Study on Inactivated Equine herpesvirus-1 Vaccine Adjuvanted with Montanide Pet Gel A

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ABSTRACT

The study reported here is a part of a long-term research effort designed in our lab to select the best adjuvant, which can be used with Equine herpesvirus-1 (EHV-1) inactivated vaccine to induce good immune response in horses without any undesirable post vaccinal reaction. This study was carried out on Montanide pet gel A, which is an innovative adjuvant developed for companion animals. To evaluate the safety and potency of EHV-1 inactivated vaccine adjuvanted with Montanide pet gel A, It was inoculated into Guinea pigs as a preliminary evaluation, given mean complement fixing antibody titers of 67 and 176 at 21and 35 days post inoculation (dpi) respectively, and mean ELISA antibody titers of 680 and 1322.5 were recorded at 21and 35 dpi respectively, There was no any undesirable post vaccinal reaction in horses inoculated with the prepared vaccine. The immune response of the inoculated horses was monitored up to eight months post inoculation (mpi), Found that the peak of complement fixing and ELISA antibody mean titer was 213.3 and 1335 at 2.5 and 3 mpi respectively. ELISA antibodies persisted up to 6 mp. In conclusion, the prepared EHV-1 inactivated vaccine adjuvanted with montanide pet gel A is highly safe and potent for Horses.

Keywords: montanide pet gel, Equine herpesvirus-1, inactivated Vaccine

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1- INTRODUCTION

Equine Herpes virus-1 (EHV-1) is one of the most common respiratory pathogens of the horses. EHV-1 induces several clinical signs of disease ranging in severity, from mild respiratory distress to abortion in pregnant mares, neonatal foal death and neuropathogenic disorders (Campbell and Studdert, 1983). 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. Successful vaccination against EHV-1 requires both humoral and cellular immune responses. Immunization with proteins (antigens) requires the presence of strong adjuvant to stimulate both immune responses (Paillot et al., 2008). Over the past 10 years many studies have attempted in Equine Vaccine Research Department at Veterinary Serum and Vaccine Research Institute (VSVRI) to characterize the safety and efficacy of different adjuvants used with EHV-1 inactivated vaccine e.g. DEAE-Dextran, Montanide ISA-70, ISA- 206, Mineral oil and Saponin (Nehal 2006, Safaa and Hussien, 2012, and Nehal et al., 2013). All adjuvants used gave acceptable protective humeral and cellular immune response, but most of them produce post vaccinal reaction at site of injection ranged from miled oedema to small or large sterile abscess. This reaction can be attributed to high sensitivity of horses, which need special adjuvant. Therefore, this study designed to evaluate the safety and potency of EHV-1

inactivated vaccine adjuvanted with Montanide pet gel A, which an innovative adjuvant developed for companion animals (Dog, Cat and horses).

2- Material and Methods

2.1. Virus

Freeze-dried locally isolated EHV-1 at its VERO cell passage Two (VEp_2) (Nehal et. al., 2009), was supplied by Equine vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSRI) and used for antigen and vaccine preparation.

2.2. Antisera

A) Reference freeze dried rabbit anti EHV-1 Serum was kindly supplied by Dr. Jennet Wellington, Research follow, Dept. of biological science, Maquairia, Univ, NSW Australia and used for virus identity . B) Local Rabbit anti EHV-1 hyper immune serum prepared by Safaa et.al, (2005) was used as positive serum control in serological test.

2.3. 2.3- Animals

2.3.1. Horses

Group of Five adult apparently healthy horses with low antibody titer against EHV-1, OIE.2012. Three of them were used to evaluate the immunogenicity, potency and safety of the vaccine. The other two horses were kept as control.

2.3.2. Guinea pig

Group of 15 guinea pigs weighting approximately 350- 450 g were used to evaluate immunogenicity of the prepared vaccine (Guo et al., 1989).

2.3.3. Mice

Two groups of pregnancy mice (10 / group), were used in safety test (OIE. 2012).

2.4.Cell Culture

African green monkey kidney cells (VERO) was maintained and grow in Eagl's minimum essential media supplemented with 10% newly born calf serum for growth media only, and penicillin sodium 100IU/ml. with streptomycin 100mg/ml. It was used for virus propagation.

2.5.Binary Ethyleneimine

0.1M Binary Ethyleneimine stock solution prepared from 2-bromoethylamine hydrobromide (Aldrich chemical Co., LTD) and 0.2N NaOH, according to Bahnemann 1990 and Mark 2004, was used for EHV-1 inactivation process.

2.6.Montanide pet gel A

It is an innovative, ready-to-disperse polymeric adjuvant designed to improve the safety and efficacy of vaccines for companion animals. It is a sterile adjuvant manufactured by Seppic Company. Used in ratio of 10%.

2.7.Preparation of EHV-1 vaccine

A master seed EHV-1(local isolate VEp_2) was grown on VERO cells for another three passage to prepare vaccine seed virus (VEp_5), which subjected to identity test. From the vaccine seed virus, a vaccine stock viral fluid was prepared by caring another one passage on VERO cell and the bulk virus harvest fluids was kept at -70 tell the titration and sterility test was done. Vaccine stock viral fluid having virus titer not less than 7 $\log_{10}$ $TCID_{50}$/ml (Mayer et al. , 1978) was inactivated by 0.008M BEI at 37˚C for 24 hours (Nehal, 2006). Finally the montanide pet gel A (adjuvant) was add in ratio of 10%, into the inactivated virus fluid while mixing on a magnetic stirrer in a low speed at room temperature tell ensure perfect homogeneity of the complete batch, according to manufacture direction, Seppic Company. The prepared EHV-1vaccine distributed in vials contained one dose (2ml /dose), caped and kept at 4˚C.

2.8.Identity test

Was done by SNT according to OIE, (2012) using reference anti sera against EHV-1.

2.9.Sterility Test

Samples were taken from the final product as well as the virus fluid before inactivation

process and tested on Nutrient agar medium, Sabouraud dextrose agar medium, Thioglycolate medium (Oxford, England) and PPLO (broth) medium for bacterial fungal and mycoplasma contaminations (OIE. 2012).

2.10. Safety Test

It was performed according to OIE. (2012). The residual infective virus activity was examined by inoculation of inactivated virus fluid on VERO cells which was incubated at 37ºC for seven days with daily observation. Additionally blind passage was done to ensure complete virus inactivation. Group of mice at late stage of pregnancy were inoculated intranasal (I/N) by 45 µl of inactivated virus fluid. Another group of mice at lat stage of pregnancy were inoculated subcutaneous (S/C) by two dose from the final product with one week interval (0.2 ml/dose).
All experimental mice were kept under observation until parturition.

2.11. Immunization of guinea pig

In order to determine the immunogenicity of the prepared vaccine, a Group of Ten guinea pigs received (2 ml/dose) subcutaneously (S/C) from the prepared vaccine, followed by a booster dose on the day 21. Another Five guinea pigs were kept as control. The guinea pigs were bled before the primary inoculation and on days 21 and 35 (Guo et. al., 1989). Sera were prepared and tested for the presence of EHV-1 antibodies by CFT and ELISA.

2.12. Immunization of horse

Three horses were inoculated I/M with the prepared vaccine (2ml/dose) as an initial dose, followed by a booster dose at 6 weak and 2nd dose at 6 month. Body temperature of the immunized horses was measured and the sites of inoculation were observed daily for seven days post each inoculation for detection of any undesirable post vaccinal reaction. Serum samples were collected from all horses at intervals to monitor the immunization curve.

2.13. Serological tests

2.13.1. Enzyme linked immunosorbent assay (Solid phase ELISA):

Indirect ELISA (Single-dilution) was carried according to Crabb and Studdert, (1993) and Sugiura et al., (1997). It was done to monitoring the immune response of inoculated Guinea pigs and horses; the end titer of the tested sample was calculated according to Williams (1987) as follow:
OD of tested sample – OD of negative control
X titer of positive control
OD of positive control – OD of negative control

2.13.2. Complement Fixation test (CFT):

The CFT using polyethylene glycol (PEG) concentrated EHV-1 antigen was performed (Singh, 2002).on sera collected from inoculated guinea pigs and horses. CF antibodies titer was expressed as the reciprocal of the highest serum dilution reducing the RBCs haemolysis to 50% or less.

3- RESULTS

3.1. Identity test

Vaccine seed virus (VEp_5) was completely neutralizing (100%) by reference anti sera against EHV-1.

3.2. Virus titration

The infectivity titer of the vaccine viral fluid (EHV-1 VEp_6) was 7 $\log_{10}$ $TCID_{50}$ /ml (7.3 $\log_{10}$ $TCID_{50}$ /2ml/dose).

3.3. Sterility Test

The Vaccine viral fluid as well as the final vaccine products proved to be sterile without any bacterial, fungal and mycoplasma contaminations when tested on special bacterial and fungal media.

3.4. Safety Test

Neither CPE in two successive blind passages on VERO cells (inoculated with the inactivated virus fluid) nor abortion or untoward reaction (roughness, loss of

weight, nervous signs, death, hyper-sensitivity) were detected in pregnant mice inoculated with inactivated virus or with the final vaccine product.

3.5. Immunization of guinea pig

EHV-1 antibodies were detectable in inoculated guinea pig 21 days after the primary inoculation with mean complement fixing antibodies and ELISA titer 64 and 680 respectively. A significant increase in the antibody titer (about 2 fold) was obtained by 2 weeks after a second inoculation of the vaccine (at 35 dpi) with mean CF antibodies and ELISA titer 176 and 1322.5 respectively as shown in (table 1& 2 and Fig.1)

3.6. Immunization of horse

In the vaccinated horses the mean CF antibodies titer as shown in (table 3 and Fig.2), were increased 4- fold within the first four weeks post inoculation (wpi) of initial dose (from 10.7 at 2wpi to 42.67 at 4wpi).The peak of CF antibodies was regarded at 1 and 1.5 months post both booster and 2nd dose with mean titers 213.3 and 341.3 respectively. Positive protective CF antibody titers ≥ 20 can be detected for 2.5 months post booster dose. While the ELISA result as shown in (table 4 and Fig. 3) 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.

Table (1): EHV-1 CF antibody titers in inoculated Guinea pigs.

Time of sampling	Mean antibody titer	Non inoculated control
0Time	-ve 1st inoculation	-ve
21dpi*	64** Booster dose	-ve
35dpi	176	-ve

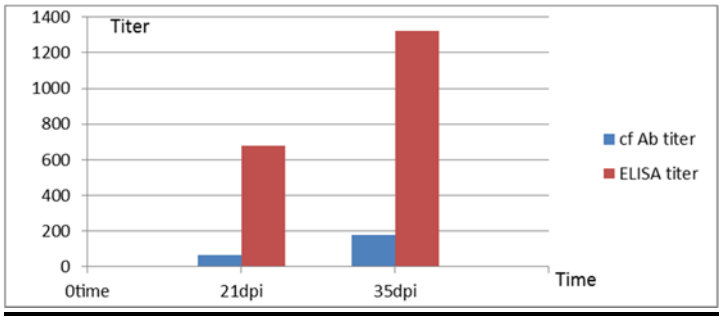
*dpi: days post inoculation. ** CF antibody titer= Reciprocal of the highest dilution of serum showed positive result.

Table (2): EHV-1 ELISA titers in inoculated Guinea pigs

Time of sampling	Mean antibody titer	Non inoculated Control
0Time	-ve 1st inoculation	-ve
21dpi*	680** Booster dose	-ve
35dpi	1322.5	-ve

*dpi: days post inoculation. **ELISA titer= Reciprocal of the highest dilution of serum showed positive result.

Fig. (1): EHV-1 CF antibodies and ELISA titer in inoculated Guinea pigs



Dpi: days post inoculation

Table (3): EHV-1CF antibody titers in inoculated horses

Time of sampling	EHV-1 CF antibody titer				
	Inoculated horses			Mean	Non inoculated Control
	H1*	H2	H3		
0 Time	-ve	-ve	--ve	-	0
	Inoculation of initial dose				
2wpi	8**	16	8	10.7	0
4wpi	32	64	32	42.67	0
6wpi	16	32	8	18.7	0
	Booster dose				
2mpi	64	128	64	85.3	0
2.5mpi	128	256	256	213.3	
3mpi	64	128	128	106.7	0
4mpi	16	32	16	21.3	0
5mpi	-ve	4	4	2.7	0
6mpi	-ve	-ve	-ve	-	0
	2nd dose				
6.5mpi	32	64	32	42.7	
7mpi	128	256	128	170.7	0
7.5mpi	256	512	256	341.3	0
8mpi	128	128	256	170.7	0

wpi : week post inoculation mpi : month post inoculation * horse number ** CF antibody titer= Reciprocal of the highest dilution of serum showed positive result.

Fig.(2):EHV-1 CF antibody titers in inoculated Horses

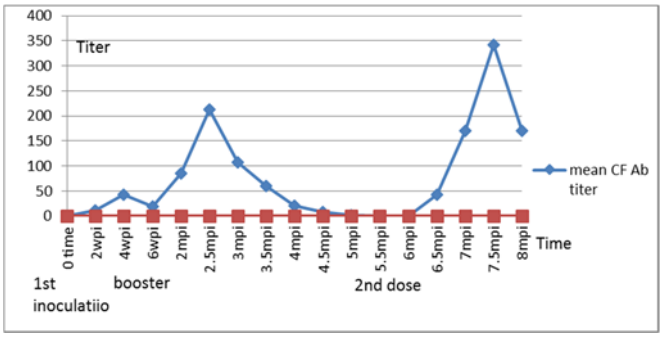
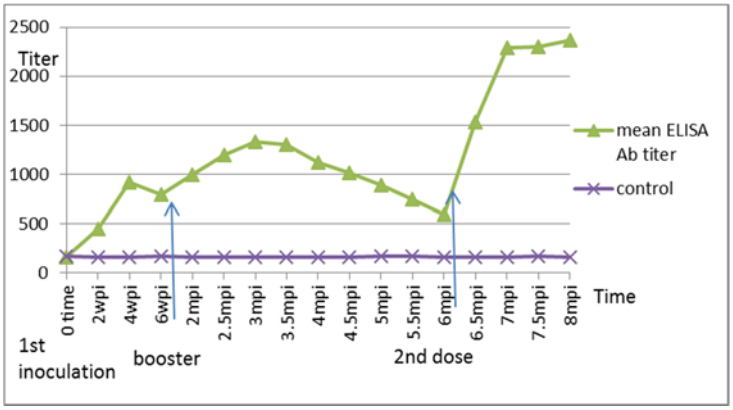


Table (4): EHV-1 ELISA antibody titers in inoculated horses

Time of sampling	EHV-1 ELISA titer				
	Inoculated horses			Mean	Non inoculated Control
	H1*	H2	H3		
0Time	165**	170	150	161.7	170
	Inoculation of initial dose				
2wpi	430	480	440	450	160
4wpi	900	950	920	923.3	160
6wpi	700	810	880	796	170
	Booster dose				
2mpi	950	1050	1000	1000	160
2.5mpi	1070	1230	1300	1200	160
3mpi	1265	1450	1290	1335	160
4mpi	1000	1200	1160	1120	160
5mpi	820	900	960	893.3	170
6mpi	650	700	630	660	160
	2nd dose				
6.5mpi	1300	1750	1540	1530	160
7mpi	2000	2570	2300	2290	160
8mpi	2020	2530	2560	2370	160

wpi : week post inoculation mpi : month post inoculation * Horse number
**ELISA titer= Reciprocal of the highest dilution of serum showed positive result.

Fig. (3): EHV-1 ELISA titer in inoculated Horses



4- DISCUSSION

Successful vaccination against EHV-1 with inactivated vaccine require presence of strong adjuvant to maximize the vaccine potency, on the other hand horse are highly valuable sensitive animals which need special adjuvant, that don't produce undesirable post vaccinal reaction either locally nor systemic. This paper studies the safety and potency of EHV-1 inactivated vaccine adjuvanted with Montanide pet gel A in horses and guinea pigs. To prepare this vaccine, the EHV-1(vaccine seed virus) was subjected to identity test by using SNT (OIE, 2012) then propagated on VERO cells and titrated, The infectivity titer of the EHV-1 VEp_6 (vaccine viral fluid) was 7 $\log_{10}$ $TCID_{50}$ /ml (7.3 $\log_{10}$ $TCID_{50}$ /2ml/dose. This titer exceeds the

immunizing dose (6.1 log_{10} $TCID_{50}$ /2ml) that reported by Charles, et. al., (1977). Vaccine viral fluid has been completely inactivated by 0.008 M BEI at 37°C for 24 hours (Nehal, 2006). The immunogenicity of the prepared vaccine was pre-estimated in guinea pig elicited an obvious immune response (table 1 & 2 and Fig.1). EHV-1 antibodies were detectable 21 days after the primary inoculation with mean complement fixing antibodies and ELISA titer 64 and 680 respectively. A significant increase in the antibody titer (about 2 fold) was obtained by 2 weeks after a second inoculation of the vaccine (at 35 dpi) with mean CF antibodies and ELISA titer 176 and 1322.5 respectively, This result was similar to the result recorded by Guo et al., (1989) and revealed that the prepared vaccine is immunogenic. Respecting to horses immunization there was no detectable adverse reaction observed in any horses after inoculation of the prepared vaccine. The immune response of the inoculated horses was monitored up to eight months post inoculation (mpi).It was found that the mean CF antibodies titer as shown in (table 3 and Fig.2), were increased 4- fold within the first four weeks post inoculation (wpi) of initial dose (from 10.7 at 2wpi to 42.67 at 4wpi).The peak of CF antibodies was regarded at 1 and 1.5 months post both booster and 2nd dose with mean titers 213.3 and 341.3 respectively. Positive protective CF antibody titers ≥ 20 can be detected for 2.5 months post booster dose. These results come parallel to results obtained by Burrows et al., (1984) and Singh et al.,(2006), who stated that CF antibodies titer increased 2 to 4 fold following each injection of the vaccine and can be detected for 2 to 3 months, Although horses with CF antibodies titer 20 showed reduction in the incidence and duration of clinical signs after challenge. In concerning to the ELISA result as dedicated in (table 4) and illustrated in (Fig. 3) 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. 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. In conclusion, the prepared EHV-1 inactivated vaccine adjuvanted with montanide pet gel A is highly safe and potent for Horses.

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