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EFFECT OF INACTIVATED SHEEP POX VACCINE AND PARAPOX OVIS VIRUS AS IMMUNOMODULATORS ON RESPONSE OF EQUINES TO INACTIVATED HERPES VACCINE

By

Ibrahim, M.M*; Fatma F. Warda; Nermeen, G. Shafik*; Abdel-Baky, M. H.*; and Magda A. Kalad****

*Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt. (CLEVB)

clevb@tedata.net.eg

** Veterinary Serum and Vaccines Research Institute, Abbasia, Cairo, Egypt.

(VSVRI)svri@idsc.gov.eg

ABSTRACT

In the present study, a trial was carried out to maximize the immune response of horses against inactivated equine herpes virus vaccine (EHV-1) immunomodulated by pre-vaccination with inactivated Parapox Ovis virus (iPPOV) and inactivated sheep pox virus. The role of these formulae of vaccines is enhancing non-specific innate immunity to reduce the immune suppression of horses due to exposure to different stressors especially transportation and respiratory infections. Serum samples for application of serological tests and whole heparinized blood for lymphocyte cell proliferation assay were taken before and after the administration of both immunomodulator (2ml/horse, I/M injection on days 0, 2 and 9) and also through different intervals after vaccination with inactivated equine herpes virus vaccine(EHV-1). The results of serological investigations (ELISA and VNT) and cellular assay (XTT) clarify the changes in immune responses of two groups of horses (B, C) that prevaccinated by inactivated Parapox Ovis virus (iPPOV) and inactivated sheep pox virus followed by vaccination with inactivated equine herpes virus vaccine (EHV-1) in comparing with the third group (A) which vaccinated with inactivated equine herpes virus vaccine (EHV-1) alone. As the duration of immune responses increased to eight months' post vaccination and also the levels for group (B and C) detected with ELISA antibody titers (72, 1105 and742) and neutralizing index (1.2, 2.0 and 1.8) in groups A, B, C, respectively. The results of this study throw the light on effect and importance to use immunomodulators for improving the immune response against inactivated equine herpes virus vaccine (EHV-1) either by elongation of intervals for revaccination or by raising the level of cellular and humoral immune responses. Also this

study presented inactivated sheep pox virus that prepared locally without adjuvant as another effective, safe immunomodulator for equines.

INTRODUCTION

The alpha herpes virus, equine herpesvirus-1 (EHV-1) is the causative agent for major economic losses and welfare problems in horses. It is among etiological agents causing respiratory and neurological diseases as well as abortion **(Patel and Heldens 2005)**. The virus has 9 serotype from 1to 5 belong to domestic horse but from 6 to 9 are belonging to wild life **(OIE, 2012).** Vaccination in conjunction with good animal management is the best way to prevent and control outbreaks of this disease especially inactivated vaccine in non-endemic areas. Inactivation using binary ethelenimine within 2 hours with final concentration 0.008M was established **(Nehal, 2006)**. Many adjuvants are used in combination with specific antigen enhance the immune response. Re hydra gel (Aluminum hydroxide gel) and Pet gel are used acting by slow release of the viral antigen **Eman, 2005 and Nehal *et al.,* 2013)**. Since the immunity following vaccination appears to be short Lasts (4-6 months) so the use of immunomodulators (immunostimulants) are of beneficial value to improve the immune response. The present study was designed as a trial to maximize the immune response of equines to inactivated herpes virus vaccine by using of two immunomodulators, inactivated Parapox Ovis virus (iPPVO) and inactivated sheep pox virus prior the use of inactivated EHV-1 vaccine.

MATERIAL AND METHODS

1- Virus:

Freeze dried local strain of EHV-1 (EP2, EP3) was used for vaccine preparation by Equine Vaccine Research Dept., Veterinary Serum and Vaccine Research Institute (VSVRI),Abbasia, Cairo according to **Hassanein *et al.* (2002) and Magda *et al*. (2013).**

2- Tissue culture:

African green monkey kidney cells (Vero) was obtained from FADDL, Plum Island and used for virus propagation and virus neutralization test (VNT).

3- Freeze dried rabbit anti EHV-1 serum:

It was kindly supplied by Dr. Jennet wellington Research Fellow, Department of Biological Science, Macquarie Uni., NSW and Australia. It was used in virus identity test.

4 - Immunomodulatory agents:

1- Inactivated Parapox Ovis virus (iPPOV) strain D107 (commercially known as zylexis Pfizer Animal Health) was used for non-specific immunization. **(Ulgen *et al.* 2014).**

2- Inactivated sheep pox virus was prepared according to **Manal *et al.* (2003)**, but without using any adjuvant to confirm efficacy of sheep pox as immunomodulator.

5- Propagation and Titration of Equine Herpesvirus-1 (EHV-1):

The locally isolated EHV-1 (EP3) with titer of 7 $\log_{10}$ ED_{50} / ml was propagated on Vero line for 7 successive passages according to **Safaa (2007).** Virus passages No. 5, 6 and 7 on cell line was titrated and the $TICID_{50}$ /ml was calculated by the method of **Reed and Muench (1938).**

6- Preparation of (EHV-1) Antigen:

EHV-1 antigen was prepared through the inoculation of locally isolated strain on the chorioallantoic membrane of 11-13day old specific pathogen free (SPF) embryonated chicken eggs (ECE) that were obtained from Koum Oshiem farm, fayoum, Egypt) according to the method described by **Dutta *et al.,* (1983) and Azmi and Field, (1993)**. This antigen was used in serological tests.

7- Preparation of (EHV-1) Hyperimmune Antisera:

Horse anti EHV-1 hyperimmune serum was prepared by **Safaa *et al.* (2005)** in Equine Vaccine Research Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, and used as positive control in serological tests (immunosorbent Assay "ELISA").

8- Preparation of inactivated equine herpes virus (EHV-1) Vaccine:

To prepare the vaccine, the EHV-1(vaccine seed virus) was subjected to identity test by using reference anti-EHV-1 serum according **(Bernhardt, 1993)** to apply VNT according to **(OIE, 2012)** then propagated on VERO cells and titrated, The infectivity titer of the EHV-1 Vero cells (passage 6) VEp6 (vaccine viral fluid) with titer of 7 log10 TCID50 /ml (7.3 $\log_{10}$ TCID50 /2ml/dose). This titer exceeds the immunizing dose (6.1 $\log_{10}$ TCID50 /2ml) that reported by **Charles *et al.,* (1977)** and inactivated with BEI 0.008M. Virus fluid- inactivator mixtures and control virus were incubated with continuous stirring at 37°C for 24hrs **(Bahnemann 1990).**

9- Quality Control and Evaluation of inactivated equine herpes virus (EHV-1) Vaccine:

Sterility, safety and Potency tests were applied according (**Egyptian Standard Regulation for Evaluation of Veterinary Biologics (CLEVB) 2nd Edition,Sec. I and III, Ch. 1, 2009).**

As the vaccine is commercially produced and supplied by Equine Vaccine Research Dept., Veterinary Serum and Vaccine Research Institute (*VSVRI).*

10- Experimental Design:

Six healthy susceptible horses with low neutralizing antibody titers (< 4) against EHV-1 **(Charles *et al.*, 1977*)*** were divided into three groups.The first group was vaccinated I/M with the prepared inactivated EHV-1 vaccine only, where each horse received 2 doses one month apart (2ml/ dose /horse). The second group was inoculated I/M firstly with iPPOV at (0, 2and 9 days-2ml/dose/ horse) then vaccinated I/M with inactivated EHV-1 vaccine. The third one was inoculated I/M firstly with prepared inactivated sheep pox virus without adjuvant in the same schedule at (0, 2and 9 days - 2ml/ dose/ horse), then vaccinated I/M with inactivated EHV-1 vaccine. Also one healthy susceptible horse with low antibody titer against EHV-1 was kept as negative control. The serum were collected at 14 and 28 days then monthly for serological tests and alsowhole blood samples were collected through (0,2 and 9 days) of immunomodulation then at 5,7,10,14,21and 28 days post vaccination.

11- Evaluation of Humoral immune response:

a) Virus Neutralization Test (VNT):

Serum samples were tested for the EHV-1 antibodies using (SNT), and expressed as neutralizing index according to **Shanker and Yadav(1986) andSenthil and Parames (2014).**

b) Enzyme Linked Immunosorbent Assay (ELISA):

The test was performed according to **Kirisawa *et al*. (1995) and Goldsby *et al.* (2000)** and the results were expressed as antibody titers.

12- Evaluation of Cellular immune response:

Lymphocyte cell proliferation assay (XTT) :

The peripheral mononuclear cells (PMNCs) separation was done from heparinized whole blood samples according to **(Macpherson and Stocker (1962) and Mayer *et al.* (1974)** using cell proliferation kit with Tetrazolium salts (XTT) reagent (AppliChem GmbH cat # A8088 Germany). The optic density (O.D) of the developed colons was then measured by ELISA reader at 450° A with reference of 630° A and this method according to **(Lucy 1974).**

RESULTS AND DISCUSSION

Table (1): Lymphocyte cell proliferation assay (XTT) (of horses inoculated by EHV-1 inactivated vaccine using iPPVO and sheep pox inactivated virus.

Time of sampling	Group A Mean value	Group B Mean value	Group C Mean value	Control
Pre. IM (day0)	**0.085**	**0.110**	**0.095**	**0.103**
2ds. IM (day2)	**0.086**	**0.312**	**0.130**	**0.102**
7 ds. IM(day 9)	**0.087**	**0.395**	**0.303**	**0.104**
Vaccination by EHV-1 inactivated vaccine (10days after 1st immunomodulation)				
5DPV	**0.401**	**0.597**	**0.539**	**0.120**
7 DPV	**0.509**	**0.830**	**0.640**	**0.100**
***10 DPV**	**0.682**	**1.083**	**0.889**	**0.089**
14 DPV	**0.651**	**0.845**	**0.720**	**0.091**
***21 DPV**	**0.402**	**0.701**	**0.682**	**0.093**
28 DPV	**0.319**	**0.512**	**0.478**	**0.082**

Group A: EHV-1 inactivated vaccine alone

Group B: EHV-1 inactivated vaccine + immunomodulator (**iPPOV)**

Group C: EHV-1 inactivated vaccine + immunomodulator (**sheep pox inactivated vaccine)**

Control: Negative control without injection

0 d. IM: 0 day 1st injection of the immunomodulator (Sample Zero = Pre IM)

2 ds. IM: 2 days after 1st injection of the immunomodulator

(2nd injection of the immunomodulator)

7 ds. IM: 9 days after 1st injection of the immunomodulator

(3rd injection of the immunomodulator)

DPV: Days post vaccination

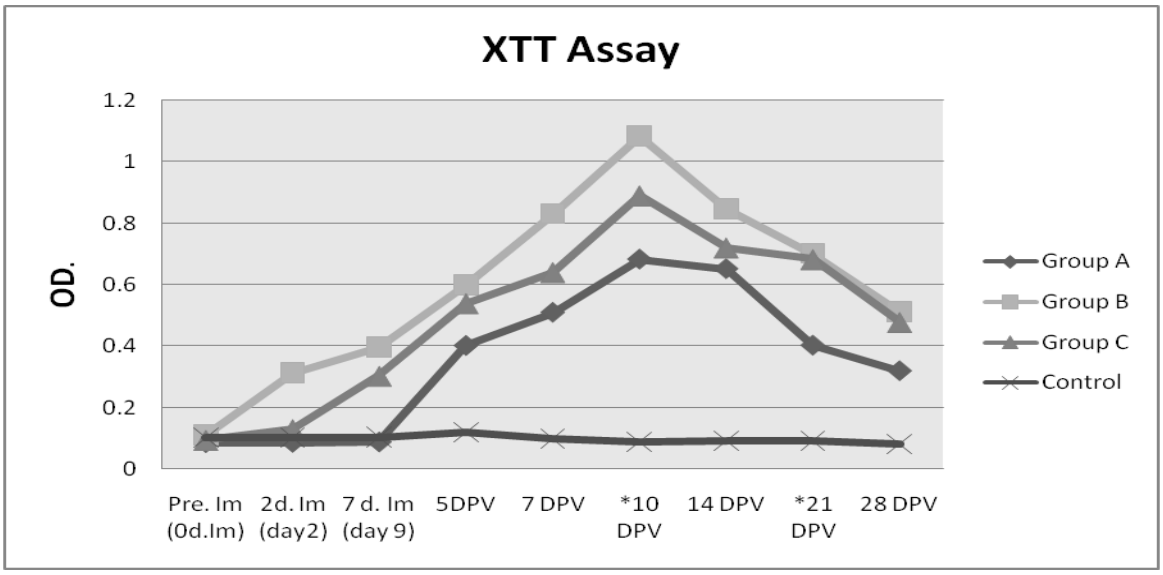

Fig. (1): Lymphocyte cell proliferation assay (XTT) of inoculated horses.

Table (2): Seroconversion of horses inoculated by EHV-1 inactivated vaccine using iPPVO and sheep pox inactivated virus tested by VNT- (NI).

Time of sampling	Group A	Group B	Group C	Control
Pre	**0.8**	**0.4**	**0.6**	**0.5**
Vaccination (ZERO DAY)				
14d PV	**0.9**	**1.4**	**1.2**	**0.6**
28d PV	**1.1**	**2.9**	**2.6**	**0.5**
Boostering				
2 MPV	**1.8**	**3.3**	**3.0**	**0.4**
***3 MPV**	**3.3**	**4.2**	**3.8**	**0.5**
4 MPV	**2.8**	**3.8**	**3.5**	**0.4**
***5 MPV**	**2.1**	**3.5**	**3.3**	**0.4**
6 MPV	**1.6**	**3.0**	**2.8**	**0.3**
7 MPV	**1.4**	**2.9**	**2.6**	**0.3**
***8 MPV**	**1.2**	**2.0**	**1.8**	**0.2**

Group A: EHV-1 inactivated vaccine alone.

Group B: EHV-1 inactivated vaccine + immunomodulator **(iPPOV).**

Group C: EHV-1 inactivated vaccine + immunomodulator **(sheep pox inactivated vaccine).**

Control: Negative control without injection.

DPV: Days post vaccination.

MPV: Months post vaccination.

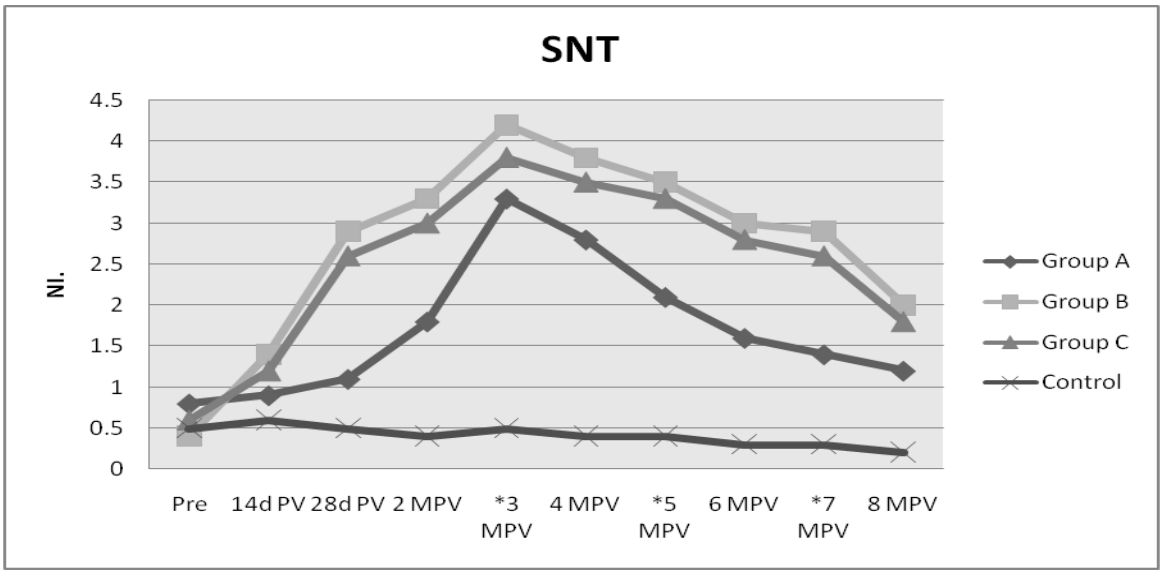

Fig. (2): Seroconversion of inoculated horses tested by VNT- (NI).

Table (3): Seroconversion of horses inoculated by EHV-1 inactivated vaccine using iPPVO and sheep pox inactivated virus tested by ELISA.

Time of sampling	Group A	Group B	Group C	Control
Pre	**46**	**78**	**56**	**70**
Vaccination (ZERO DAY)				
***14D PV**	**97**	**142**	**129**	**65**
28D PV	**387**	**508**	**498**	**62**
Boostering				
2 MPV	**522**	**1797**	**1552**	**61**
***3 MPV**	**709**	**2276**	**1853**	**60**
4 MPV	**623**	**1943**	**1603**	**59**
***5 MPV**	**325**	**1903**	**1420**	**56**
6 MPV	**109**	**1443**	**1052**	**55**
7 MPV	**98**	**1394**	**995**	**54**
***8 MPV**	**72**	**1105**	**742**	**54**

Group A: EHV-1 inactivated vaccine alone

Group B: EHV-1 inactivated vaccine + immunomodulator (**iPPOV)**

Group C: EHV-1 inactivated vaccine + immunomodulator (**sheep pox inactivated vaccine)**

Control: Negative control without injection

DPV: Days post vaccination

MPV: Months post vaccination

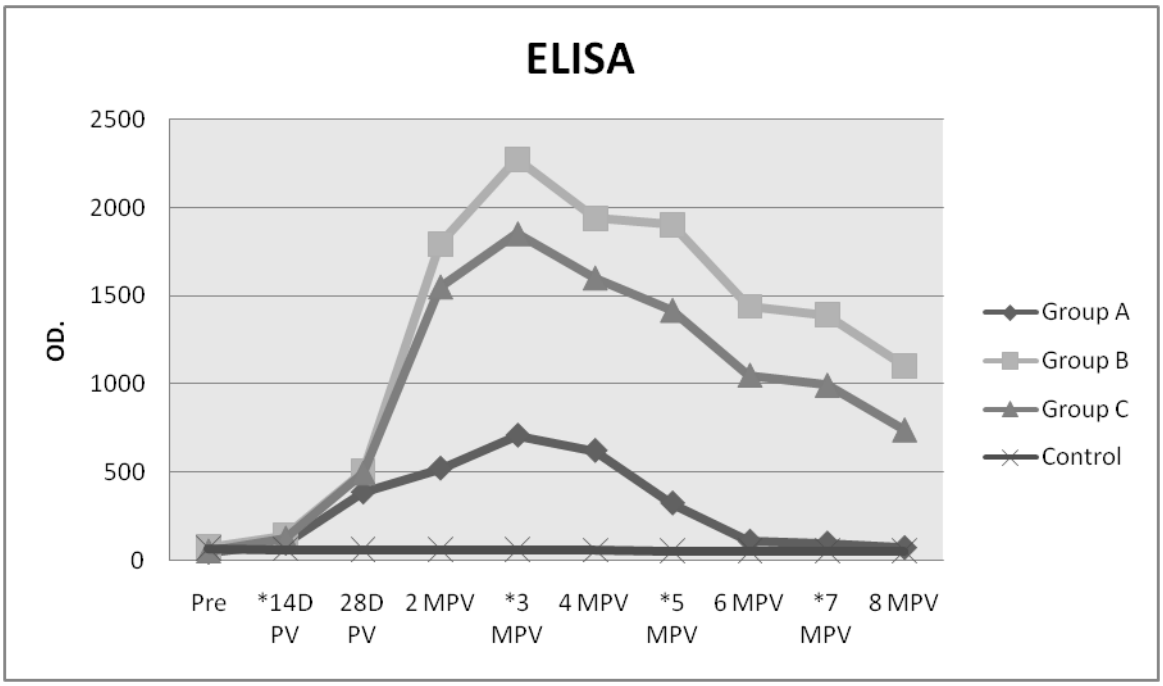

Fig. (3): Seroconversion results tested by ELISA.

There is a great interest with equine industry which plays an important role in national income all over the world especially in Egypt and Arabian countries.

Equine Herpes virus type1 is a disease of stress (stress mediated diseases) causing epidemic abortion in mares, the birth of weak non-viable foals, or a sporadic paralytic neurologic disease (equine herpes virus myeloencephalopathy-EHM) secondary to vasculitis of the spinal cord and brain **(Patel and Heldens 2005)**. As immunity following vaccination appears to be short lived and it is recommended that foals and young horses be revaccinated at 4 - 6 months' intervals. Regrettably protection induced by vaccination is not always optimal. This study highlights the beneficial use of immunomodulators (iPPOV and sheep pox inactivated virus) to evade immunity to produce a variety of immunomodulatory proteins that support viral replication in spite of an active immune response. **(Paillot, 2013) iPPOV** is currently used in equine medicine as an immunomodulator to improve immune system. The administrator of iPPOV to horses has a supportive effect on their cellular immunity and an immunomodulatory effect against equine viral infections **(Ulgen *et al.*, 2014)**. Their activity is based on the activation of innate cells and consequent cytokine production **(Anzilero *et al.* 2014).** Also using of another type as inactivated sheep pox virus without adjuvant may improve

immune system and has a supportive effect on cellular immunity and an immunomodulatory effect against equine viral infections without any biological risk for equines or zoonotic effect for humans **(Johnston, and McFadden, 2003)**.Concerning the cellular assay, it is clear from (Table 1), Fig.(1) that, the use of iPPOV and sheep pox inactivated vaccine immunomodulators before the vaccination with inactivated equine herpes virus vaccine (EHV-1) evoke higher cellular responses for inoculated horses than that not immunomodulated. These responses elevated to the peak at (10 DPV) (0.682, 1.083 and 0.889) and persisted to (21 DPV) (0.402, 0.701and 0.682) as show respectively to confirm the effect of immunomodulators. The results were agreeable with **Ulgen *et al.,* (2014) and Fachinger *et al.* (2000)** who reported that immunomodulator activate innate immune cells and support animals where immune function and response are suboptimal or stressed (suppressed) as EHV is a disease of stress. (Table 2), Fig. (2) Showed increasing of the neutralizing index (NI) started from two weeks (14DPV) (0.9, 1.4 and 1.2) to reach the peak at 3MPV (3.3, 4.2 and 3.8) respectively with great elevation of the immunomodulated groups (B and C) than non immunomodulated group (A). This superiority persisted to more than 8MPV (1.2, 2.0 and1.8) while the group A started to decrease after (5MPV) in comparing with group (B and C) (2.1, 3.5 and 3.3). These results are in agreement with **Shanker and Yadav (1986) and Senthil and Parames (2014)** who reported that, the virus neutralizing indices of vaccinated equines with EHV-1 was ranging from (1.5 to 3.5). (Table 3), Fig. (3) Illustrated the ELISA antibody titer of immunomodulated horses in group (B, C) are much higher than group (A) in the same manner of that obtained by (NI) as the results started to increase from (14DPV) (97,142 and 129) respectively to reach the peak at 3MPV (709, 2276 and 1853). Also the superiority persisted to 8MPV (72, 1105, 742) and the group A mean titer started to decrease after (5MPV) in comparing with group (B and C) (325, 1903 and 1420). These results are in agreement with (OIE 2015) which approved that, the vaccine is considered immunogenic where there is a significant increase of neutralizing antibody titer of vaccinated horses in parallel to the ELISA antibody titer (IgG). The cellular and humoral investigations showed that elevation in nearly parallel manner for groups B and C that immunomodulated before EHV-1 vaccination in comparing with group A that vaccinated with inactivated EHV-1 alone. While the results of the group (B) which immunomodulated by (iPPOV) showed greater elevation than that of the group (C) which immunomodulated by inactivated sheep pox virus. These may be attributed to the preparation

of inactivated sheep pox virus without using of adjuvant as both immunomodulators are poxviruses as cited by **Johnston and McFadden (2003).**

CONCLUSION

In conclusion, it is obvious that, the administrations of both immunomodulators potentiate the immune response and enhance the efficacy and duration of immunity against inactivated EHV-1 vaccine with decreasing of other immune suppressive stressors. Also there is the ability to use of locally prepared inactivated sheep pox virus to act as safe and effective equine immunomodulator as it is used without any adjuvant also without any biological risk for equines or Zoonotic effect for humans.

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