Received: 27 April 2023, Accepted: 22 May 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 3, 483-491

Efficacy of Freeze Dried Inactivated Equine Influenza, Equine Herpesvirus-1 and Tetanus Toxoid Vaccine

Nashwa K. Madkour[1], Eman M. Ebeid[1], Nehal S. Saleh[1], Fatma F. Warda[1*], Hamed A. El-Helw[2]. Aliaa Abd El-Bakey[2]

[1]Equine Vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Agriculture Research Center (ARC), Egypt.

[2]Anaerobic Bacterial Vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Agriculture Research Center (ARC), Egypt.

***Correspondence**
Corresponding author: Fatma F. Warda
E-mail address: dr_fa_fa@yahoo.com

Abstract

Vaccination has a very important role in controlling the most infectious diseases in horses especially Equine Influenza Virus (EIV), Equine Herpesvirus-1 (EHV-1) and Tetanus. The keeping quality of the locally prepared combined inactivated vaccine which contains EIV, EHV-1 and Tetanus Toxoid (TT) adjuvanted with saponin and Alhydrogel was improved through preparing the lyophilized inactivated EIV, EHV-1 and TT vaccine that reconstituted with adjuvant (saponin) and then the potency and keeping quality of the two vaccines (liquid and lyophilized) were compared. The two vaccine batches were inoculated into groups of guinea pigs and horses for potency and immunogenicity measurement. The immune response in guinea pigs and horses were measured by HI test for EIV and ELISA for EHV-1 and Toxoid Neutralizing antibody test (TN) for TT which proved that antibody was detected at 2 weeks post vaccination reached its peak at two-month post inoculation (2MPI) then declined gradually until 7MPI. There were no significant differences between all vaccines, as they were potent, efficient, and immunogenic. Regarding to the keeping quality, the tested vaccine vials were kept at 4ºC for various interval times (1, 2, 2.5, and 3 years) then inoculated into guinea pig groups. Finally, the lyophilized vaccine was proved to be stable and potent for 3 years while the liquid vaccine was stable for 2 years.

KEYWORDS
EIV, EHV-1, Tetanus toxoid, Guinea pig, ECE, Lyophilized vaccine, TN

INTRODUCTION

Equine Influenza (EI), Equine Herpesvirus-1 (EHV-1) and Tetanus are three of the most concerning diseases of horses. Equine Influenza Virus (EIV) is a commune respiratory virus of horses and other Equidae worldwide (Chambers, 2020). EIV strain is capable of existing outbreaks belonging to H3N8 Subtype A2, while H7N7 Subtype A1 hasn't isolated since 1979 (Cullinane *et al.*, 2020). Equine Herpesvirus-1 infection causes several clinical manifestations as respiratory symptoms, abortion in pregnant mares, neonatal death and neuropathogenic disorders (Laval *et al.*,2021). Tetanus, caused by *Clostridium tetani* toxin, is a fatal contagious disease of domestic animal species. There is a significant susceptibility variation to neurotoxin of *C. tetani* among the animal species, but horses are the most susceptible (Jansen and Knoetze, 1979).

Hygienic measure and vaccination are the best methods to control infectious diseases (Mohamed *et al.*, 2022). The locally combined inactivated EI, EHV-1 and TT vaccine adjuvanted with both saponin and Alhydrogel showed high antibody titer, exceeding the clinical protection level against the previous diseases (Bayoumi *et al.*, 2018).

Lyophilization of product extended its shelf life and its stability is enhanced and thus the marketability. In process of lyophilization the water is removed without heat which prevents product degradation. Dissolution of the reconstituted product is both easy and rapid (Sarah Kinney, 2020).

In the present study, we investigated the potency and keeping quality of the lyophilized inactivated Equine Influenza, Equine Herpesvirus-1 and Tetanus Toxoid vaccine reconstituted in saponin as dissolvent and adjuvant, in comparable to the locally combined inactivated EI, EHV-1 and TT vaccine adjuvanted with saponin and Alhydrogel.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Animal Ethics of the Veterinary Serum and Vaccine Research Institute Committee (VSVRI). All the experiments were conducted in accordance with the VSVRI guideline for animal research.

Virus

Equine Influenza virus (EIV)

Locally isolated freeze dried EIV (A/equi-2/ Egypt/ 6066 NAMRU3- VSVRI 2008), at its egg passage three (Ep3). As the infectivity titer was 10 $\log_{10}$ EID_{50}/0.1ml and HA titer was 11 $\log_2$

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
ISSN: 2090-6277/2090-6269/ © 2011-2023 Journal of Advanced Veterinary Research. All rights reserved.

/0.05ml.

Equine Herpesvirus-1 (EHV-1)

Freeze dried isolated EHV-1 of Vero cell passage 5 (VCp5) with $TCID_{50}$ 7.5 log_{10}/ml.

EIV and EHV-1 were obtained from Equine Vaccine Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Egypt.

Tetanus Toxoid

Harvard strain, 49205 of *C. tetani* strain was supplied by anaerobic bacterial Vaccine Research Department, VSVRI, Abasia, Cairo, Egypt. It was cultivated and propagated in specific toxin production medium according to El-Helw (2007) and incubated for 7 days at 35ºC. The obtained tetanus toxin was estimated by determination of MLD/ml and limit of flocculation (Lf) according to Ramon (1922) and Fu *et al.* (2004). Each dose included 40 Lf/ horse dose.

Antisera

Reference antisera against A/ equi-1/ parague/56(H7N7) and A/eque-2/ Miami 63 (H3N8) obtained from National Veterinary Laboratories, United States, Department of Agriculture and Veterinary Services were used.

Lyophilized rabbit anti EHV-1 and 4 sera was supplied by Dr. Jennet Wellington, Research follow, Department of Biological Science Aquaria, Univ, NSW, Australia.

Commercial antitetanic serum was applied by the Egyptian Organization of Biological Products and Vaccine, Tetanus Department, Egypt. Each vial contained 1500 IU/ml.

Animals

Horses

To evaluate the immunogenicity and potency of the vaccines, six fully-grown apparently healthy horses with low antibody titer against EHV-1and EIV were used in this study (OIE, 2019b, c). Four pregnant mares were used for safety test of inactivated EHV-1 vaccine batches (2 mares / batch).

Guinea pig (G. pig)

Groups of G. pigs (5/group) weighting nearly 350- 450 g were used to evaluate immunogenicity, keeping quality and potency of the vaccines (Guo *et al*., 1989; OIE, 2019b).

Mice

Swiss Albino mice (n.= 50) of an age 4-6 weeks, were used in Tetanus Toxoid safety test. The Toxin Neutralization (TN) test applied for evaluating of Tetanus antitoxin potency, and other 2 groups of pregnant mice (8/group) were used for safety test of inactivated EHV-1 in vaccine batches.

Specific pathogen free embryonated chicken eggs (SPF-ECE)

SPF-ECE were purchased from Fayoum, Egypt (Koum Oshiem Farm), then was used in EI virus propagation, infectivity titration and determination of the residual infective virus activity.

Cell culture

African green monkey kidney cells (VERO) were grown in Eagle's minimum essential media with 10% fetal calf serum, streptomycin 100 mg/ml and penicillin sodium 100 IU/ml. The cells used for EHV-1 propagation, titration, and determination of the residual infective virus activity.

Inactivator

Binary Ethyleneimine (BEI) stock solution (0.1M) prepared according to Bahnemann (1990) and Mark and Mellencamp (2004) from 2-bromoethylamine hydrobromide (Aldrich chemical Co., LTD) and 0.2N NaOH. Formalin of concentration 37% was used at 1% (v/v) for conversion of tetanus toxin into tetanus toxoid.

Adjuvant

Aluminum Hydroxide gel was purchased from Alhydrogel, Superfos Biosectoe, Denmark.

Quillaja saponaria molina extract (saponin) was purchased from ACROS Co. (New Jersey, USA). It was used as vaccine diluent and adjuvant.

Stabilizer

Lactose peptone tris buffer (LPTB), pH 7.4, that was prepared according to Hazrati and Ozawa (1965) and Ebied (2005).

Identity test

Hemagglutination inhibition (HI) test was carried out to confirm EI (A/equi-2/Egypt/6066 NAMRU3-VSVRI/2008) EP3 as vaccine seed virus, using reference antisera for EIV subtype 1 and 2 (OIE, 2019b).

Serum neutralization test (SNT) was performed by using reference antisera for EHV-1and 4 (OIE, 2019c) to confirm the EHV-1VERO P2 as vaccine seed virus.

Limit of Flocculation (Lf) was performed by using commercial tetanus antitoxin (1500 IU/ml) for tetanus toxin production evaluation and for determination of tetanus toxoid dose used in vaccine.

SPF-ECE (9-11 days) were inoculated intra-allantoic with 0.1ml of infected egg fluid (5 ECE / dilution) and incubated at 35˚C for 72 hours, then chilled overnight (Tyerell and Valentine, 1957). The infectivity titer was known as log10EID50/ 0.1ml (Reed and Muench, 1938).

Titration of EHV-1

Virus fluid (tenfold serial dilutions) was made in sterile PBS and inoculated into VERO cells (4 wells/ dilution) and incubated for 5 days at 37˚C. The titer was mentioned as $log_{10}TID_{50}$/ml (Reed and Muench, 1938).

Titration of Tetanus Toxin

Titration of tetanus toxin was performed by determination of MLD/ ml in mice, sample was obtained from toxin production medium after period of incubation and subjected to a serial dilution. Each dilution was inoculated into 2 mice and left for observation for 4 days, the reciprocal of highest dilution that cause death of all mice was recorded.

Limit of flocculation

It was done on both toxin and toxoid to determine the dose used in vaccine. It was performed by adding different amounts of antitetanic serum (100 IU/ml) to series of tubes containing 1 ml of tetanus toxin or toxoid incubated at 45ºC then the first tube that showed flocculation was recorded.

Vaccine Preparation

EI seed virus was propagated through the allantoic sac route in SPF-ECE 9-11 days old for two successive passages (Ep5). The incubation of inoculated eggs was done at 35°C for 3 days. The amnio-allantoic fluids (virus fluid) were collected and centrifugated at 1500 rpm for 15 minutes and tested for sterility, haemagglutinating activity and infectivity titer.

EI virus inactivation: Virus fluid (HA titer 11 $\log_2$ and infectivity titer 10 $\log_{10}$ EID_{50}/0.1 ml) was inactivated with BEI at 0.003 M concentration with continuous stirring at 37°C for one day (Ebied, 2005).

EHV-1 seed virus (VEp2) was propagated on VERO cells (3 successive passages). Vaccine virus fluid (VEp5) was titrated to estimate $TCID_{50}$.

EHV-1 inactivation: Virus fluid with 7.5 $\log_{10}$ $TCID_{50}$/ ml was inactivated by BEI of 0.008M concentration at 37°C with continuous stirring for one day (Saleh, 2006).

Sodium thiosulphate (2% concentration) was for stopping BEI action on (EI and EHV-1) and neutralizing the residual BEI toxicity on target host.

Tetanus Toxoid

C. tetani strain was propagate on toxin production medium (El-Helw, 2007) at 35ºC/ 7 days, then the obtained samples used for evaluation of MLD/ml and Lf. Toxin was inactivated by adding of Formalin 37% at (1% v/v) left for 21 days at 37 ºC.

Vaccine Batches

Inactivated EI and EHV-1fluids (equal volume) and Tetanus Toxoid were mixed then divided into two batches.

Combined lyophilized vaccine

The 1st Batch(A) of vaccine viral fluid (EI, EHV-1) and tetanus toxoid was mixed with the stabilizer (equal volume) then put into vials (6 ml/vial) and freeze dried. At the time of inoculation, the formula of vaccine was reconstituted with 3 ml saponin as adjuvant (1mg/dose).

Combined liquid vaccine

The 2nd Batch (B) of vaccine viral fluid (EI, EHV-1) and tetanus toxoid was mixed with 20% Alhydrogel and saponin (1mg/dose) then kept on magnetic stir at 4ºC for one day. Then distributed into vials (3 ml/vial). Vaccine vials of the two batches representing one horse dose were caped and kept at 4ºC.

Vaccine quality control

Sterility

Samples from each inactivated virus fluids and the final products were inoculated on various media (Nutrient Agar, Sabouraud Dextrose Agar and thioglycolate) to exclude contaminations as bacterial, mycoplasma and fungal types (OIE, 2019a).

Safety

Safety test of EI and EHV-1

Residual infective virus activity

Applied on the inactivated virus fluid to insure complete virus inactivation. Two successive passages of inactivated EIV were inoculated in ECE (9-11 day) intra-allantoic (ten eggs/group) and incubated at 35ºC for three days. Allantoic and amniotic fluids of the inoculated eggs were collected, and pooled. Hemagglutination activity should not be determined in harvested egg fluid of the 1st nor the 2nd passage (OIE, 2019b).

Inactivated EHV-1 was inoculated on:

Chorioallantoic membrane (CAM) of ECE (11-13 days) then incubated at 37ºC for five days with daily observation. Pock lesion shouldn't be determined on CAM (Saleh, 2006).

VERO cells incubated at 37ºC for 7 days with daily examination. To ensure a complete EHV-1 inactivation, another blind passage was done (Madkour *et al*., 2016).

Safety test in animals

Safety in pregnant mares

Each vaccine batch was inoculated I/M into two pregnant mares at the last 3rd of pregnancy. Every mare received two doses (3ml/dose) with 4 weeks interval (OIE, 2020).

Safety in pregnant mice

The two vaccine batches were inoculated subcutaneously (S/C) into two groups of pregnant mice. Each group received two doses (0.2 ml/dose) with one week interval (Slater *et al*., 1993). All mice and mares were kept under examination in a good hygienic condition until childbirth.

Safety test of tetanus toxoid

It was performed by inoculating 5 mice with 0.5 ml of the vaccine batches intraperitoneally and noticed for one week (European Pharmacopoeia, 2009).

Immunogenicity and potency

In Guinea pigs (G. pig)

Three groups of seronegative G. pig (5 G. pig/ group), first 2 groups were inoculated S/C with the horse dose of each prepared vaccines batch in two doses with 21 days apart and the third one left as a control (Guo *et al*.,1989).

Group A was inoculated with the combined lyophilized vaccine reconstituted in saponin (1 mg/dose).

Group B was inoculated with the combined liquid vaccine adjuvanted with saponin and Alhydrogel.

Group C was kept as a control group under the similar conditions of the experiments.

AT 3-week post inoculation (3WPI), the 1st serum samples were harvested from all previous groups, and groups (A and B) were inoculated with the 2nd dose of their vaccine. Then the 2nd serum samples were harvested from all groups on 14 days later (5WPI).

HI test was applied on the 1st serum samples of all groups to determine HI antibody titer for EI virus (OIE, 2019b). While ELISA, were carried on the 1st and 2nd serum samples of all groups to determine EHV-1 antibodies titer (Guo *et al*., 1989). Also, toxin neutralization titer (TN) test carried out on the 2nd serum samples of all groups (Barile *et al*., 1970).

In horses

Six local horses, 2-4 years old, seronegative or with low antibody titer against EIV and EHV-1 less than 4 neutralizing antibodies were subdivided into 3 groups.

Group A was inoculated with batch A, combined lyophilized vaccine reconstituted in saponin (1mg/dose).

Group B was inoculated with batch B, combined liquid vaccine with Alhydrogel and saponin adjuvant.

Group C was kept as a control group in the similar conditions of the experiments.

Each group was inoculated (I/M at lower third) in the neck with two doses of the previous prepared vaccines one month apart (Wilson, 1999).

Serum samples were harvested at various interval times from each group for checking the immune response using HI test (OIE, 2019b) for EIV, ELISA for EHV-1 (Mumford and Bates, 1984; Sugiura *et al*., 1997; Singh *et al*., 2006). Toxin neutralization test used to determine antibody titer against tetanus toxin according to Barile *et al*. (1970).

Keeping quality (for vaccine storage evaluation)

Vials from each vaccine batch (1 and 2) were kept at 4˚C for three years. Immunogenicity of these vaccine samples were assessed in groups of guinea pigs (as mentioned before).

Statistical analysis

The antibody titer normality against EIV, EHV-1 and tetanus toxin of *C. tetani* in two groups of vaccinated guinea pigs was as well as two groups of vaccinated horses in the prepared combined vaccine lyophilized and liquid (A & B) were checked by ANOVA test. The titers show a normal distribution in different groups. T- test is used to differentiate between the two groups of vaccinated guinea pigs (lyophilized and liquid batches). The significant difference between groups was set at $P \leq 0.05$. The calculation was performed by R program for Windows ver. 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Sterility and safety test

Concerning safety of vaccine, no residual virulent virus was detected in any of the inactivated viral fluids which was proved by absence of haemagglutinating activity, pock lesion and CPE in both ECE and VERO cells for EIV and EHV-1. Also, all inoculated horses showed normal body temperature and no undesirable local or systemic reaction observed following primary and booster injections. There was no abortion in pregnant mares, or mice. The prepared vaccines were proven to be free from any contamination by bacteria, fungi, or other viruses.

Immunogenicity in guinea pigs

Guinea pigs inoculated with both combined vaccine batches lyophilized and liquid (A and B). Data in Tables 1, 2 and 3 revealed that the mean EI-HI antibody titer were 9.6 $\log_2$ and 10.2 $\log_2$, respectively. The mean ELISA antibody titer against EHV-1 were detected 3 WPI as 422 and 322.8, respectively. The antibody titer has a considerable increase of about 2-fold at two weeks post the 2nd dose with mean ELISA titer of 936.6 and 939.6, respectively. The mean antibody titers against tetanus toxin after 2 weeks post second dose (5WPI) were 37 and 37.6 IU/ml inoculated with vaccine A and B, respectively.

Table 1. EI-HI antibody titer in sera of guinea pigs inoculated with both combined vaccine batches lyophilized and liquid (A and B).

Guinea Pigs No.	Group A inoculated with Lyophilized vaccine		Group B inoculated with Liquid vaccine		Group C non- inoculated control	
	0 Time	3WPI	0 Time	3WPI	0 Time	3WPI
1	0	10*	0	10*	0	0
2	0	9	0	10	0	0
3	0	10	0	10	0	0
4	0	9	0	10	0	0
5	0	10	0	11	0	0
Mean	0	9.6	0	10.2	0	0

WPI: week post inoculation; *: EI-HI antibody titers expressed as log2

Table 2. EHV-1 antibody titer in sera of guinea Pigs inoculated with both combined vaccines lyophilized and liquid (A and B).

Guinea pigs (No.)	Group A inoculated with Lyophilized vaccine			Group B inoculated with Liquid vaccine			Group C control non inoculated		
	0 Time	3WPI	5WPI	0 Time	3WPI	5WPI	0 Time	3WPI	5WPI
1	0	510**	1142	0	164	563	0	0	0
2	0	600	1240	0	100	415	0	0	0
3	0	260	461	0	550	1560	0	0	0
4	0	430	1130	0	380	950	0	0	0
5	0	310	710	0	420	1210	0	0	0
Mean	0	422	936.6	0	322.8	939.6	0	0	0

WPI: week post inoculation; **: ELISA antibody titer= Reciprocal of the highest dilution of serum showed positive result.

Immunization of horse

Immune response against EIV

The antibody titer was estimated using HI test against EIV (Table 4). EIV-HI mean antibody titer in horse sera groups inoculated with both combined vaccine batches lyophilized and liquid (A and B) were 6.25 log_2 and 6.5 log_2, respectively, at two-week post inoculation (WPI). Then it increased to 8 log_2 and 7 log_2 respectively, at 2 weeks post booster dose and reach the peak at 2MPI as 9.5 log_2 and 9.75 log_2, respectively. EIV-HI mean antibody titer in sera of horse groups A and B began to decline gradually until reach 6 log_2 and 6.5 log_2, respectively, at 7MPI.

Immune responses against EHV-1

The antibody titer level against EHV-1 in horse sera inoculated with both combined vaccine batches, (A-lyophilized and B-liquid) were detected using ELISA (Table 5). The 1st dose of both vaccines' batches stimulated the horse's immune response and produced mean ELISA antibody titer at two WPI as 629 and 515, respectively. Moreover, there was a significant increase in antibody titers 2-fold or more at 2MPI of 2700 and 2767.5, respectively. Then decreased gradually to 530 and 575, respectively at 7MPV.

Immune responses in horses to tetanus

Evaluation of antibody titer against Tetanus Toxoid (Table 6) shown that the maximum antibody titer was reported at 3MPI as 9 IU and 10 IU, respectively, then the titer reached to lower level after 7 MPI (0.5IU) for vaccines together.

Regarding the statistical analysis using T-test between groups in Tables 1-6, there were no significant differences between previous groups in which P≥ 0.05.

Assessment of keeping quality of the prepared vaccine batches (A and B) in guinea pigs

The immune responses of guinea pig inoculated with the prepared vaccine batches (A and B) kept for different intervals (Zero, 1, 2, 2.5 and 3 years) at 4°C shown at Figs. 1, 2 and 3. The mean EI-HI antibody titer produced by the two vaccine batches didn't change after one year of incubation at 4°C as it was stable at 9.6 log_2, 10.2 log_2. Then decreased gradually till 8.4 log_2 at 3 years for batch A and 6.6 log_2 at 2.5 years for batch B. Also, there was no considerable change in mean ELISA antibody titer against EHV-1 produced by the two vaccine batches after one year of incubation at 4°C. It begins at zero time with 422 and 322.8 at 3WPI; respectively, then increased to 936.6 and 939.6 at 5WPI. It was 410 and 320 at 3WPIat one year after incubation at 4°C; respectively, then it increased to 930, 830 for both at 5WPI. The produced antibodies titer decreased gradually to 200 at 3WPI and 510 at 5WPI after 3 years of incubation for batch A and 280 at 3WPI and 600 at 5WPI after 2 years of incubation for batch B. On the other hand, the antibody titer against tetanus toxin TN produced by the two vaccine batches didn't change after one year of incubation remain at 37IU/ml and 37.6IU/ml, respectively then decline gradually till 36.2 IU/ml after 3 years of incubation for batch A, and 32.5IU/ml after 2 years of incubation for batch B.

Focusing on the statistical analysis performed using Student's T-Test between groups in Figs. 7, 8 and 9, when the prepared vaccines (lyophilized, liquid) were evaluated for their keeping quality, it was found that there were significant differences (P < 0.05) between the groups that were vaccinated with lyophilized and liquid vaccines, where lyophilized vaccine gave a significant in-

Table 3. Tetanus antitoxin titer in sera of guinea pigs inoculated with both combined vaccines lyophilized and liquid (A and B) tested by TN test.

Guinea pigs No.	Group A inoculated with Lyophilized vaccine		Group B vaccinated with Liquid vaccine		Group C non- inoculated control	
	0 Time	5WPI	0 Time	5WPI	0 Time	5W PI
1	0	38	0	39	0	0
2	0	37	0	36	0	0
3	0	36	0	37	0	0
4	0	39	0	40	0	0
5	0	35	0	36	0	0
Mean	0	37	0	37.6	0	0

Table 4. EI-HI antibody titers in horses vaccinated with both combined vaccines lyophilized and liquid (A and B).

Time of sampling	EI-HI antibodies titer in sera of horse inoculated with						
	Lyophilized Vaccine (A)			Liquid Vaccine (B)			Control Non inoculated
	H1*	H2	Mean	H3	H4	Mean	
0Time	0	0	0	0	0	0	0
2WPI	6.5**	6	6.25	6	7	6.5	0
(B)4WPI	6	6	6	6	6	6	0
1.5MPI	7.5	8.5	8	6.5	7.5	7	0
2MPI	9	10	9.5	9	10.5	9.75	0
3MPI	9	9.5	9.25	9.5	10	9.75	0
4MPI	8	9	8.5	9	9	9	0
5MPI	7.5	8.5	8	8	8.5	8.25	0
6MPI	6	8	7	7	8	7.5	0
7MPI	6	6	6	6	7	6.5	0

WPI: week post inoculation; MPI: month post inoculation; *: Horse number, B: Booster dose; **: EI-HI antibody titers expressed as log2

crease in HI, ELISA and Tetanus antibody titers when it was compared with the liquid vaccine results.

DISCUSSION

Egypt is one of the most famous countries in breeding and exportation of pure Arabian horses, which constitute a remarkable addition support to national income. The best approach for maintenance of horses breeding is fighting of disease by applying a good vaccine protocol against the more endemic infectious disease. Three of the more important infectious horse diseases are Equine Influenza (EI), Equine Herpesvirus-1 and Tetanus.

In this study, two types of vaccine were prepared, the first form (A) was a lyophilized inactivated Equine Influenza, Equine Herpesvirus-1 and Tetanus Toxoid vaccine that reconstituted with saponin as adjuvant and dissolvent. The second form(B) was a liquid inactivated EI, EHV-1 and TT vaccine reconstituted with saponin and Alhydrogel as adjuvant.

Regarding quality control measures on each type of vaccines, results revealed that absence of residual virus activity in each of the inactivated viral fluids of EIV and EHV-1. This result was proved by either hemagglutinating activity or pock lesion or cytopathic effect in both ECE and VERO cells, respectively (Madkour *et al*., 2016; Saleh *et al*., 2017). Moreover, no abnormal clinical findings were recorded including abortion, roughness, loss of weight, deaths, nervous signs and allergy on inoculated mice with TT (European Pharmacopoeia, 2009). In addition, there was normal body temperature, absence of undesirable local or systemic reaction in all inoculated horses after primary and booster injections. Also, no abortion presented in pregnant mares was recorded, these results confirm vaccine safety as mentioned by

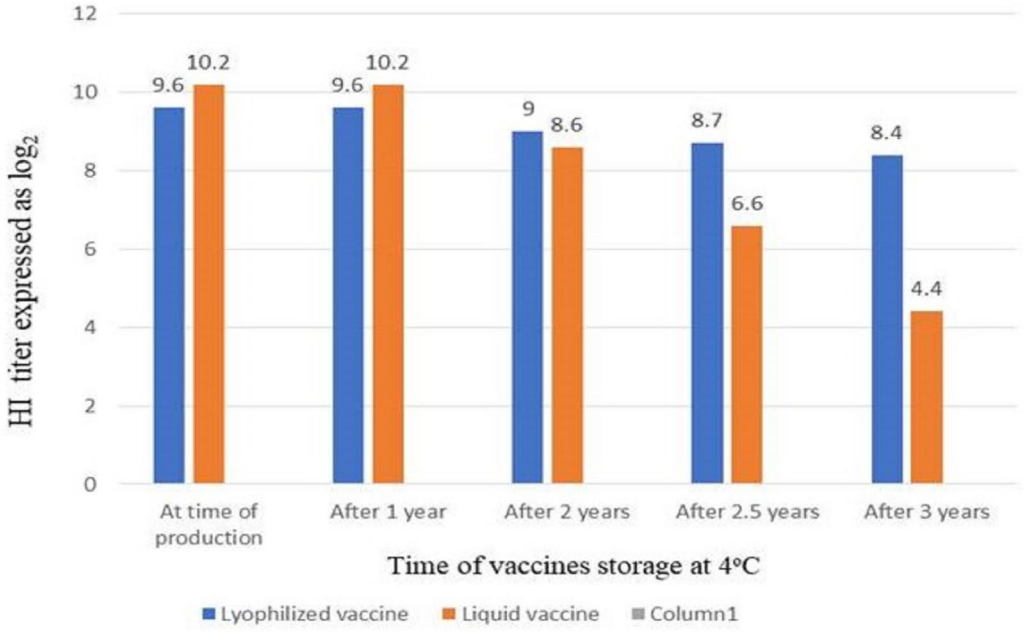

Fig. 1. EI-HI antibody titers in G. pigs inoculated by prepared vaccine batches (A and B) to evaluate its keeping quality at 4o C for different time.

Table 5. EHV-1 antibody titers in horses vaccinated with both combined vaccines lyophilized and liquid (A and B) tested by ELISA.

Time of sampling	EHV-1 antibodies titer in sera of horse inoculated with						Control Non inoculated
	Lyophilized Vaccine batch (A)			Liquid Vaccine batch (B)			
	H1*	H2	Mean	H3	H4	Mean	
0Time	95**	95	95	90	90	90	100
2WPI	658	600	629	530	500	515	100
(B)4WPI	1885	1700	1792.5	1800	1680	1740	95
1.5MPI	1950	1850	1900	1910	1830	1870	100
2MPI	2760	2640	2700	2675	2560	2767.5	95
3MPI	2100	1900	2000	2040	1840	1940	90
4MPI	1780	1690	1735	1750	1700	1725	100
5MPI	1500	1480	1490	1560	1510	1535	95
6MPI	1160	1100	1130	1250	1130	1190	90
7MPI	560	500	530	620	530	575	95

WPI: week post inoculation; MPI: month post inoculation; *: Horse number; B: Booster dose; ELISA antibody titer= Reciprocal of the highest dilution of serum showed positive result.

Table 6. Mean Tetanus antitoxin titer in sera of horses vaccinated with both combined vaccines lyophilized and liquid (A and B).

Time of sampling	0-Day	1MPI	1.5MPI	2 MPI	3 MPI	4 MPI	5 MPI	6 MPI	7 MPI
Lyophilized Vaccine A	0	0.5 IU	1.5 IU	7 IU	**9 IU**	7 IU	2 IU	1 IU	0.5 IU
Liquid Vaccine B	0	0.5 IU	1.5 IU	8IU	**10** IU	7 IU	3 IU	1 IU	0.5 IU

OIE (2020).

The potency and immunogenicity of the prepared vaccine batches evaluated in G. pig were clarified in Tables 1-3. The mean EIV antibody titer which tested by HI after 3 weeks post inoculation was 9.6 log_2 in group A and 10.2 log_2 in group B, that exceed the protective level against EIV (6 log_2) as reported by OIE (2019b) and Ebied *et al.* (2009). The mean EHV-1 antibodies titer tested by ELISA were 422 in group A and 322.8 in group B at 3 weeks post inoculation, which had a significant increase about 2-fold 5 WPI (two week post the 2nd dose) with mean ELISA titer of 936.6 in group A and 939.6 in group B. This result agrees with Guo *et al.* (1989) and Madkour *et al.* (2016). These results confirmed that the prepared combined vaccine batches were potent and immunogenic.

Comparing the potency of the two types of vaccines against tetanus toxin in guinea pigs clarified that the mean antibody titer was 37 IU/ml and 37.6 IU/ml for vaccine A and B, respectively (Table 3), and these results indicated that there was no significant difference between the two types of vaccine. Also, these results achieved the minimum allowable limit required for approving of tetanus toxoid according to the Code of Federal Regulations (2005) and European Pharmacopoeia (2009) who recorded that the tetanus vaccine used in horses should be produce an average antibody titer not less than 30 IU/ml in G. Pigs.

The immunogenic response of the prepared vaccine batches tested in horses (Tables 4, 5 and 6). Group A was inoculated with lyophilized inactivated vaccine and group B was inoculated with liquid vaccine showed that EIV antibody titer were detectable at 2WPI with mean titer of 6.25 log_2 and 6.5 log_2, respectively, which reached peak at 2MPI (9.5 log_2,9.75 log_2). Then it decreased

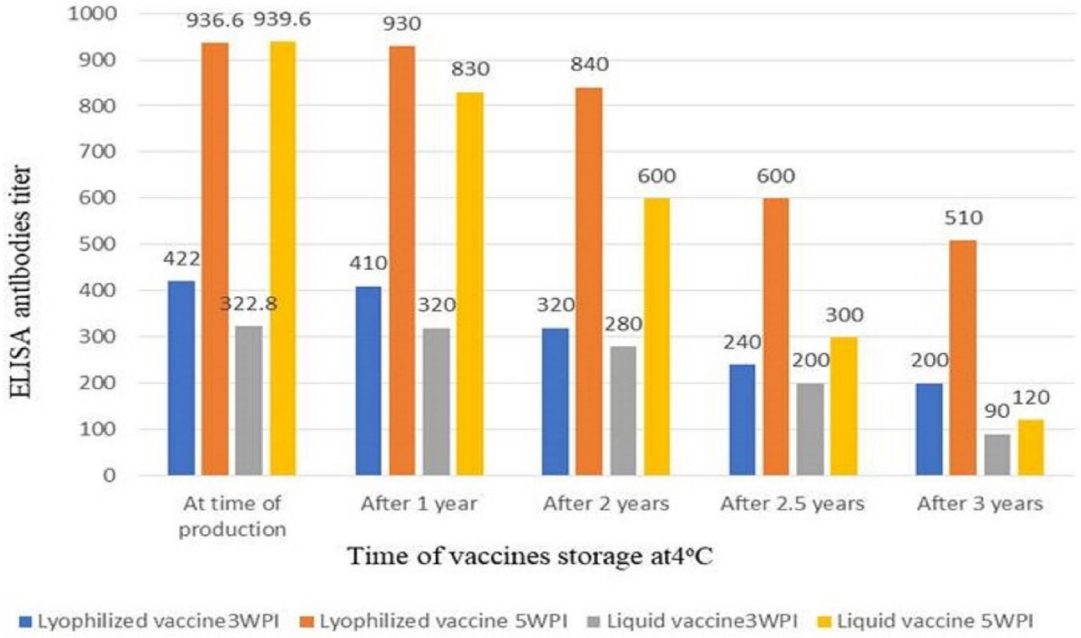

Fig. 2. Mean EHV-1 ELISA antibodies titer in G. pigs inoculated by prepared vaccine batches (A and B) to evaluate its keeping quality at 4o C for different time. WPI: week post inoculation; **ELISA antibody titer= Reciprocal of the highest dilution of serum showed positive.

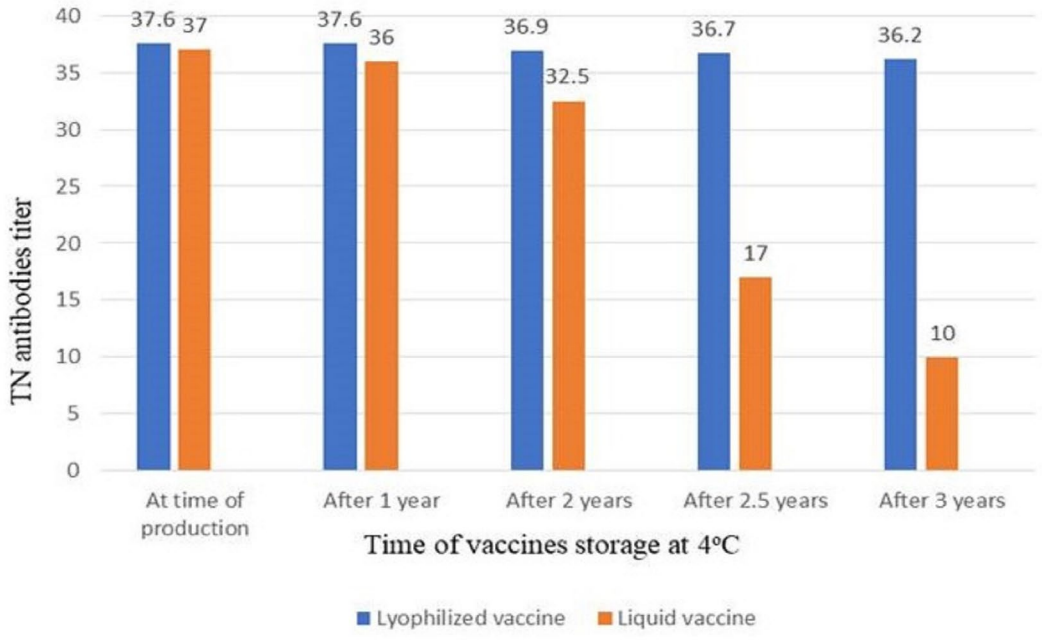

Fig. 3. Mean Tetanus antitoxin titer in sera of G. pigs vaccinated by different types of vaccines (A and B) to evaluate its keeping quality at 4ºC at different periods after production.

gradually from 4MPI, and remains within the protective level until 7MPI with mean titer of 6 log_2 and 6.5 log_2, respectively. This result agrees with OIE (2019b) who reported that the protective level of EI-HI antibody titer must not be lower than (64) 6 log_2.

Serum samples in horses of groups A and B, which inoculated with the prepared vaccine showed that EHV-1 antibodies with mean ELISA titer of 629 and 515, respectively at 2WPI, reached the peak at 2MPI with 4 fold increasing to 2700 and 2767.5, respectively. Then it decreased to 7MPI with mean titer of 530 and 575, respectively. This result agrees with Bannai *et al.* (2014) who reported that ELISA antibody titer begins to increase in the 14th day post injection then achieved its peak at two months with 4-fold increasing, which means a valuable immune response.

Evaluation of the antibody titer against tetanus toxin in horses inoculated by both vaccine batches (A and B), data in Table 6 showed that the maximum TN antibody titer was noticed at 3MPI as 9IU/ml and 10IU/ml, respectively (Barile *et al*., 1970). Tetanus antitoxin titers stays at the protective level for up to seven PI (0.5 IU/ml) for both vaccines as reported by Löhrer *et al*. (1970) and Heldens *et al*. (2001) who mentioned that TN antibody titer of 0.01 IU/m1 is considered protective.

The stability of the prepared vaccine determined through measuring the immune response in G. pigs inoculated with the prepared vaccine batches (A and B) after being kept at 4°C for interval time (0-1,5-2-2,5-3 year) (Figs. 1, 2 and 3). The mean EI-HI antibodies titer produced by the two vaccine batches don't change after one year of storage at 4°C, and remain stable at 9.6 log_2 and 10.2 log_2, respectively. Then it decreased gradually till 8.4 log_2 at 3 years for lyophilized vaccine and till 6.6 log_2 at 2.5 years for liquid vaccine that exceed the protective level (6 log_2) against EIV (OIE, 2019b), and while in batch B, EI HI antibodies titer was 4.4 log_2 after 3 years of storage at 4°C which is less than the protective level.

Also, there was no significant change in mean ELISA antibody titer against EHV-1 produced by the two vaccine batches after one year of storage at 4°C, begin at zero time with 422 and 322.8 at 3WPI: respectively. Then it increased to 936.6 and 939.6 at 5WPI, respectively. Furthermore, at one year after storage at 4°C, the titer were 410 and 320 at 3WPI, respectively, then it increased to 930 and 830 for both at 5WPI. The produced antibodies titer decreased gradually till 200 at 3WPI and 510 at 5WPI after 3 years of storage for batch A and (280 at 3WPI and 600 at 5WPI) after 2 years of storage for batch B. In this study, there was more than 2-fold increase in ELISA antibodies titer between the serum samples of the inoculated G. pigs at 3WPI and 5WPI. In case of lyophilized vaccine, its keeping was at 4°C for 3 years, but in case of liquid vaccine for 2.5 years only. This result agrees with Guo *et al.* (1989) and Madkour *et al.* (2016).

A near result was reported in test of antibody titer against tetanus toxoid produced by the two vaccine batches after storage for various interval at 4°C. The TN remain at 37 IU/ml and 37.6 IU/ml, respectively, after one year then declines gradually till 36.2 IU/ml after 3 years of storage for batch A, and 32.5 IU/ml after 2 years of storage for batch B. The lyophilized vaccine was stable, and it produced a protective range of humeral response when kept in 4°C for 3 years while, liquid vaccine was stable for 2 years (Plowright *et al*., 1970; Ebied, 2005).

CONCLUSION

The first vaccine batch (A) which contains freeze dried inactivated EIV, EHV-1, TT reconstituted in time of inoculation with saponin as adjuvant gives a high level of stability and more keeping quality for 3 years compared to batch (B) liquid form which was stable for 2 years. The two batches give the same level of potency and immunogenicity in G. pigs and Horses.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest to disclose.

REFERENCES

Bahnemann, H.G., 1990. Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethyleneimine. Vaccine 8, 299-303.

Bannai, H., Mae., N., Ode, H., Nemoto, M., Tsujimura, K., Yamanaka, T., Kondo, T., Matsumura, T., 2014. Successful Control of Winter Pyrexias Caused by Equine Herpesvirus Type 1 in Japanese Training Centers by Achieving High Vaccination Coverage. Clin. Vaccine Immunol. 21, 1070–1076.

Barile, M.F., Hardegree, M.C., Pittman, M., 1970. Immunization against neonatal tetanus in New Guinea. 3 The toxin neutralization test and the response of guinea pigs to the toxoids as used in immunization schedules in New Guinea. Bull. WHO 43, 453-459.

Bayoumi, M., Abdelfattah,S., Abdelmoneim, M., Moustafa, N., Madkour, N.K., El-Helw H.A., 2018. Field Evaluation of the prepared combined equine influenza, equine herpesvirus-1and tetanus toxoid vaccine in horses. Benha Vet. Med. J. 34, 289-299.

Chambers, T.M., 2020. A brief introduction to Equine influenza and equine influenza viruses. Methods Mol. Biol. 2123, 355-360

Code of Federal Regulations, 2005. Title 9, Part 113.114, U.S. Government Printing Office, Washington, DC, training centers by achieving high vaccination coverage. Clin. Vaccine Immunology 21, 1070-1076.

Cullinane, A., Gahan, J., Walsh, C., Nemoto, M., Entenfellner, J., Olguin-Perglione, C., Garvey, M., Huang, Fu T.Q., Venner, M., Yamanaka, T., 2020. Evaluation of Current Equine Influenza Vaccination Protocols Prior to Shipment, Guided by OIE Standards. Vaccines 8, 107.

Ebied, E.M., 2005 Trials for preparation of killed adjuvated vaccine for equine influenza. Thesis PhD, Vet. Med, University of Cairo, Egypt.

Ebied, E.M., Saleh, S.N., Madkour, N.K., Elkabbany, M.M.A., Soliman, I.M.A., 2009. Trials for preparation of monovalent inactivated Equine influenza vaccine. The Third Sci. Conf., Banha and RasSudr, Egypt.

El-Helw, H.A., 2007. A new medium for production of tetanus toxin. J. Egypt. Vet. Med. Assoc. 67, 125-131.

European Pharmacopoeia, 2009. *Clostridium tetani* vaccines (Veterinary suppl) ISBN; 9780113227990

Fu, S.W., Xue, J., Zhang, Y.L., Zhou, D.Y., 2004. Simplified purification method for *Clostridium* difficile toxin A. World J. Gastroenterol. 10, 2756- 2758.

Guo, P., Goebel, S., Davis, S., Perkus, M.E., Languet, B., Desmet'r, A.E.P., Allen, G.P., Paoletrt, E., 1989. Expression in recombinant vaccinia virus of the equine herpesvirus 1 gene encoding glycoprotein gpl3 and protection of immunized animals. J. Virol. 63, 4189-4198.

Hazrati, A., Ozawa, Y., 1965. Monovalent live horse sickness vaccine. Bull. Int. Epiz., 64, 688.

Heldens, J., Kersten, A., Weststrate, M., Van Den Hoven, R., 2001. Vaccinology: Duration of immunity induced by an adjuvanted and inactivated equine influenza, tetanus and equine herpesvirus 1 and 4 combination vaccine. Vet. Q. 23, 210-217.

Jansen, B.C., Knoetze, P.C., 1979.The immune response of horses to tetanus toxoid. Onderstepoort J. Vet. Res. 46, 211–216.

Laval, K., Poelaert, K.C.K., van Cleemput, J., Zhao, J., Vandekerckhove, A.P., Gryspeerdt, A.C., Garré, B., van der Meulen, K., Baghi, H.B., Dubale, H.N., Zarak, I., Crombrugge, E.v., Nauwynck, H.J., 2021. The Pathogenesis and Immune Evasive Mechanisms of Equine Herpesvirus Type 1. Front. Microbiol. 12, 662686.

Löhrer, J., Radvila, P., 1970. Active tetanus prophylaxis in the horse and the duration of immunity. Schweiz. Arch. Tierheilkd. 112, 307-314.

Madkour, N.K., Ebeid, E. M., Saleh, N.S., Warda, F.Y., El-Kabbany, M.M.A., Soliman I.M.A., 2016. Duration of immunity induced by combined vaccine against Equine influenza and Equine herpesvirus-1Veterinary Medical Journal – Giza, Faculty of Veterinary Medicine, Cairo University, Egypt.

Mark, W., Mellencamp, J., 2004. Chemically inactivated ehv-1 kya virus; and an adjuvant which includes cross-linked olefinically unsaturated carboxylic acid polymer. Patents, publication number US 6803041 B2.

Mohamed, S.A., Khafagy, H.A., Shafik, N.G., Yahia, A.I.S., 2022. Detection of humoral immune response induced in horses vaccinated with inactivated Equine Herpes virus vaccine. Investigation 7, 21.

Mumford, J.A., Bates, J., 1984. Trials of an inactivated equine herpesvirus-1 vaccine; challenge with a subtype 2 virus. Vet. Rec. 114, 375-381

OIE, 2019b. Equine influenza (infection with equine influenza virus). Chapter 7,1-19.

OIE, 2019c. Equine Rhino Pnemonities. Manual of standards diagnostic tests and vaccines. Chapter 12-8.

OIE, 2020. OIE expert surveillance panel on equine influenza vaccine composition, OIE head quarter, 16 April 2020 conclusions and recommendation. Available online. https://www.OIE Int /scientific –expertise /specific-information-and-recommendation equine-influenza / accessed on 1 June 2020.

OIE, 2019a. Tests for sterility and freedom from contamination of biological materials Chapter 1, 1-9.

Plowright, W., Rampton C.S., Taylor W.P., Hernimank, A.J., 1970. Studies on RinderPest Culture Vaccine: lll. Stability of the Lyophilized Product. Res. Vet. Sci. 11, 71-81.

Ramon, G.A., 1922. Propos du titrage in vitro du serum antidiphtérique par la flocculation. Compt. Rend. Soc. Biol. 86, 813-815.

Reed, L.T., Munech, H., 1938. A simple method of estimating 50 % end points. Am. J. Hyg. 27, 493.

Saleh, N.S., 2006. Preliminary trials for production of equine viral abortion inactivated vaccine. Thesis PhD. Fac. Vet. Med, Virology Department, Benha University, Egypt.

Saleh, N.S., Ebeid, E.M., Warda F.F., Eldakhly, A.T., Madkour N.K., Elkabany, M.A., Soliman, I.M.A., 2017. Lyophilized EHV-1 vaccine inactivated and adjuvanted by Quillaja saponaria Molina extract. J. Vet. Sci.13, 123-134.

Sarah-kinney. J., 2020. Benfits of lyophilization and how to validate our aseptic process for lyophilized products. Brek shire sterile manufacturis. https://berkshiresterilemanufacturing.com/news/insights/benefits-of-lyophilization-and-how-to-reduce-contamination-risk/

Singh, B.K., Tandon, S.N., Virmani, N., 2006. Immune response to oil inactivated Equine herpesvirus -1 using different emulsifiers in horses. Indian J. Biotec. 5, 42-46.

Slater, I.D., Gibson J.S., Field, H.J., 1993. Pathogenicity of thymidine kinase - deficient mutant of EHV-1 in mice and specific pathogen free foals. J. Gen. Virol. 74, 819-828.

Sugiura,T., Kondo, T., Matsumura, T., Imagawa, H., Kamada, M., Ihara, T., 1997. Evaluation of enzyme linked immunosorbent assay for titration of antibodies to EHV-1. J. Equine Sci. 8, 57-61.

Tyrrell D.A., Valentine R.C., 1957. Assay of influenza virus particles by hemagglutination and electron microscopy. J. Gen. Microbiol. 16, 668-675.

Wilson, D.W., 1999. Vaccination program for foals and weanlings. Proceed. Anim. Conv. AHEP. 45, 254-252.