

**Original Paper****Immunogenicity of inactivated Equine Influenza (H3N8) virus vaccine with different adjuvants in equine**Fatma Fadel Warda<sup>1</sup>, Eman abd El-Menum Shosha<sup>2\*</sup>, Aml M. Abdel-Ra'ouf<sup>3</sup> and Magda Anes Kalad<sup>1</sup><sup>1</sup>Agriculture Research center (ARC); Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.<sup>2</sup> Faculty of Veterinary Medicine, New Valley University, Egypt.<sup>3</sup>Department of Animal Medicine (Infectious Dis.), Faculty of Veterinary Medicine, Aswan University, Egypt.**ARTICLE INFO****Keywords**

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**ABSTRACT**

Equine influenza virus (EIV) is of the important common and important respiratory infectious diseases of horses. Vaccination can reduce the prevalence and severity of disease. In the current study, a trial to improve the strength and duration of immune response through using of montanide oil ISA 206, saponin and *Mycobacterium pheli* extract as adjuvants for an inactivated EIV [A/equine 2/Alexandria (1-2008) (H3N8)] vaccine. The vaccine was inactivated by 0.003M binary ethyleneimine at 37°C for 18 hours. Safety and potency of the prepared vaccine were studied by inoculation into guinea pigs and horses then evaluated using haemagglutination inhibition (HI) test for humeral immune response. Cellular immune response evaluated in horses inoculated with inactivated EIV vaccine adjuvanted with *Mycobacterium pheli* extract using lymphocyte blastogenesis. The vaccine was safe and potent for guinea pigs and horses which inducing HI antibodies titer that persist for 10 months post-inoculation in vaccinated horses. EIV vaccine adjuvanted with montanide oil ISA 206 was more potent (antibody titer persisted up to 10 months) than the other vaccines adjuvanted either with saponin or *Mycobacterium pheli* extract. In conclusion, EIV vaccine adjuvanted with montanide oil ISA 206 can keep at 4°C for over one year and at room temperature for 6 months without effect on its immunogenic characters.

**1. INTRODUCTION**

Equine influenza virus (EIV) is one of the most highly contagious respiratory disease affecting horses worldwide. EIV is caused by viruses belong to a family *orthomyxoviridae*, genus influenza A virus which grouped two antigenically distinct subtypes represented by two reference strains, A/equine/Prague/1/56 (H7N7) (Equine influenza virus-1), and A/equine/Miami/1/1963 (H3N8) (Equine influenza virus-2) (Elton and Bryant, 2011; Pouwels et al., 2014 and Pavulraj et al., 2017). Equine influenza (EI) disease is an OIE-listed important disease in its Terrestrial Animal Diseases (OIE, 2020). EIV considered an important cause of respiratory diseases in breeding and racing horses (Perglione et al., 2016 and Gahan et al., 2019).

In Egypt, the first outbreak of EI disease recorded in October, 1989, as EIV subtypes-1 and 2 were isolated. The second outbreak appeared in winter, 1999-2000 as subtype-2 was isolated (Soliman et al., 2008). Also, the third outbreak occurred in June, 2008, as EIV subtype-2 was isolated (Soliman et al., 2008). EI leads to epithelial

destruction in the upper respiratory tract resulting in coughing, fever, nasal discharge, performance impairment and dyspnea with high morbidity rate (Laabassi et al., 2015; Gildea et al., 2016 and Favaro et al., 2018). Diseased horses and recovered ones without any complications must give a supportive care for one week/every feverish day to allow regeneration of the mucociliary apparatus (Bonnie and Dacvim, 2014).

Vaccination is the most efficient defense mechanism against EI disease (Cullinane et al., 2020). The vaccination aims to decreasing clinical signs and the convalescence period of the disease as well as reduction of the likelihood of secondary infection (Paillot et al., 2013 and Fougerolle et al., 2019). Mostly, the currently commercially equine influenza vaccines included, an inactivated whole virus and live attenuated vaccine, still restricted to the requirement of an accurate match among the produced vaccine and virus field strain (Paillot et al., 2013 and Kapoor et al., 2014). Vaccine formulation that enables a reduction in the number of different

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vaccinations required per year, will provide a protection from the most critical pathogens as EIV (Nashwa et al., 2016). In order to enhance the potency and longevity of humeral and cellular immune responses for inactivated vaccines, the antigen was mixed with an adjuvant to avoid insufficient immunogenicity of antigen (Paillot et al., 2006 and Steven et al., 2008). Furthermore, electing a right adjuvant is one of the successful keys for adjuvanted vaccine in the field (Parker et al., 2009). The right adjuvant should achieve the correct balance between efficacy & safety, animal species and its sensitivity (Steven et al., 2008 and Kinsley et al., 2016)., thus, in this study we try to improve the strength and duration of immune response by using of montanide oil ISA 206, saponin and non-specific immune stimulator of *Mycobacterium phlei* extract as adjuvant for inactivated locally prepared EIV vaccine .

## 2. MATERIAL AND METHODS

### 2.1 Ethical approval

The experiments and sampling done without making any exertion or hurt to the animals. This study carried out according to the regulation and procedures approved by the ethics committee on animal experimentation of Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo and the guide for the care and use of animals, NO.8023.

### 2.2 Equine influenza virus H3N8, antisera and adjuvants

#### 2.2.1 .Seed virus and antisera

Locally identified freeze dried EIV which designated as (A/equine-2/ Alexandria (1-2008) H3N8) (Magda et al., 2011 and OIE, 2019) egg passage number 3 (EP<sub>3</sub>) with haemagglutinating (HA) titer (11 log<sub>2</sub>/0.05 ml) and infectivity titer 9.3log<sub>10</sub> EID<sub>50</sub>/0.1 ml; used for vaccine preparation. Reference antiserum against A/equine-1/parague/1/56 (H7N7) and A/equine-2/ Miami/1/63 (H3N8) obtained from National Veterinary Laboratories, United States Department of Agriculture, veterinary services (NVSL, USDA, VS); used in the identity test .

#### 2.2.2 Binary ethyleneimine (BEI)

0.1M binary ethyleneimine (Aldrich chemical Co. LTD), dissolved in 100ml of 0.2 NaOH solution and used as virus inactivator according to (Eman, 2005).

### 2.2.3 Adjuvants

#### 2.2.3.1 Montanide oil ISA 206 (W/W) 50/50

Montonide ISA 206 water in oil in water (w/o/w) obtained from SEPPIC Company, Paris, France).

#### 2.2.3.2 Saponin

It obtained as power from E. Merck Company, 2mg/ ml of purified saponin in distilled water and autoclaved, then added to inactivated virus as (V/V) 2cm / horse (Eman et al., 2009 and Soliman et al., 2011).

#### 2.2.3.3 *Mycobacterium phlei* (*M. Phlei*) extract

Non-specific immune potentiator *M. Phlei*, 200 mg/ml gave as 2cm/horse was supplied by Bacterial Diagnostic Department, (VSVRI), Abbasia.

### 2.3 Animals

#### 2.3.1 Horses

Ten apparently healthy EIV seronegative horses (2-4years old), seven of them used to evaluate immunogenicity of the prepared vaccine and three were used as control (OIE, 2019) .

#### 2.3.2 Guinea pigs (*g. pig*)

Four groups of guinea pig (300-400) gm/weight (5/group): the first three groups determine the potency and the stability of the prepared vaccine, but the last one kept as a control.

### 2.4 .Inactivated egg adapted EIV vaccine Preparation

#### 2.4.1 .Embryonated chicken eggs (ECE)

Specific pathogen free (SPF-ECE) 9-11 days old used for virus propagation, egg infectivity titration and to detect the residual infective virus in the inactivated virus fluid (OIE, 2019).

#### 2.4.2 .Identity test

The identity of the vaccine seed EIV (A/equine-2/Alex. (1-2008) H3N8 EP<sub>3</sub> confirmed by HI test using reference antiserum against EIV subtype-1 and subtype-2 (OIE, 2019).

#### 2.4.3 Virus inactivation

Vaccine virus suspension of EIV subtype-2 with HA titer 11 log<sub>2</sub>/0.05 ml and infectivity titer 9.3 log<sub>10</sub> EID<sub>50</sub>/0.1 ml incubated with BEI in a final concentration (0.003 M) at 37oc for 24 hours (Eman, 2009).

### 2.5 Vaccination protocols

2 .5.1 .Addition of montanide oil ISA 206 adjuvant (W/W 50/50)Prepare separately 50 ml of inactivated virus and 58 ml of oil phase (W/W) in 250 ml beakers then worm up both phases at 30 °C± 2 °C .

#### 2.5.2 .Addition of saponin adjuvant

Equal volume of saponin solution and vaccine is mixed (V/V) then dispensed into vials, each of them contain 2ml that representing one horse dose. Each horse dose contains 2mg saponin (Eman et al., 2009 and Soliman et al., 2011).

#### 2.5.3 .Additional *Mycobacterim phlei* extract adjuvant

*M. phlei* prepared in trypsin (0.25%) solved in veronal buffer PH 7.2. Each 1ml contains 100mg *M. phlei* extract to be used as immune stimulant and each horse dose was 200mg of *M. phlei* extract (Mallick et al., 1985).

## 2.6 Vaccine quality control

### 2.6.1 Sterility test

Samples taken from the inactivated virus fluid then tested on different media for detection of bacterial, fungal and Mycoplasma contaminations (OIE, 2019).

### 2.6.2 Residual infective virus activity in ECE

0.4 ml of the undiluted inactivated virus fluid inoculated into the allantoic cavities of a group of SPF-ECE (9-11) days old and incubated at 35°C for 3 days. The allantoic fluid was harvested from the inoculated eggs then 0.2 ml of the harvested undiluted fluid was inoculated into a further group of SPF-ECE (OIE, 2019).

### 2.6.3 Safety test of the prepared inactivated EIV adjuvanted vaccine

Three horses, each one inoculated I/M with the vaccine dose (2ml/dose/horse) repeated 4 weeks later. The horses kept under observation for ten days after the second set of injection for recording any abnormal local or systemic reactions (OIE, 2019).

### 2.6.4 Potency test of the prepared inactivated EIV adjuvanted vaccine

Four seronegative groups of guinea pigs (5 guinea pigs/group); group A, B, C were inoculated subcutaneously (S/C) with 2ml of the of inactivated EIV adjuvanted vaccine prepared vaccine. Group (D) kept as control at the same condition of the experiment. Serum samples collected twenty one days post-inoculation from groups and tested for HI antibody titer using HI test (OIE, 2019).

### 2.6.5 Immune response of horses vaccinated with the prepared inactivated EIV adjuvanted vaccine

Four seronegative horses, each one inoculated intramuscular (I/M) with the prepared EIV vaccine (dose/2ml). Then they received a booster dose at 4<sup>th</sup> weeks after primary dose. Last group (D) was kept as control at the same condition of the experiment. Serum samples collected from horses at different intervals and screened for the immune response using HI test (OIE, 2019).

## 2.6.6 Lymphocyte blastogenesis assay

### 2.7.1 Cell-mediated immune response (CMI) evaluation using lymphocyte blastogenesis assay

Whole jugular blood samples collected with heparin anticoagulant from experimental horses vaccinated with inactivated EIV vaccine adjuvanted with *M.phlei* extract at 3,5,7,10,15,21 and 25 days post-vaccination (DPV) for evaluation of CMI response using XTT Cell Viability Assay Kit according to the Charles et al., (1978) and Lee (1984). The cell proliferation expressed as optical density (OD).

## 2.7 Haemagglutination test (HA) and Haemagglutination Inhibition test (HI)

For titration of vaccine viral fluid and evaluation of the humeral immune response of immunized g. pigs and horses according to (OIE, 2019).

## 2.8 Vaccines stability

### 2.8.1 Stability of the prepared inactivated EIV adjuvanted vaccine

Vials of inactivated EIV adjuvanted vaccine divided into 3 groups. Group (A) kept as liquid inactivated EIV vaccine adjuvanted with montanide oil ISA 206 at 4°C for over 18 months and at (25-28) °C for 6 months. Group (B), the liquid inactivated EIV vaccine adjuvanted with saponin kept at 4°C for 1 year. Group (C) kept as control at the same condition of the experiment.

## 2.9 Statistical analysis

Statistical analysis was carried out using the SAS/STAT guide, version 9. The one-way analysis of variance test compares the serology results between each group in guinea pigs and horses. The difference between the mean values was performed using the least significant difference as  $p \leq 0.05$ . T test analyze the results of stability test and CMI response evaluation between each group as  $p \leq 0.05$  (Gomez and Gomez, 1984).

## 3. RESULTS

### 3.1 Identity test

Vaccine seed EIV (A/equine-2/Alex. (1-2008) H3N8, EP<sub>3</sub>) was completely neutralizing (100%) by reference antiserum against EIV subtype-1 and subtype-2.

### 3.2 Virus inactivation

The infectivity titer of the prepared vaccine virus suspension obtained after the 3 passages in ECE as ( $9.3 \log_{10}$  EID<sub>50</sub>/0.1ml) and its haemagglutinating titer (1024 HA units/0.05 ml). The vaccine virus fluid inactivated with BEI at a final concentration 0.003M within 18 hours then the HA titer of the virus was (11  $\log_2$  HA units).

### 3.3 Residual infective virus activity test

Residual infective virus activity showed no haemagglutinating activity when inoculated into ECE 9-11 days old.

### 3.4 Sterility Test

Free of vaccine virus fluid as well as the final vaccine products from contamination with any biological materials confirmed by culturing on the different bacteriological and mycological media.

### 3.5. Inactivated EIV vaccine induces humoral immune response in g.pig

#### 3.5.1 HI antibody titers in g. pigs inoculated with the prepared inactivated EIV adjuvanted vaccine

Guinea pig's serum samples of group (A) Showed mean HI antibodies titer at 3weeks post inoculation of 2457, while in group (B) was (973) and group (C) was (192). But group (D) used as a control (Table 1 & Figure 1).

#### 3.5.2 Immunogenic potency of the prepared inactivated EIV vaccine

Serum samples of each g.pig group tested for HI antibody titer using HI test. Group (A) which inoculated with inactivated EIV vaccine adjuvanted with montanide oil ISA 206 showed HI antibody titer with a mean value (2457), and group (B) that inoculated with the inactivated EIV vaccine adjuvanted saponin was (973).while, the mean of HI antibody titer of group (C) was (192) which inoculated with the inactivated EIV vaccine adjuvanted *M.phlei* extract. Also, the analysis of variance test compares the serology results between each group. HI antibody titers in g.pigs sera gave a significant result at 0.05 and P-value was 0.01026 towards group A.

### 3.6 Inactivated EIV vaccine induces humoral immune response in horses

#### 3.6.1 Immunization of horse

Four groups of horses have no prior immunity to EIV, were inoculated with the different types of prepared vaccines and one group kept as control (Table 3 and Figure 2). Group (A) inoculated with inactivated EIV vaccine adjuvanted with montanide oil ISA 206, the 1<sup>st</sup> dose stimulated HI antibodies in the vaccinated horse's sera at 2weeks post-vaccination (WPV) with a mean value (64), by booster at 4<sup>th</sup> week post-inoculation, a peak of HI antibodies titer was obtained at 4.5<sup>th</sup> month with a mean titer (4096), then the titer decline gradually till 10<sup>th</sup> month post-vaccination with a considerable protective HI antibody level (64). Group (B) inoculated with inactivated EIV vaccine adjuvanted with saponin, the 1<sup>st</sup> dose stimulated the antibody response in the vaccinated horses sera at 2 WPV with a mean value (32), by booster at 4<sup>th</sup> week post- inoculation, a high level of HI antibodies titer at 3.5<sup>th</sup> month with a mean titer (1024), then the titer decreased gradually till 8<sup>th</sup> month

post-inoculation with a considerable protective HI antibody level (6), Group (C) inoculated with inactivated EIV vaccine adjuvanted with *Mycobacterium phlei* extract, the 1<sup>st</sup> dose stimulated HI antibodies in the vaccinated horses sera at 2 WPV with a mean value (24), by booster at 4<sup>th</sup> week post-inoculation, a high level of HI antibodies titer at 2.5<sup>th</sup> month with a mean titer (192), then the titer began to decline gradually till 4.5<sup>th</sup> month post-vaccination with a considerable protective HI antibody level (64). Also group (C) tested for CMI response. According to statistical analysis, one-way analysis of variance test compares the serology results of each group. HI antibody titers in horse's sera showed a significant result at 0.05 and the P-value was 0.000937 towards group A.

#### 3.6.2 Safety Test

The prepared inactivated EIV vaccine was safe for inoculated horses with neither abnormal local nor systemic reaction following initial and boosting injections.

### 3.7. Inactivated EIV vaccine induces cellular immune response in horses

There is a significant increase in CMI by lymphocyte proliferation assay which reaches its maximum level at 10<sup>th</sup> DPV with a mean of (0.68) in horses inoculated with inactivated EI vaccine adjuvanted with *M.phlei* extract (Table 4, Figure 3). Then, it decreased gradually till 25<sup>th</sup> DPV with a mean of (0.13). CMI response of horses showed a significant result at 0.05 as  $p \leq 0.05$  towards treatment group using T-Test

#### 3.8 Stability of inactivated EIV adjuvanted vaccine

The vaccine adjuvanted with montanide oil ISA 206 in group (A) can keep at 4° C for 18 months and at (25-28) °C for 6 months. Also, in group (B) the vaccine adjuvanted with saponin kept at 4° C for 1 year and can't keep at room temperature (28° C) (Table 2). Stability test results between each group compared using T test which demonstrated a significant result at 0.05 as  $p \leq 0.05$  towards treatment group A at 4° C. Also, there is a significant result at (25-28) °C at 0.05 towards treatment group A.

Table (1): HI antibody titers in guinea pigs sera inoculated with the prepared inactivated EIV adjuvanted vaccine.

No. of guinea pigs	* HI antibody titer Vaccinated Groups							
	Group (A)		Group (B)		Group (C)		Group (D) Control Group	
	Pre-inoculated samples	21 days post inoculation	Pre-inoculated samples	21 days post inoculation	Pre-inoculated samples	21 days post inoculation	Pre-inoculated samples	21 days post inoculation
1	zero	4096	zero	2048	zero	256	zero	zero
2	zero	1024	zero	1024	zero	128	zero	zero
3	zero	2048	zero	512	zero	256	zero	zero
4	zero	1024	zero	1024	zero	64	zero	zero
5	zero	4096	zero	256	zero	256	zero	zero
Mean	zero	2457	zero	973	zero	192	zero	zero

Group (A): Guinea pigs inoculated with inactivated EIV vaccine adjuvanted with montanide oil ISA 206. Group (B): inoculated with inactivated EIV vaccine adjuvanted with Saponin. Group (C): inoculated with inactivated EIV vaccine adjuvanted *Mycobacterium phlei* extract. Group (D): control group.

Table (2): Stability of the prepared inactivated EIV adjuvanted vaccine in guinea pigs.

Temperature	Time Stage	Mean HI antibody titer in sera of g. Pigs		
		Group (A)	Group (B)	Group (C)
4 °C	0 times	1433	716	zero
	6 month	1428	435	zero
	12 month	1400	102	zero
	15 month	1228	25	zero
	18 month	1225	15	zero
	0 times	1433	716	zero
Room temperature (25-28) °C	3 month	358	15	zero
	6 month	89	zero	zero
	7 month	50	zero	zero
	8 month	22	zero	zero

- Mean dilution giving the complete Heamagglutination.

- Inhibition antibody titer expressed as the reciprocal of serum inhibition of heamagglutination.

-Group (A): liquid inactivated EIV vaccine adjuvanted with montanide oil ISA 206 kept at 4°C and room temperature (25-28°C). Group (B): liquid inactivated EIV vaccine adjuvanted saponin was kept at 4°C. Group(C): control group.

Table (3): Seroconversion of horses inoculated with prepared inactivated EIV adjuvanted vaccine.

Time of sampling	HI Titer in sera of vaccinated horses									Group (D) control
	Group (A)			Group (B)			Group (C)			
	H1	H2	mean	H1	H2	mean	H1	H2	Mean	
Pre-vaccination	zero	zero	zero	zero	zero	zero	zero	zero	zero	
2wpv	64	64	64	32	32	32	16	64	24	
3wpv	256	128	192	64	128	96	64	64	64	
4wpv	128	64	96	32	64	48	32	32	32	
6wpv	512	256	384	64	128	96	64	64	64	
8wpv	512	512	512	128	512	112	64	128	96	
10wpv	1024	1024	1024	512	512	512	128	256	192	
12wpv	1024	1024	1024	1024	1024	1024	128	128	128	
3.5mpv	2048	2048	2048	1024	1024	1024	64	128	96	----ve
4mpv	4096	2048	3072	1024	512	768	64	64	64	
4.5mpv	4096	4096	4096	512	512	512	64	64	64	
5mpv	2048	2048	2048	512	128	320	32	32	32	
6mpv	2048	1024	1536	256	128	192	32	32	32	
7mpv	1024	512	768	256	64	160	32	16	24	
8mpv	512	256	384	128	64	96	16	16	16	
9mpv	256	128	192	64	32	48	zero	zero	zero	
10mpv	64	64	64	32	32	32	zero	zero	zero	
11mpv	zero	zero	zero	zero	zero	zero	zero	zero	zero	

Wpv: week post-vaccination. Mpv: month post-vaccination. (H): horse. HI titer: Heamagglutination antibody titer expressed as the reciprocal of serum dilution giving an inhibition of heamagglutination.

--ve: Negative results.

Table (4): Evaluation of CMI response of horses inoculated with the inactivated EIV vaccine adjuvanted with *Mycobacterium phlei* extract and control none vaccinated (The cell proliferation expressed as optical density).

Vaccinated horse groups	Days post-vaccination						
	3dpv	5dpv	7dpv	10dpv	15dpv	21dpv	25dpv
Group A	0.234	0.301	0.502	0.7816	0.531	0.221	0.11
Group B	0.211	0.22	0.281	0.581	0.42	0.181	0.15
Mean	0.223	0.261	0.392	0.681	0.476	0.201	0.13
Control group	0.186	0.183	0.172	0.16	0.14	0.14	0.13

W: weeks. M: months. Dpv: Days post-vaccination.

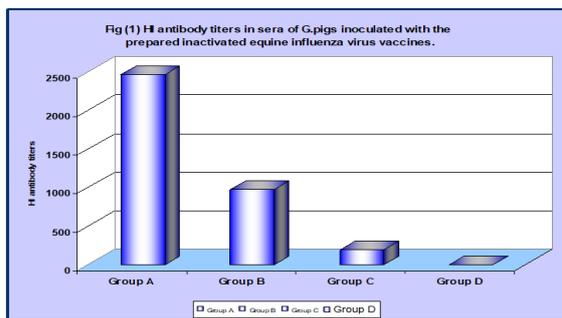


Fig. (1): HI antibody titers of guinea pigs sera inoculated with the prepared inactivated EIV adjuvanted vaccine.

Group (A): Guinea pigs inoculated with inactivated EIV vaccine adjuvanted with montanide oil ISA 206. Group (B): Inoculated with inactivated EIV vaccine adjuvanted with Saponin. Group (C): Inoculated with inactivated EIV vaccine adjuvanted *Mycobacterium phlei* extract. Group (D): Control group.

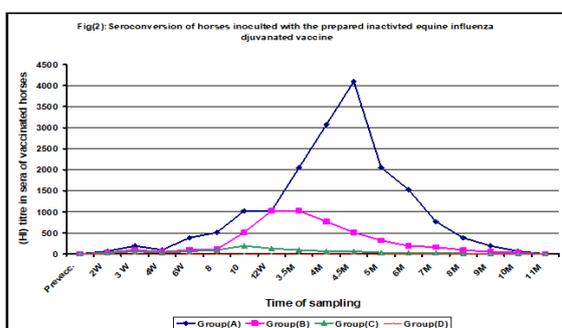


Fig. (2): Seroconversion of horses inoculated with inactivated EIV vaccine adjuvanted tested by HI test.

Prevacc: prevaccination. W: week. M: month. (H): horse. HI titer: Hemagglutination antibody titer expressed as the reciprocal of serum dilution giving an inhibition of hemagglutination.

Group (A): Guinea pigs inoculated with inactivated EIV vaccine adjuvanted with montanide oil ISA 206. Group (B): Inoculated with inactivated EIV vaccine adjuvanted with Saponin. Group (C): Inoculated with inactivated EIV vaccine adjuvanted *Mycobacterium phlei* extract. Group (D): Control group.

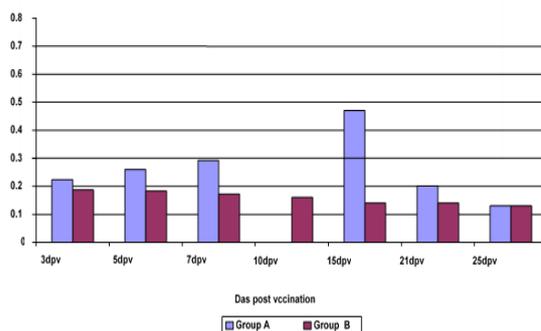


Fig (3): CMI response of horses inoculated with the inactivated EIV vaccine adjuvanted with *Mycobacterium phlei* extract and control none vaccinated (The cell proliferation expressed as optical density).

Group A: Horses inoculated with the inactivated EIV vaccine adjuvant with *Mycobacterium phlei* extract.

Group B: Control none vaccinated.

### 3. DISCUSSION

The enzootic nature of EI disease is maintained by developing clinical cases or unapparent infections in

susceptible horses especially after movement from different areas or countries.

Thus, vaccination remains a vital quick strategy and an effective long-term protection to overcome EI in conjunction with good Hygienic precautions (Kapoor et al., 2014 and Paillot et al., 2017).

Vaccination against EIV is widely used to prevent and control the disease. Despite the intensive use of EIV vaccines, outbreaks still occur. An efficient vaccine requires a good antigens and a preferable adjuvant to enhance the antigen immunogenicity (Steven et al., 2008 and Daly and Murcia, 2018). So, the current study shows a method to improve the strength and duration of immunogenicity using Montanide oil ISA 206, saponin and *M.pheli* extract as adjuvants for EIV vaccine. The infective titer of EIV (A/equine-2/ Alex. (1-2008) H3N8) which used for vaccine preparation was  $9.3 \log_{10} \text{EID}_{50}/0.1 \text{ ml}$  and its hemagglutinating titer (1024 HA units/0.05 ml). The vaccine seed virus should contain HA unites not less than  $9 \log_2 \text{ HA unites } /0.05 \text{ ml}$  and  $7 \log_{10} \text{ EID}_{50}/ 0.1 \text{ ml}$ , this result was in agreement with (Eman et al., 2009). The vaccine virus fluid inactivated with BEI at a final concentration 0.003 M within 18 hours at 37°C. HA titer of the virus after inactivation was not demonstrated any changes. BEI acts directly on the viral nucleic acid without any effect on the antigenic properties and these results agreed with (Hassanein, 1992). There are no any local or systemic reactions appeared on horses vaccinated with the prepared vaccine which confirming vaccine safety as recorded by (OIE, 2019). Concerning the vaccine sterility, the vaccine virus fluid proved to be sterile, free from all contaminant. The vaccine potency in g.pigs; group (A) serum samples which inoculated with inactivated EIV vaccine adjuvanted with montanide ISA 206 Showed mean HI antibodies titer at 3weeks post-inoculation of (2457). In group (B), the mean HI antibodies titer was (973) and group (C) was (192). While, the control group (D) showed negative results as the protective HI antibodies titer shouldn't less than (64) according to (OIE, 2019). In order to compare and evaluate the immunizing capacity of each prepared vaccine, 4 groups of horses inoculated with different types of adjuvanted vaccine. The results agreed with other previous studies as (Eman et al., 2009) and (OIE, 2019) who stated that EIV vaccine induces high antibodies level by adding a suitable adjuvant to the antigenic media. Also, (Soliman et al., 2011) who stated that saponin induces protective antibody levels with prolonged duration of immunity and can be safely used associated with various antigenic media. Abdelhady, (2016) mentioned that saponin based adjuvants can enhance the cell mediated immunity and humeral immunity with a low dose required for adjuvant activity. kamal et al., (2004) and Marcoss et al., (2005) decided that *M.pheli* extract based vaccines in horses is a potential use in enhancing CMI. There is a considerable protective antibody titer persisted up to (10) months for EIV montanide ISA 206 adjuvent vaccine and up to eight months for EIV saponin adjuvant vaccine, but it reaches up to 4.5 months for *M.pheli* extract adjuvant vaccin.

## 5. CONCLUSION

Immunization of horses with montanide ISA 206 adjuvant vaccine gives an adequate level of immunity better than the other adjuvants. *M.pheli* extract gives an immune response less than montanide ISA 206 and saponin .

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