antibodies. The main type of vaccine commercially available. Successful vaccination against EHV-1 requires both humoral and cellular immune responses **(Kapoor et. al., 2014).**

The first vaccination attempts started shortly after the virus could be propagated in cultured cells in the late 1950s and early 1960s **(Patel and Heldens, 2005; Rosas et al., 2006).** The low compliance with recommended EHV-1/EHV-4 vaccination regimes, which required a basic scheme of two immunizations in 4–6-week intervals followed by 6-monthly booster vaccinations for most commercial vaccines, it is even more worrisome as vaccines are considered to be at least partly responsible for the reduction of abortions in many horse populations such as the thoroughbred population in Kentucky and Australia **(Allen and Powell, 2003; Foote et al., 2006).** While the levels of neutralizing antibodies seem to be no correlate of protection, the frequency of EHV-1- specific precursor cytotoxic T lymphocytes (CTL) were shown to be higher in horses that were protected from clinical disease compared to those that were not **(Allen et al., 1995; Paillot et al., 2005).** Consistent with such findings, marketed and experimental MLV vaccines

offer at least partial protection against disease development **(Goodman et al., 2006; Rosas et al., 2006).** However, the only vaccine with a claim against EHV-1-induced abortion is an inactivated EHV-1/EHV-4 combination vaccine **(Heldens et al., 2001)** that is to be applied in months 5, 7, and 9 of gestation.

Currently available vaccines do not reliably block infection, the development of viremia or the establishment of latency and EHM has been observed in horses regularly vaccinated against EHV-1 at 3–5 monthly intervals with inactivated or modified-live vaccines (MLV); **(Friday et al., 2000; Henninger et al., 2007).** Thus, vaccination can be expected to reduce nasopharyngeal virus shedding during an outbreak and thereby limit the spread of infection. While vaccination also increases serum antibody titer, this fails to alter the duration of cell-associated viremia or the outcome of pregnancy following challenge infection with EHV-1 **(Kydd, et al., 2003).**

2. Material And Methods:

2.1. Material

2.1.1. Animals:

Six horses and six donkeys were used for evaluation of immune response against equine herpes virus-1 vaccine in addition four mares and two horses for safety test of the prepared vaccine.

2.1.2. Mice:

Healthy adult mice (3-4 weeks old) were used to study the immunogenic potency and safety of the locally prepared EHV-1 inactivated freeze-dried vaccine.

2.1.3. Virus:

Freeze dried local strain of equine herpesvirus-1 (EHV-1), which isolated from cases of aborted foal by **Hassanein et al., (2002); Safaa, (2003)** in Equine Research Department, Veterinary serum and vaccine research Institute, Abbasia, Cairo.

2.1.4. Vaccine:

Locally prepared EHV-1 Alum gel inactivated freeze-dried vaccine was kindly supplied by **Prof. Dr. Safaa A. Mohamed Ali Warda;** Professor and Head of Equine Viral Disease Dept., Veterinary Serum & Vaccine Research Institute, Abbasia, Cairo.

2.1.5. Antigen:

A partially purified antigen of EHV-1 local isolate propagated on Vero cell line was prepared according to the method described by **Dutta et al., (1983) and Azmi and Field (1993)** used in serological test.

2.1.6. Antisera:

A. Freeze-dried rabbit anti- EHV-1 serum produced kindly by Dr. Jannet Wellington, Research Fellow, Department of Biological Science, Macquarie Univ, NSW and Australia. It was used in virus identity test (serum neutralization test, SNT).

B. Horse anti-EHV-1 hyperimmune sera, prepared in Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, used as positive control in serological test.

2.1.7. Sterility media:

Nutrient agar medium (bacteriologically-aerobic), thioglycolate medium (Oxford, England) (bacteriologically-anaerobic), sabouraud's dextrose agar medium (fungal) and PPLO (broth) medium (mycoplasma) were used to determine the purity of the vaccine.

2.1.8. Aluminum hydroxide gel:

Aluminum hydroxide with low viscosity (Rehydragel LV) was made in Denmark by Superfos Biosector a/s. The following table provides the analytical description of the product:

Dry matter as AL2O3		2.0%
Equivalent to AL (OH)3		3.0%
Nitrogen	max	0.005%
Sulphate (free)	max	0.05%
Sulphate (total)	max	0.1%
PH		6.5 ± 0.5

Alum hydroxide was used as an adjuvant and stored at 4 – 20°c

2.1.9. Embryonated chicken eggs (ECE)

Specific pathogen free (SPF) embryonated chicken eggs, 11-13 days of incubation old were obtained from SPF Koum Osheim Farm, Fayoum. They were used for viral re-isolation from challenged mice and detection of infective residual virus activity of the prepared inactivated viral fluids by inoculation onto the chorioallantoic membrane (CAM).

2.1.10. Reagent and solution for serological test:

2.1.10.1. Reagent for agar gel precipitation test (AGPT):

1.5 % of Noble agar (BDH Chemical LTD, Poole, England) was prepared in normal physiological saline pH 7.2 containing sodium merthiolate at a final concentration of 1: 10,000.

2.1.10.2. Reagent and solutions used in solid phase ELISA;

2.1.10.2.1. Phosphate buffered saline (PBS):

Sodium chloride	8.0 gm
Potassium chloride	0.2 gm
Potassium dihydrogen phosphate	0.2 gm
Di-sodium hydrogen phosphate 2 H2O	3.1 gm
DDw to	1000 ml

2.1.10.2.2. Coating buffer:

Na_2CO3	1.86 gm
NaHCO3	2.93 gm
DDW	1000 ml

2.1.10.2.3. Washing buffer:

PBS + 0.05 % Tween 20

2.1.10.2.4. Blocking buffer:

PBS + 2.3 % bovine serum albumin

2.1.10.2.5. Diluting buffer:

PBS + 0.05 % Tween 20 + 0.1 % bovine serum albumin

2.1.10.2.6. Substrate solution:

Citric acid	7.74 gm
Sodium dihydrogen phosphate	17.3 gm
DDW	1000 ml
pH	5.5-6.0

2.1.10.2.7. Substrate working solution:

This was freshly made by addition of ortho-phenelene diamine-2 HC1 (OPD), Sigma in a concentration of 1 gm per 1 ml of substrate stock solution and 0.8 ul of H_2O_2 (30%) per 1 ml buffer added just before use and kept in a dark brown bottle.

2.1.10.2.8. Anti-horse IgG:

Anti-horse IgG whole molecule conjugated with peroxidase produced by binding site Birmingham Research Park, UK.

2.1.9.2.9. Anti-mice IgG:

Anti-mice IgG whole molecule conjugate with peroxidase produced by binding site Birmingham Research Park, UK.

2.1.10.2.10. Stopping solution: ' •

2 N sulphuric acid solution prepared by mixing 1 ml concentrated sulphuric acid with 6.5 ml water to be used immediately.

2.2. Methods:

2.2.1. Collection of Blood Samples from horses and donkeys:

Volumes of 6 to 8 mL blood samples were collected from each of the Egyptian horses and donkeys, using external jugular venipuncture method.

2.2.2. Preparation of blood serum samples:

The serum samples were prepared according to the standard procedures after blood clotting at ambient environment and overnight incubation at 18 - 26°C controlled room temperature. Serum samples were separated to stock tubes and kept in a freezer (-20 °C) until use.

2.2.3. Evaluation of the prepared Alum gel inactivated EHV-1 vaccine:

2.2.3.1. Residual infective virus activity test:

I- In Embryonated Chicken Egg (ECE) according to (Safaa and Hussien, 2012).

This test was performed on the inactivated virus suspension just after inactivation process as 0.3 ml of undiluted inactivated virus was inoculated on the CAM of ECE 11-13 day old (group of five eggs) and incubated at 37°C for 5-6 days. The CAMs of inoculated ECE were collected and examined for the presence of pock lesion. If there is no pock lesion appeared, 10% suspension from this negative CAMs blind was made and re-inoculated on another group of ECE (5 eggs).

II- On cell culture (VERO cell line): that inoculated for 7 days at 37c with daily observation for the presence of cytopathic effect (CPE). Blind passage was done to ensure complete virus inactivation according to **(OIE, 2015).**

2.2.3.2. Safety test:

To assure safety of each inactivated vaccine batch of EHV-1 must demonstrate complete inactivation of virus as well as “safety field trial: in horses and safety test in laboratory animals according to **(OIE, 2015).**

2.2.3.2.1. In mice: (Awan et al., 1990)

This test was performed on the inactivated virus suspension just after inactivation process as well as on the final Alum gel inactivated EHV-1 vaccine in two groups of mice 3-4 weeks old. The first group of mice (10 mice) inoculated with 0.5 ml inactivated virus fluid I/P and kept under observation for 15 days. Five mice were killed after 5 days and another after 10 days. Liver and lung tissues were collected from killed mice.

The 2nd group of mice (10 mice) were inoculated S/C with the final inactivated EHV-1 vaccine. Each mouse inoculated with two doses (0.2 ml/dose) 5 mice were killed after 5 days from the first dose and another 5 mice were killed after 5 days from the 2nd dose. Also, liver and lung tissues were collected from all killed mice.

10% tissues suspension from all liver and lung samples collected from the two groups of mice were inoculated on CAMs of ECE 11-13 days old and incubated at 37°C for 5 days. The CAMs of inoculated ECE were collected and examined for the presence of pock lesion.

2.2.3.2.2. Safety test in horses: (OIE, 2015)

Four mares included in this study in addition to another two horses present in stables of the Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo were inoculated with the locally prepared inactivated EHV-1 freeze dried vaccine, reconstituted in DEAE-Dextran (100 mg/ml). Each horse received two doses I/M (3ml/dose/horse) with interval 1 month. All horses were kept under observation and body temperatures were recorded.

2.2.3.3. Potency of the locally prepared EHV-1 inactivated vaccine in mice:

Mice were used as murine models of EHV-1 according to **(Awan et al., 1995)** and **Walker et al. (1998).**

Two groups of mice, 3-4 weeks old (10 mice/group); Group (A) inoculated by the prepared EHV-1 inactivated freeze-dried vaccine, reconstituted in DEAE-Dextran (l00mg/ml), each mouse take two doses S/C (0.2 ml/dose) with one-week interval. Group (B) was kept as control.

Serum samples were taken from the two mice groups after 10 days from the second dose. The obtained serum samples were inactivated at 56°C for

30 minutes then screened for EHV-1 antibodies by ELISA and AGPT.

The two mouse groups were challenged I/P by EHV-1 egg adopted with titer of 9 log10 EID50/ml (0.5 ml /mice). Three mice from each group were killed at 6 and 9 days post challenge.

Liver and lung were collected from killed mice and ground in sterile sand to prepared 10% tissue emulsion and inoculated intra-yolk in ECE7 days old, to detect the presence of EHV-1 in these organs.

2.2.4. Immunization of horses and donkeys:

Horses and donkeys were inoculated I/M with the prepared vaccine (EHV-1 inactivated freeze-dried vaccine) (2ml/dose) as an initial dose, followed by a booster dose at 4 weeks. Immune response of vaccinated animals was traced by serum samples collected from horses and donkeys at 2weeks intervals to monitor the immunization curve using ELISA, AGPT and VNT.

2.2.5. Clinical studies:

Horses and donkeys were thoroughly monitored for presence of post vaccinal reactions throughout the entire experimental period as nasal discharge, cough, changes in submandibular lymph nodes, local inflammation responses at the site of injection (swelling, hotness, pain); systemic manifestations as fever, lethargy, anorexia and hypersensitivity reactions as urticaria, anaphylaxis. Pulse rate, respiratory rate and rectal temperature were monitored.

2.2.6. Serological techniques:

2.2.6.2. Agar gel precipitation test (AGPT): according to (Tewari and Prasad, 1968)

The agar medium (section 2.1.9.1.) was poured into Petri dishes. Sets of wells were punched in each dish. Each set consisted of one central well containing partially purified EHV-1 virus antigen (section 2.1.4.).

Six peripheral wells filled with the tested sera. The dishes were incubated at 37°C in a humid chamber. The results were read after 24-48 hours where clear sharp precipitating line could be seen between the central well containing antigen and peripheral wells containing tested sera control positive and negative sera were also included in the test which gives negative results.

2.2.6.3. Enzyme linked immunosorbent assay (Solid phase ELISA): according to (Sugahara et al., 1997).

The cut -off point of optical density was 0.1

2.2.6.4. Virus neutralization test (VNT): according to Reed and Muench (1938).

3. Results:

3.1. Titration of the stock seed virus:

Propagation of EHV-1 local isolate was subjected for further passages (P4, 6 and 8) on VERO cells. The infectivity titer of the stock virus was 8 $\log_{10}$ $TCID_{50}$/ ml according to **Reed and Muench (1938)** as shown in table (1). The recommended titer for preparation of EHV-1 vaccine must be not less than 10^7 $TCID_{50}$/dose.

Table (1): Titration of the stock seed virus fluid on tissue culture

Passage number	**Virus titer Expressed as $\log_{10}$ $TCID_{50}$/ml**
T.C P4	6.5
T.C P6	7.0
T.C P8	8.0

T.C P: Tissue culture passage.

3.2. Evaluation of the locally prepared tissue culture inactivated EHV-1 vaccine:

Tissue culture Alum gel inactivated EHV-1 vaccine was prepared according to **(Nehal, 2006 and OIE, 2015)**. Steps for evaluation were as follows:

3.2.1. Purity test:

The vaccine viral fluid as well as the final vaccine products were tested on special medium for bacterial, fungal and mycoplasma (Nutrient agar, Sabouraud's dextrose, thioglycolate, and PPLO) and incubated at 37°C for 10 days at least. The results proved the negative growth of any bacterial, fungal and Mycoplasma contaminants of the prepared vaccine.

3.2.2. Identity test

The freeze dried local isolate of EHV-1 propagated in tissue culture as well as vaccine stock virus was identified by serum neutralization test using reference rabbit anti EHV-1 and EHV-4 Sera. Complete neutralization (100%) against reference anti-EHV-1 was achieved.

3.2.3. Safety test:

3.2.3.1. Safety test in mice:

I/P and S/C inoculation of pregnant mice with inactivated virus fluid or the final vaccine product showed neither abortion nor untoward symptoms as loss of weight, nervous signs, death, hypersensitivity and respiratory distress in mice. 10% of liver and lung tissues suspension collected from scarified mice of were inoculated onto CAMs of ECEs. There is no pock lesion appeared on collected CAM inoculated, that proved complete virus inactivation and the prepared vaccine is safe to use.

3.2.3.2. Safety test in horses:

Vaccinated horses with first and booster doses of Alum gel EHV-1 inactivated vaccine, there were no increases in body temperature and post vaccinal abnormalities (shock, toxicity, and hypersensitivity systemic or local reaction at the site of injection).

3.3. Residual infective virus activity:

Inoculation of embryonated chicken eggs (ECE) onto chorioallantoic membrane (CAMs) with 0.3 ml of

undiluted inactivated virus fluid, the result showed there was no residual virulent virus which achieved by the absence of pock lesion on inoculated ECE by the two blind passages onto CAMs. Also, there is no CPE was observed in two successive blind passages on inoculated VERO cells (1 ml/rowae) of undiluted inactivated virus after 7 days of incubation at 37°C.

3.4. The potency of tissue culture (T.C) Alum gel inactivated EHV-1 vaccine in mice:

Two groups of mice (7mice/group), 4-6 weeks old were used for evaluation of the potency of tissue culture (T.C) Alum gel inactivated EHV-1 vaccine. The first group was inoculated subcutaneously with 0.2 ml of Alum gel EHV-1 inactivated vaccine. After one week, it was inoculated with the booster dose of the same vaccine and the second group was unvaccinated used as a control. The result in **table (2)** showed increase in the ELISA antibodies titer against EHV-1 in inoculated mice group with mean antibodies titer 599.8 while EHV-1antibodies wasn't registered in control group.

Also, in AGPT, precipitin line was observed in-between the central virus antigen and peripheral collected sera sample of inoculated mice group.

Table (2): Immune response of mice inoculated with Alum gel inactivated EHV-1 vaccine by ELISA and AGPT.

Mice No.	Inoculated mice		Control	
	ELISA	AGPT	ELISA	AGPT
1	600	-	-	-
2	590	+	-	-
3	612	+	-	-
4	580	+	-	-
5	605	-	-	-
6	613	+	-	-
7	599	+	-	-
Mean	599.8	+	-	-

(-): Negative result. (+): Positive precipitin line.

3.5. Results of clinical observation:

None of the vaccinated horse showed nasal charge, swelling at the site of injection or increase in body temperature post vaccination. It is clear from **table (3)** and **figures (1), (2)** and **(3)** that all animals was in the normal range of temperature, pulse and respiration pre-vaccination and up to 7days post-vaccination without any respiratory symptoms.

Table (3): The mean values of temperature, pulse and respiration of horses and donkeys pre and post vaccination.

Animal No.	**Temperature** (°C)		**Pulse** (/min)		**Respiration** (/min)	
	Pre vaccination	Post vaccination	Pre vaccination	Post vaccination	Pre vaccination	Post vaccination
Horse 1	37.8°c	37.7°c	37	35	14	14
Horse 2	38.3°c	38.6°c	35	36	11	13
Horse 3	38.2°c	38.5°c	40	39	13	12
Mean	**38.1°c**	**38.3°c**	**37.3**	**36.7**	**12.7**	**13**
Donkey 1	37.7°c	37.7°c	34	36	10	11
Donkey 2	38.1°c	38.1°c	37	36	11	11
Donkey 3	37.9°c	37.8°c	33	38	10	12
Mean	**37.9°c**	**37.8°c**	**34.7**	**36.7**	**10.3**	**11.3**

D: donkey H: horse

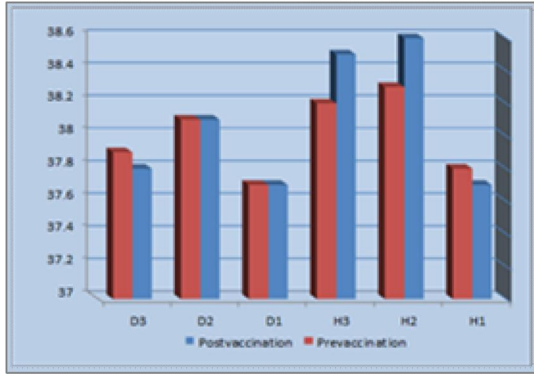

Fig. (1): The mean values of temperature of horses and donkeys pre and post vaccination.

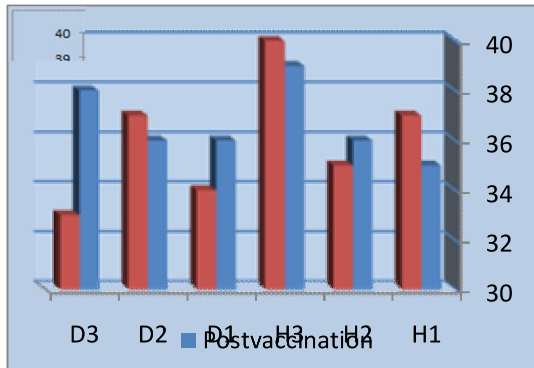

Fig. (2): The mean values of pulse of horses and donkeys pre and post vaccination.

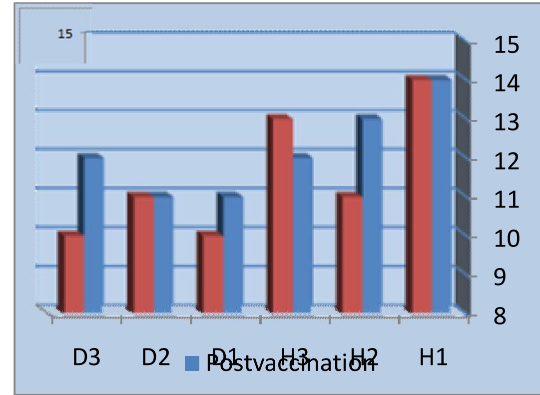

Fig. (3): The mean values of respiration of horses and donkeys pre and post vaccination.

3.6. The immune response of horses vaccinated with Alum gel inactivated EHV-1 vaccine:

Vaccination of horses and donkeys as shown in **tables (4 & 5)** and **fig. (4)** revealed, an increase in the antibodies response against EHV-1 at two weeks post vaccination (2WPV) with the mean ELISA antibodies titer 335.3 and 352 in horses and donkeys respectively. After boostering, antibodies titer is continuing to increase until reaching to its peak at the 12th weeks post vaccination (12WPV) with mean antibodies titer 1269.7 and 1153 in horses and donkeys respectively, then the antibodies titer decreased gradually till to the 26th WPV with a considerable protective antibody level. Also AGPT showed precipitin line in collected samples having high antibodies.

Table (4) Immune response of horses vaccinated with Alum gel inactivated EHV-1 vaccine using ELISA and AGPT.

time of sampling / weeks	EHV-1 antibodies titer of vaccinated horses							Control	
	Horse 1		**Horse 2**		**Horse 3**		**Mean ELISA**		
	ELISA	AGPT	ELISA	AGPT	ELISA	AGPT		ELISA	AGPT
Prevacc.	60	-	70	-	40	-	56.7	20	-
Vaccination									
2 WPV	380	+	410	+	266	-	335.3	40	-
4	205	-	280	-	180	-	221.7	50	-
Boostering									
6	695	+	740	+	532	+	622.3	60	-
8	961	+	ND	+	899	+	930	50	-
10	1021	+	1378	+	1056	+	1151.7	44	-
12	1238	+	1293	+	1278	+	1269.7	84	-
14	1200	+	1390	+	1084	+	1224.7	58	-
16	1150	+	1200	+	998	+	1116	77	-
18	989	+	ND	+	944	+	966.5	80	-
20	ND	+	860	+	710	+	785	70	-
22	660	+	702	+	ND	+	681	55	-
24	491	+	493	+	278	+	420.7	64	-
26	264	+	213	+	186	+	221	68	-

WPV: Weeks post vaccination.
(-): Negative result. (+): Positive precipitin line.

Table (5) Immune response of donkeys vaccinated with tissue culture inactivated EHV-1 vaccine using ELISA and AGPT.

Time of sampling / weeks	EHV-1 antibodies titer of vaccinated donkeys							Control	
	Donkey 1		**Donkey 2**		**Donkey 3**		**Mean ELISA**		
	ELISA	AGPT	ELISA	AGPT	ELISA	AGPT		ELISA	AGPT
Prevacc.	30	-	30	-	20	-	25.7	20	-
Vaccination									
2 WPV	380	+	410	+	266	-	352	30	-
4	205	-	280	-	180	-	221.7	33	-
Boostering									
6	695	+	740	+	532	+	655.7	40	-
8	951	+	1000	+	999	+	983.3	37	-
10	1021	+	1278	+	1056	+	1118.3	25	-
12	1238	+	1193	+	1028	+	1153	45	-
14	1200	+	1190	+	1047	+	1145.7	25	-
16	1150	+	1100	+	ND	+	1125	38	-
18	ND	+	1140	+	944	+	1042	40	-
20	801	+	860	+	ND	+	830.5	28	-
22	660	+	605	+	544	+	603	35	-
24	491	+	ND	+	428	+	459.5	35	-
26	264	+	213	+	186	+	221	42	-

WPV: Weeks post vaccination; (-): Negative result. (+): Positive precipitin line.

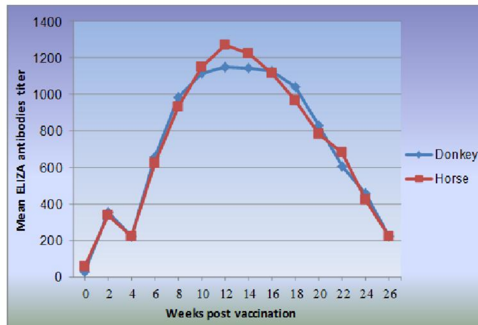

Fig. (4): Immune response of horses and donkeys vaccinated with Alum gel inactivated EHV-1 vaccine using ELISA

The results in **table (6)** & **fig. (5)** revealed an increase in the neutralizing antibody titer at two weeks post vaccination (2WPV) with the mean antibodies titer 1 and 1.26 in horses and donkeys respectively. After boostering, antibodies titer is continuing to increase until reaching to its peak at the 12th weeks post vaccination (12WPV) with mean antibodies titer 3.1 and 3.16 in horses and donkeys respectively, then the antibodies titer decreasing gradually till to the 26th WPV with a considerable protective antibody level.

Table (6): Immune response of vaccinated horses and donkeys with tissue culture Alum gel inactivated EHV-1 vaccine using virus neutralization test (VNT).

time of sampling / weeks	Vaccinated animals								Control
	Horse				**Donkey**				
	H1	**H2**	**H3**	**Mean**	**D1**	**D2**	**D3**	**Mean**	
Prevacc.	-	-	-	-	-	-		-	-
Vaccination									
2 WPV	0.9	1.1	1.0	1.0	1.0	1.5	1.3	1.26	-
4	0.8	0.9	0.8	0.83	0.9	1.0	1.0	0.97	-
			Boostering						
6	1.5	1.7	1.7	1.6	1.5	1.7	1.5	1.6	-
8	2.5	2.1	2.2	2.27	2.5	2.4	2.2	2.4	-
10	2.5	2.3	2.5	2.4	2.5	2.8	2.3	2.5	-
12	2.75	3.5	3.0	3.1	3.0	3.5	3.0	3.16	-
14	2.5	3.0	3.0	2.8	3.0	3.4	2.9	3.1	-
16	2.3	2.5	2.4	2.4	2.9	3.0	2.8	2.9	-
18	1.9	2.0	2.0	1.97	2.4	2.0	2.5	2.3	-
20	1.5	2.0	2.0	1.8	2.0	1.9	2.0	1.96	-
22	1.5	1.8	1.8	1.7	1.5	1.5	1.4	1.46	-
24	1.0	1.5	1.6	1.37	1.3	1.2	1.1	1.2	-
26	0.9	1.2	1.3	1.1	1.0	1.0	0.9	0.96	-

WPV: Weeks post vaccination. (-): Negative result.
H: Horse D: Donkey

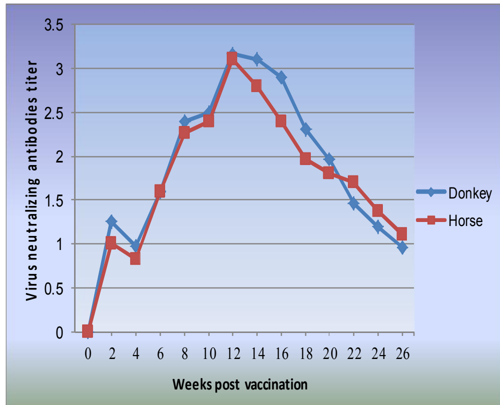

Fig. (5): Immune response of vaccinated horses and donkeys with tissue culture Alum gel inactivated EHV-1 vaccine using virus neutralization test (VNT).

4. Discussion:

Vaccination remains today one of the best options to fight EHV-1 infection in combination with management measures. Whole inactivated EHV-1 vaccines, which provide variable levels of protection against the disease through the induction of antibodies, have been the main type of vaccine commercially available.

There are three important factors determining response to inactivated vaccines: antigen content of the vaccine; adjuvant; and vaccination regime **(Mark, et al., 2006).** Propagation of EHV-1 local isolate was subjected for further passages (Passage 4, 6 and 8) on VERO cells. The infectivity titer of the stock virus was 10^8 $TCID_{50}$ /2ml/dose according to **Reed and Muench (1938)** as shown in Table (8). The recommended titer for preparation of EHV-1 vaccine must be not less than 10^7 $TCID_{50}$/dose **(Mayr et al., 1978)**. The freeze dried local isolate of EHV-1 propagated in tissue culture as well as vaccine stock virus was identified by serum neutralization test using reference rabbit anti EHV-1 and EHV-4 Sera. Complete neutralization (100%) against reference anti-EHV-1 was achieved **(OIE, 2015).**

In concerning to the sterility test, it proved the negative growth of any bacterial, fungal and Mycoplasma contaminants in the vaccine viral fluid or in the final prepared vaccine product when tested on bacterial and fungal media **(OIE, 2015).** Determination of the residual live virus by inoculation of embryonated chicken eggs (ECE) onto chorioallantoic membrane (CAMs) and on VERO cell line with undiluted inactivated virus fluid revealed that, there was no living infected virus in both of them which achieved by the absence of pock lesion by the two blind passages onto CAMs. Also, there is no CPE was observed in two successive blind passages on inoculated VERO cells **(OIE, 2015).**

Regarding the safety of the final vaccine product, I/P and S/C inoculation of pregnant mice with inactivated virus fluid or the final vaccine product showed neither abortion nor untoward symptoms as loss of weight, nervous signs, death, hypersensitivity and respiratory distress in mice. Also 10% of liver and lung tissues suspension collected from scarified mice was inoculated onto CAMs of ECEs revealed that, there is no pock lesion appeared that proved complete virus inactivation and the prepared vaccine is safe to use. Also, the prepared vaccine was safe in horse and there were no increases in body temperature and post vaccinal abnormalities (shock, toxicity, and hypersensitivity systemic reaction or locally at the site of injection). Safety test must be applied to each EHV-1 vaccine batch as recommended by **(OIE, 2015).**

Regarding the results of clinical observation, none of the vaccinated horse showed nasal charge, swelling at the site of injection or increase in body temperature post vaccination. It is clear from **table (10) & figures (11), (12) and (13)** that all animals was in the normal range of temperature, pulse and respiration pre-vaccination and up to 7days post-vaccination without any respiratory symptoms. This came in accordance with **Gundasheva and Tsachev (2015)** reported in his experiment that, booster vaccination did not cause alterations at injection site except for one animal presented with slight and rapidly resolving oedema and observed also acceleration of pulse rate (within the reference range) and lack of statistically significant changes in respiratory rate and rectal temperature. Also **Singh et al., (2006)** have observed no swelling at the site of intramuscular injection against EHV-1.

The ELISA test is useful in disease surveillance, confirmation of clinical cases, monitoring of population or individual animal freedom from an infection prior to movement and monitoring of immune status in individual animals or populations post vaccination **(OIE, 2015).** In concerning to the ELISA results as dedicated in **Tables (11 & 12)** and illustrated in **(Fig.14)** that there is an increase in the antibody response at two weeks post vaccination (2WPV) with the mean ELISA antibodies titer 335.3 and 352 in horses and donkeys respectively. After boostering, antibodies titer is continuing to increase until reaching to its peak at the 12th weeks post vaccination (12WPV) with mean antibodies titer 1269.7 and 1153 in horses and donkeys respectively, then the antibodies titer decreasing gradually till to the 26th WPV with a considerable protective antibody level. This result came in similarity with results of **(Nehal, 2015)** who reported that, the first dose of the prepared vaccine was able to stimulate reasonable antibody response detected at 2wpi with a mean

ELISA titer 450 and reached its peak at the 4wpi with a mean ELISA titer 923.3. By booster and 2nd dose a much higher level of antibodies was developed, reached their maximum at the 1.5 and 2 months post each dose with a mean value 1335 and 2370 respectively. Then the ELISA antibodies titer began to decline gradually till 6th month post vaccination, with a considerable protective antibody level. Also these results agree with **Bannai et al., (2014) and Rusli et al., (2014)** who found that ELISA antibodies begin to increase by day 14 post injection then reached its peak at 2 months with 4 fold increasing which indicate good immune response.

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