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# IMMUNE RESPONSE OF HORSES TO INACTIVATED EQUINE INFLUENZA TISSUE CULTURE VACCINE

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## SUMMARY

Equine influenza is a common disease of the horse, causing significant morbidity worldwide. Vaccination is the most effective method of prophylaxis against influenza, designed to elicit a protective antibody response and resistance to reinfection. Cell-derived influenza vaccines are capable of providing equivalent protection as those obtained from egg-derived vaccines. In this study, inactivated equine influenza tissue culture (EITC) vaccine was prepared. Two different adjuvants were used, the first was saponin (2 mg / 1ml /dose) and the second was mineral oil (ISA-206, 50%W**:** W). The potency test of the prepared vaccines in Guinea pigs showed that the mean HI antibody titres at 3 weeks post inoculation were (665.6) and (1638.4) respectively. Testing the immunogenicity of the prepared vaccines in horses revealed that Primary dose of inactivated EITC vaccine adjuvanted with saponin and ISA -206 resulted in an initial increase in circulating HI antibodies in horses at 2 weeks post vaccination, reached the peak at 3 to 4 weeks post vaccination then decrease but remain within the protective level until 4 and 6weeks respectively. After the second vaccinal dose, there were much higher HI antibody titres, reached their maximum titre at 4 months (1024) and 6 months (2o48), with much longer duration of immunity within the protective level for 8 months (96) and 12 months (64) respectively. Testing the keeping quality of the prepared Inactivated EITC vaccine adjuvanted with ISA-206 showed that it is was stable at 4°C up to 15 months and for 6 months at room temperature. While the other vaccine adjuvanted with saponin was found to be stable at 4°C for 12 months and for 1 month at room temperature.

**Key words**: Equine influenza virus, MDCK, adjuvant, saponin, ISA-206, vaccine, Potency test, Immunogenicity.

## INTRODACTION

Equine influenza viruses (EIV) are major respiratory pathogens of horses, causing high morbidity and occasional mortality. Equine influenza (EI) is a highly contagious disease contracted by inhalation of infectious virus aerosols. EI is not only an important welfare issue but

can have a profound economic impact on the equine industry with major epidemics disrupting horse racing and breeding **(Bryant et al., 2010).** Antigenically, EI viruses are classified as influenza type A viruses belonging to the family Orthomyxoviridae. Two EIV subtypes have been recognized, A/Equi-1/H7N7 firstly isolated in 1956 **(Sovinova et al., 1958)** and A/Equi- 2/H3N8, firstly isolated in 1963 **(Wadell et al., 1963).** However, the H7N7 subtype has not been isolated from horses for over 25 years and is presumed not to circulate at present. The H3N8 subtype is responsible for widespread outbreaks in vaccinated and unvaccinated horses, and continue to be isolated to this day **(Martella et al., 2007, Bryant et al., 2009, Bountouri et al.,2011 and Elton and Bryant,2011).**

In Egypt, the first record of an unusual outbreak of equine influenza disease among equine populations was in October 1989, where influenza virus type A subtypes 1 and 2 were detected ***(*Ismail et al., 1990 and Esmat et al., 1992).** The second outbreak was recorded in 2000**,** where influenza virus type A subtype 2 was isolated and designated as A/equi-2/Cairo-2/2000(**Hamoda et al., 2001 and Nashwa, 2004)**. The third outbreak was recorded in June (2008) where subtype 2 was isolated and designated as A/equi/Egypt/6066 NAMRU3-VSVRI/2008 (**Soliman et al., 2008 and Magda et al., 2011)**.

Vaccination is the most cost-effective strategy to control and prevent influenza pandemics and seasonal epidemics. It reduces clinical signs of the disease, with subsequent improved animal welfear leading to a shortened convalescent period and reducing secondary infection. Also, it reduces shedding of the virus which has important implications for the spread of the infection **(paillot et al., 2006).** For decades, vaccine supply relied on embryonated chicken eggs as a substrate for influenza propagation. However, to cope with a potential shortage of eggs in a pandemic situation, to increase the flexibility of production campaigns and to avoid problems related to egg derived vaccines, large-scale mammalian cell culture systems were developed for human and veterinary influenza vaccines. **(Nayak et al., 2005; Widjaja et al., 2006, Schulze-Horsel et al.,2008; Audsley and Tannock, 2008 and Gregersen et al., 2011).**

Significant efforts of authorities and vaccine manufacturers over the last decade have led to the establishment of cell culture-derived influenza vaccines. **(Michael et al., 2011, Shao-Zhen Feng, et al., 2011 and Kun Liu et al., 2012).** An effective vaccine needs not only good antigens but also preferable adjuvant to enhance the immunogenicity of antigen. The adjuvant was used to enhance humoral and cellular immune responses **(Harold et al., 2005).**W/O/W emulsions have low viscosity, easily injectable, safe, stable and have the ability to enhance short and long term immune response, they induce humoral mediated immune response. The antigen in the external aqueous phase is immediately available to the immune system and antigen in the internal aqueous phase is slowly released **(Aucouturier and Ganne, 2000).**

ISA-206, mineral oil adjuvant used in vaccine since it is able to induce not only a specific humoral immune response but also a specific cellular immune response. **(Langellotti et al., 2011).** Saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity **(Oda et al., 2000, Rajput et al., 2007 and Xiaoming Songa & Songhua Hu 2009).** In a previous study the locally isolated equine influenza virus (**A/equi-2/Egypt/6066 NAMRU3-VSVRI/2008)** was successfully propagated and adapted to MDCK cell line and yielded a high titre enough for vaccine preparation, two adjuvants and different doses were used. A preliminary study conducted to test the prepared vaccine in G. pigs, proving it to be safe and potent and further work is needed for tracing the immunoefficiency of the prepared vaccine in horses (target animal**) (Nashwa et al.,2012).** So, the present study was planned for monitoring the immune response of horses to inactivated equine influenza tissue culture vaccine adjuvanted either with saponin or ISA-206.

## MATERIALS AND METHODS

### Virus

The locally identified EIV (A/equi- 2/Egypt /6066 NAMRU3-VSVRI/2008) egg passage three (EP3) – $MDCK_3$ with HA titre 10 $\log_2$

and infectivity titre ٩.5 $\log_{10}$ $TCID_{50}$/0.1 ml was used for vaccine preparation (**Nashwa et al; 2012**).

**Antisera**

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

**Identity test**

Identity of the selected vaccine seed virus EI (A/equi/Egypt/6066 NAMRU3-VSVRI/2008) EP3 $MDCK_2$ was confirmed by HI test using reference antisera against EI virus subtype 1 and 2 **(OIE, 2012).**

**Haemagglutination test (HA) & Haemagglutination Inhibition test (HI)**They were conducted according to the method described by **OIE (2012).**

**Virus infectivity assay**

Titration of infectious virus was performed on MDCK cells according to **Smith et al. (2008)**. The 50% tissue culture infective dose ($TCID_{50}$) was determined via the (**Reed and Muench method, 1938)**.

**Preparation of inactivated equine influenza tissue culture vaccine**

The virus fluid of EI- $EP_3$ $MDCK_3$ with HA titre 10 $\log_2$ and infectivity titre 9.5 $\log_{10}$ $TCID_{50}$/0.1 ml was inactivated with binary Ethyleneimine (BEI) at a final concentration 0.003 M with continuous stirring at 37°C for 24 hours, followed by immediate addition of sterile sodium thiosulphate at a final concentration 2% to stop the action of BEI on the virus and neutralize the toxic action of residual inactivator on target host **(U.S. Pat. NO. 6803041 B2 & Nashwa et al; 2012).**Inactivated EITC virus fluid was examined for residual infective virus activity in ECE and MDCK cells **(OIE, 2012).**

**Embryonated chicken eggs (ECE)**

Specific pathogen free (SPF), 9 to 11 day old embryonated chicken eggs (ECE) were obtained from Koum Oshiem Farm, Fayoum, Egypt and used for detection of the residual infective virus activity.

**Madin-Darby Canine Kidney cells (MDCK)**
Obtained from National Research Center and used for detection of the residual infective virus activity.

## Addition of adjuvant

Two equal parts of inactivated EITC virus fluid were mixed with either one of two different adjuvants, the first was saponin (2 mg / dose) **(Soliman et al., 2011)** and the second was mineral oil (ISA-206, 50%W**:** W) **(Aucouturier and Ganne, 2000),** as described by Seppic protocols and dispensed in convenient bottle and stored at 4-8 $^{o}$C.

## Sterility and safety testing of the prepared vaccine

They were performed according to **OIE (2012).**

## Potency test of EITC vaccine in G. pigs

Thirty seronegative G. pigs were divided into 3 groups (10 G. pigs/group). Group (A) was inoculated subcutaneously (S/C) with 1 ml of the prepared EITC vaccine adjuvanted with saponin (2mg/dose), while group (B) was inoculated subcutaneously (S/C) with 1 ml of the prepared EITC vaccine adjuvanted with mineral oil ISA-206. Group (C) was kept as control under the same conditions of the experiment. Twenty one days post inoculation, serum samples were collected from all groups and tested for HI antibody titre using HI test **(OIE, 2012).**

## Testing the immunogenicity of EITC vaccine in horses

Six seronegative horses were divided into 3 groups:

### Group (A)

Two horses were inoculated deep intra muscularly (I/M) with two doses (1ml) of the prepared EITC vaccine adjuvanted with saponin (2mg/dose), four weeks apart.

### Group (B)

Two horses were inoculated with two doses of the prepared EITC vaccine adjuvanted with mineral oil (ISA-206) four to six weeks apart.

Each dose (1ml) was injected deep (I/M) in the lower third of the neck (**Nashwa et al; 2012**).

### Group (C)
Two horses were kept at the same conditions as a control.

Serum samples were collected at different intervals till one year and screened for the immune response using HI test. The horses were also observed for signs of disease and untoward reactions to vaccination.

### Stability of the prepared EITC vaccine
Random vials of each vaccine were divided into two groups. The first group was kept at 4°C. The second group was kept at room temperature (25-28°C). Samples from each group of the vaccine were taken separately every three months and tested for its potency in Guinea pigs.

## RESULTS AND DISCUSSION
Equine influenza is a common disease of horses, causing significant morbidity worldwide. Vaccination is the most effective method of prophylaxis against influenza, designed to elicit a protective antibody response and resistance to reinfection. The WHO recommended the use of established mammalian cell culture lines as an alternative to egg-based substrates in the manufacture of influenza vaccine since 2006 **(Alexander and Scott 2009).**

Cell-derived influenza vaccines are capable of providing equivalent or even better protection in animal models than those obtained from egg-derived vaccines. In addition, these vaccines were found to be safe and highly efficacious in humans. Cell-based flu vaccines offer a number of advantages over the traditional method: (a) cell lines are fully characterized and in compliance with regulatory guidelines; (b) the raw materials for production are defined and can be easily produced in a short period. There are two licensed and regulatory-approved continuous cell lines being used for influenza vaccine production: MDCK (Madin-Darby canine kidney) cells and Vero (African green monkey kidney) cells. Vero cells have been widely approved for manufacturing other viral vaccines but MDCK cells are only licensed for influenza vaccines. **(Tseng et al. 2011 and Hu et al. 2011)**

In this study, EI-EP3-MDCK3 of HA titre 10 log2 /0.05 ml and infectivity titre 9.5 log10 TCID50/0.1 ml was used for preparation of

inactivated vaccine, Table (1).The virus was inactivated with Binary ethyleneimine (BEI) at a final concentration 0.003 M at 37°C for 24 hours **(nashwa, 2012)** HA titre of the virus after inactivation not show any changes (10 log2 HA unit). This was in agreement with **Kucera and Beckenhaur (1977)** who prepared EI (A/equi-2) inactivated vaccine from the field strain with 9 log2 to 10 log2 haemagglutinating units. In the present study, saponin and ISA- 206 were used as adjuvants for the prepared vaccine.

The potency of the prepared vaccines in Guinea pigs was demonstrated by the obtained data in table (2) and Fig. (1),Guinea pigs serum samples of group (A) which inoculated with (1ml) of the prepared EITC vaccine adjuvanted with saponin and group (B) inoculated with (1ml) of ISA-206 oil adjuvanted vaccine, showed that the mean HI antibody titres at 3 weeks post inoculation were (665.6) and (1638.4) respectively, exceeded the protective level (64) reported by **(OIE 2012)**.From the obtained results, it is clear that EITC vaccine adjuvanted with ISA-206 oil induces much higher HI antibodies than that adjuvanted with saponin.

It was found that vaccinated horses remained clinically free from signs of equine influenza during the time following vaccination; in addition no untoward local or systemic reactions were observed followed vaccination with any of the prepared vaccine formulae, confirming the vaccine safety as recommended by **(OIE 2012)**.Table (3) and Fig. (2) Clarify that the first dose of EITC vaccine adjuvanted with saponin was able to stimulate HI antibodies in horses at 2 weeks post vaccination(96) ,which reached the peak at 3 weeks (128) then decrease but remain within the protective level until 4 weeks. By boostering at the 4$^{th}$ week post inoculation higher level of HI antibodies were obtained. Antibodies reached their maximum titre at 4 months (1024), then began to decline gradually till 8 months post vaccination (96), but remain within the protective level **(Rajput et al., 2007)** .

Also, Primary dose of EITC vaccine adjuvanted with mineral oil (ISA - 206) resulted in an initial increase in circulating HI antibodies at 2 weeks

post vaccination (48) which reached the peak at 4 weeks post vaccination (384) then decrease but remain within the protective level until 6weeks.After the second vaccinal dose (6weeks) there was much higher HI antibody titre reached their maximum titre at 6 months (2048). It remains within the protective level up to 12 months (64). This was in agreement with **Chukwuedo and Nimzing (2011)** who reported that, the antibody titres obtained with single and repeated inoculations of (FMD) vaccines formulated with montanide ISA 206 adjuvant gave good responses and protection from the challenge.

It is clear that EITC vaccine adjuvanted with ISA 206 induced higher average antibody titres as well as longer lasting immune response in horses than those induced by saponin. These results agree with **Cloete et al., (2008)**who reported that, Oil (ISA- 206) based FMD vaccines can provide early protection against infection, also induced higher antibody titres in animals that received a booster vaccination, and able to protect for longer time periods than the aluminium hydroxide gel-saponin (AS) vaccine formulations.

The keeping quality of the prepared vaccines was studied at different temperature for 15 months. Inactivated EITC vaccine adjuvanted with ISA-206 was found to be stable at 4°C for 15 months and for 6 months at room temperature. While the other vaccine adjuvanted with saponin found to be stable at 4°C for 12 months and for 1 month at room temperature. These results agree with **Cloete et al., (2008)** who found that Oil (ISA- 206) based FMD vaccines more stable at 4 °C than for formulations (AS), and **Aucouturier and Ganne, (2000)** who reported that (ISA 206) based vaccines were stable at 4 °C for two years and six months at room temperature.

In conclusion, it was found that cell based equine influenza vaccine provide equivalent protection in horses as egg drived vaccine with the advantage that it is safe and easly produced with in a short time. EITC vaccine either adjuvanted with saponin or ISA-206(mineral oil) induced good immunogenic effect but the vaccine adjuvanted with ISA-206 induced higher and longer lasting antibody response .Also ISA-206 adjuvanted vaccine was more stable at 4 °C and at room temperature.

Table (1): Titre of EI virus

| EI-EP3-MDCK3 | HA titre * | Infectivity titre ** |
|---|---|---|
| | 10 | 9.5 |

* HA titre expressed as $\log_2$ /0.05 ml.

**Infectivity titre expressed as $\log_{10}$ $TCID_{50}$/0.1 ml.

Table (2): EI-HI antibody titres in sera of Guinea pigs inoculated with different formulae of inactivated equine influenza tissue culture vaccine

| Mean EI-HI antibody titre in G.pigs sera | | | | | |
|---|---|---|---|---|---|
| Group (A) | | Group ( B ) | | Group (C) | |
| Pre-inoculation | 21 days post inoculation | Pre-inoculation | 21 days post inoculation | Pre-inoculation | 21 days post inoculation |
| 0 | 665.6 | 0 | 1638.4 | 0 | 0 |

Group (A):G. pigs inoculated with 1 ml EITC vaccine adjuvanted with saponin.
Group (B):G. pigs inoculated with 1 ml EITC vaccine adjuvanted with ISA-206.
Group (C): Control group.

Table (3): EI –HI antibody titres in horses vaccinated with different formulae of inactivated equine influenza tissue culture vaccine

| Time of sampling | EI –HI antibody titres in sera of horses | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Group (A) | | | Group (B) | | | Group (C) | |
| | H1 | H2 | Mean | H3 | H4 | Mean | H5 | H6 |
| Prevacc. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 WPV | 64 | 128 | 96 | 32 | 64 | 48 | 0 | 0 |
| 3 WPV | 128 | 128 | 128 | 64 | 128 | 96 | 0 | 0 |
| (b) 4WPV | 64 | 64 | 64 | 256 | 512 | 384 | 0 | 0 |
| 5 WPV | 128 | 256 | 192 | 128 | 256 | 192 | 0 | 0 |
| (b)* 6WPV | 512 | 256 | 384 | 64 | 128 | 96 | 0 | 0 |
| 2MPV | 512 | 512 | 512 | 512 | 256 | 384 | 0 | 0 |
| 3 MPV | 1024 | 512 | 768 | 512 | 512 | 512 | 0 | 0 |
| 4MPV | 1024 | 1024 | 1024 | 1024 | 512 | 768 | 0 | 0 |
| 5 MPV | 512 | 512 | 512 | 1024 | 1024 | 1024 | 0 | 0 |
| 6 MPV | 256 | 512 | 384 | 2048 | 2048 | 2048 | 0 | 0 |
| 7MPV | 256 | 256 | 256 | 1024 | 1024 | 1024 | 0 | 0 |
| 8 MPV | 128 | 64 | 96 | 1024 | 512 | 768 | 0 | 0 |
| 9 MPV | 64 | 32 | 48 | 512 | 256 | 384 | 0 | 0 |
| 10 MPV | 32 | 16 | 24 | 256 | 64 | 160 | 0 | 0 |
| 11MPV | 16 | 16 | 16 | 128 | 64 | 96 | 0 | 0 |
| 12MPV | 8 | 8 | 8 | 64 | 64 | 64 | 0 | 0 |

Group (A):Horses inoculated with 1 ml EITC vaccine adjuvanted with saponin.

Group (B): Horses inoculated with 1 ml EITC vaccine adjuvanted with ISA-206.

Group (C): Control group

(H): Horse (WPV): week post vaccination (b): Boostering Group (A)

(MPV)month post vaccination (b)*: Boostering Group (B)

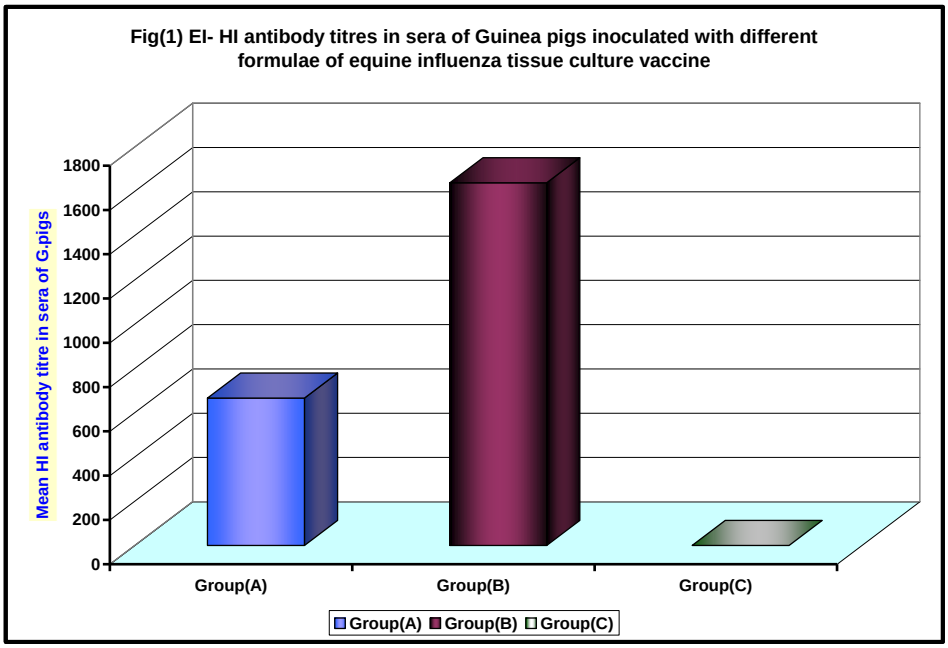
Table 4:Stability of different formulae of EITC vaccine in Guinea pigs

| Temperature of storage (°C) | Time of storage | Mean EI-HI antibody titre in G.pigs sera | | |
|---|---|---|---|---|
| | | *Group (A) | **Group (B): | ***Group(C) |
| 4°C | 0 | 665.6 | 1638.4 | 0 |
| | 3 Months | 614.4 | 1433.6 | 0 |
| | 6 Months | 512 | 1433.6 | 0 |
| | 9 Months | 128 | 1228.8 | 0 |
| | 12 Months | 89.6 | 1228.8 | 0 |
| | 15 Months | 32 | 1024 | 0 |
| Room Temperature (25°C-28°C) | 0 | 665.6 | 1638.4 | 0 |
| | 1Month | 64 | 1433.6 | 0 |
| | 3 Months | 35.2 | 614.4 | 0 |
| | 6 Months | 0 | 128 | 0 |
| | 9 Months | 0 | 35.2 | 0 |
| | 12 Months | 0 | 0 | 0 |

***Group (A):G. pigs inoculated with 1 ml EITC vaccine adjuvanted with saponin.**

****Group (B):G. pigs inoculated with 1 ml EITC vaccine adjuvanted with ISA-206.**

*****Group (C): Control group.**


Fig(1) EI- HI antibody titres in sera of Guinea pigs inoculated with different formulae of equine influenza tissue culture vaccine
Mean HI antibody titre in sera of G.pigs
1800
1600
1400
1200
1000
800
600
400
200
0
Group(A)
Group(B)
Group(C)
Group(A) Group(B) Group(C)

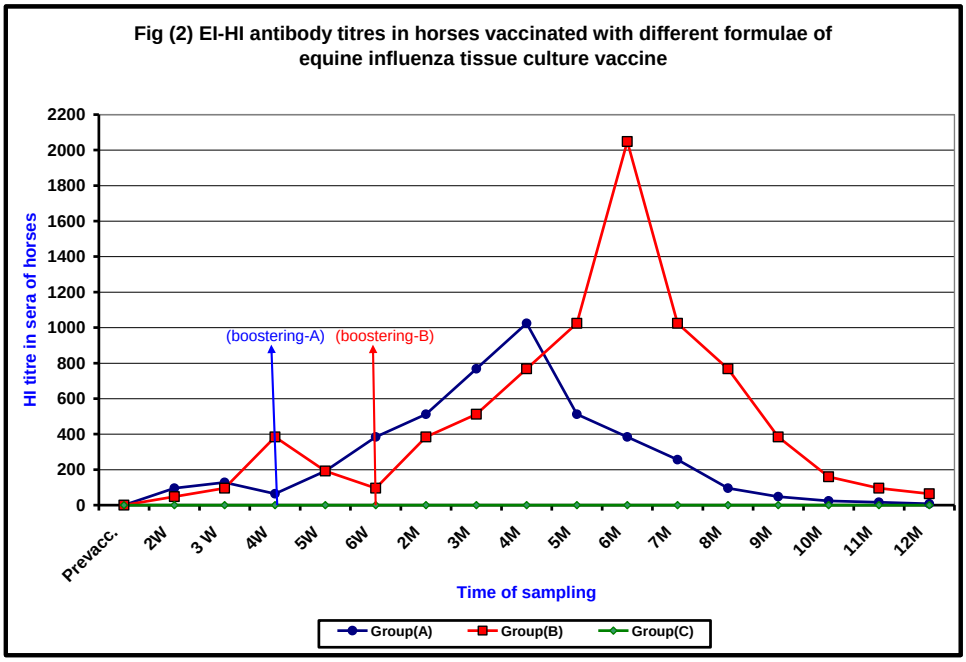


Fig (2) EI-HI antibody titres in horses vaccinated with different formulae of equine influenza tissue culture vaccine
HI titre in sera of horses
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
(boostering-A)
(boostering-B)
Prevacc.
2W
3 W
4W
5W
6W
2M
3M
4M
5M
6M
7M
8M
9M
10M
11M
12M
Time of sampling
Group(A)
Group(B)
Group(C)

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# الإستجابه المناعية للخيول المحصنة بلقاح نسيجي مثبط لإنفلونزا الخيول

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## الملخص العربى

مرض الإنفلونزا من الأمراض الشائعة فى الخيول فى العالم. يظل التحصين هو الوسيلة الأكثر فاعلية للحصول على الاجسام المضادة لتجنب المرض وتقليل الأعراض المصاحبة له.و قد وجد أن لقاح الإنفلونزا المحضر علي الخلايا النسيجية قادر علي الحماية من المرض بدرجة مساوية للقاح المنتج علي أجنة البيض. في هذة الدراسة تم تحضير لقاح نسيجي مثبط لإنفلونزا الخيول. تم استخدام نوعين من المحفزات – الأول الصابونين (٢ مجم /١ ملى/جرعة) و الثانى زيت -ISA 206( W /W%٥٠ ) وكانت الجرعة ١ ملى . وقد ثبتت كفاءة كل من اللقاحين عند حقنهم فى الخيول والأرانب الهندية وذلك بقدرتهم على إنتاج مستويات عالية من الأجسام المناعية. حيث كان متوسط الاجسام المناعية للقاحين فى الأرانب الهندى بعد ثلاث اسابيع من الحقن باستخدام اختبار التلازن الدموى المثبط (٦٦٥,٦) و( ١٦٣٨,٤) على التوالى. كانت الاستجابة المناعية للخيول باستخدام اختبار التلازن الدموى المثبط بعد الجرعة الاولى من اللقاحين عند اسبوعين(96) و(48) على التوالى، ووصلت الى اقصى معدل لها عند الاسبوع الثالث و الرابع على التوالى ثم أخذت فى النقصان مع الاحتفاظ بمعدل الحماية حتى ٤ و٦ اسابيع على التوالى . بعد الجرعة التعزيزية ظهر مستوى أعلى من الاجسام المناعية ووصلت الى اقصى معدل لها عند الشهرالرابع (١٠٢٤) فى الخيول المحفزة بمادة الصابونين، وعند الشهر السادس (٢٠٤٨) فى الخيول المحفزة بزيت ISA-206 واستمر معدل الاجسام المناعية مدة أطول فى سيرم الخيول المحصنة حتى ٨ شهور (٩٦) و١٢ شهر (٦٤) علي التوالى.

تم تقييم كفاءة اللقاحين بعد حفظهما عند درجات حرارة مختلفة لمدة خمسة عشر شهر. وقد وجد أن اللقاح المحفز بزيت ISA-206 أحتفظ بكفائتة المناعية حتى ١٥شهرعند ٤ م° و لمدة ٦ شهور عند ٢٥-٢٨ م°.بينما اللقاح المحفز بالصابونين أحتفظ بكفائتة المناعية لمدة١٢شهر عند ٤ م° و لمدة شهر عند ٢٥-٢٨م°.