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Immune Response Induced by Carbomer Adjuvanted Equine Influenza Vaccine

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Abstract

Vaccination against Equine influenza virus (EIV) is necessary to minimize and prevent spreading of the disease. There are many types of natural and synthetic adjuvants which can improve the efficacy of animal vaccines, such as aluminum compounds, emulsions, saponins, and carbomer. The current study was carried out to compare inactivated Equine influenza vaccines containing carbomer as an adjuvant in concentrations of 0.25% and 0.50% (formula 2 & 3) with an inactivated vaccine containing a combination of aluminum hydroxide gel and saponin as a conventional adjuvant (formula 1). The potency of the different vaccine formulations was tested in groups of guinea pigs. The mean HI- antibodies titer 3 weeks post inoculation of group (A) with formula-1, group (B) with formula-2 and group (C) with formula-3 were 9.2 $\log_2$, 10.4 $\log_2$ and 10.6 $\log_2$. All formulae were potent with higher HI antibodies in group B compared to group A. Groups B and C had nearly the same results. Stability testing of the prepared vaccines (formula 1&2) proved that both formulae were potent and stable at 4ºC for two years. Seroconversion of the prepared vaccines (formula 1&2) in horses revealed that EI vaccine adjuvanted with carbomer induced higher HI-antibodies titer as well as longer lasting immune response in horses than those induced by combination of aluminum hydroxide gel & saponin.

KEYWORDS
Adjuvant, Carbomer, Aluminum hydroxide gel, Saponin, Equine influenza vaccine.

INTRODUCTION

One of the costliest respiratory conditions affecting horses, equine influenza (EI) has an economic impact on the equine industry and equestrian competitions. In developing countries where equids are still often utilized as working animals, EI also exacts a hefty price. The best method for managing and preventing EI outbreaks in horse populations is still vaccination (Fatai *et al*., 2021). According to Paillot *et al.* (2013), there are three different types of equine influenza vaccinations: whole inactivated vaccines, live attenuated vaccines, and vaccines based on viral vectors. The original and still most widely used form of vaccine for many years was whole-inactivated EIV (Paillot, 2014).

The choice of an appropriate viral strain, the antigen concentration of the vaccine, the adjuvant, and the immunization schedule are some of the crucial variables that affect how the body reacts to inactivated vaccinations (Holmes *et al*., 2006). According to Daly (2011) and Cullinane (2022), the subtype H3N8 of influenza A is the parent genus of all present EIV strains.

Equine influenza virus type A subtype H3N8 was isolated in Egypt, designated as A/equi-2/Cairo-2/2000 (Hamoda *et al*., 2001: Nashwa *et al*., 2004) and A/Equi/Egypt/6066 NAMRU3-VSVRI/2008 (Soliman *et al*., 2008; Magda *et al*., 2011).

Since the 1970s, polyacrylic acid polymers known as carbomers have been used in veterinary medicine. It has effective adjuvant qualities. These polymers are currently used as adjuvants in licensed inactivated and vector-based equine influenza vaccinations. It strengthens and prolongs antibody reactions (Mumford *et al*., 1994; Paillot; 2014; Burakova *et al*., 2018).

It might improve cellular immunity. Additionally, it stimulated the production of both Th1 and Th2 cytokines, such as high titers of IFN-, IL-2, and IL-4, as well as a Th1 isotype-switched antibody response from antigen-specific T cells. Through enhanced APC antigen absorption, it can stimulate Th1 cell responses (Krashias *et al*., 2010; Dey *et al*., 2012). Previous research suggested that these crosslinked polymers (carbomers) could capture and release antigenic chemicals over time (Lai *et al*., 2012).

The absence of any evidence of local or systemic toxicity is one of carbomer's other benefits. Since there is no direct interaction with the antigen and it can be mixed with the carbomer gel by shaking, its conformation is preserved.

In this investigation, carbomer was used as an adjuvant in place of the more traditional adjuvants saponin and aluminum hydroxide gel to prepare the Equine Influenza vaccine. The produced vaccines' potency and storage quality were assessed in guinea pigs. Following a series of two vaccination doses in horses, the persistence of antibodies was observed.

MATERIALS AND METHODS

Ethical Approval

The Animal Ethics Committee of the Veterinary Serum and Vaccine Research Institute (VSVRI) approved this work. All tests adhere to the VSVRI guidelines for using animals in research.

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Virus

The vaccine was made using an egg passage three (EP3) isolate of the locally discovered freeze-dried Equine influenza virus (A/equi-2/Egypt/6066 NAMRU3-VSVRI/2008). Equine Viral Disease Research, Veterinary Serum & Vaccine Research Institute (VSVRI), Abbasia, Egypt, provided the EIV.

Antisera

The National Veterinary Laboratories, United States Department of Agriculture and Veterinary Services (NVSL, USDA, VS) provided reference antisera against A/equi-1/parague/56 (H7N7) and A/equi-2/Miami/63 (H3N8), which were used to confirm the virus identity using the Hemagglutination Inhibition (HI) test.

Animals

Horses

Six adult horses that appeared in good health and had low antibody titers against the EIV were utilized to assess the efficacy and immunogenicity of the currently manufactured vaccinations.

Guinea pigs

To assess the immunogenicity, potency, and stability of the produced vaccines, groups of guinea pigs (5 and 10) weighing roughly 350–450 g were utilized (OIE, 2019).

Specific pathogen free- Embryonated chicken eggs (SPF-ECE)

Koum Oshiem Farm, Fayoum, Egypt provided the SPF-ECE, which were used for EIV propagation, infectivity titration, and the detection of residual infective virus activity.

Adjuvant

Aluminium hydroxide gel with low viscosity (Alhydrogel LV) was used as an adjuvant then stored at 4-8°C (Eman *et al*., 2009).

The adjuvant saponin (Quillaja extract) was purchased from ACROS Co. (New Jersey, USA). It is created by dissolving 1 g in 100 ml of PBS, filtered and thwn sterilized to create a stock of 10 mg/ml (Bayoumi *et al*., 2018).

Carbomer

It was purchased as a fluffy white powder from Lubrizol Co., To create two different quantities of aqueous stock solutions (0.5 and 1%), it was dissolved in hot water. According to the United States Pharmacopeial Convention (1990), the produced solutions were sterilized by autoclaving at 121°C for 20 min. before being stored at 4°C until further use.

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

They were conducted according to the method described by OIE (2019).

Egg infectivity titration for EIV

SPF-ECE of 9-11 days old were inoculated with 0.1ml of serial tenfold dilution of the infected egg fluid (4 ECE / dilution) inta-allantoic sac route. The eggs were incubated at 35˚C for 72 hours, then chilled overnight according to Tyerell and Valentine (1957). The infectivity titer given as $\log_{10}EID_{50}$/ 0.1ml according to the method established by Reed and Muench (1938).

Preparation of EI vaccine with different formulations

The allantoic sac method was used to propagate the EI seed virus for two further passages (Ep5) in SPF-ECE that was 9–11 days old. The eggs were incubated at 35°C for 3 days. The amnio-allantoic fluids (virus fluid) were harvested and clarified by centrifugation at 1500 rpm for 15 minutes then tested for sterility, haemagglutinating activity which expressed as $\log_2$ (HA unit/0.05 ml) by using HA test and infectivity titer also expressed as $\log_{10}EID_{50}$/0.1 ml.

Virus inactivation

Binary ethyleneimine (BEI) (with a final concentration 0.003 M) was used to inactivate vaccine virus fluid (EI-Ep5 with HA titer 10 $\log_2$ and infectivity titer 10 $\log_{10}$ EID_{50}/0.1 ml) while stirring continuously at 37°C for 24 hours. After inactivation, sterile sodium thiosulphate (of a final concentration of 2%) was applied right away to end BEI's impact on the virus and neutralize any remaining toxicity of the inactivator on the target host (Eman, 2005).

Residual infective virus activity test

To ensure full virus inactivation, this test was run on the inactivated virus fluid shortly after the inactivation process.

A set of ECE 9–11 day old (ten eggs) were injected with undiluted inactivated EIV and placed in an incubator at 35 °C for three days. The inoculated eggs' allantoic and amniotic fluids are harvested, pooled, and passed into new egg groups (ten eggs), where they are incubated for three days at 35 degrees Celsius. These egg groupings shouldn't exhibit any haemagglutinating activity (OIE, 2019).

Vaccine formulation

Three formulae of EI vaccine were prepared as follow:

Formula (1), inactivated EIV suspension was adjuvanted with combination of saponin (1 mg/horse dose) and 20% Alhydrogel using magnetic stirrer at 4ºC for 24 hours (Eman *et al*., 2009; bayoumi, 2018).

Formula (2), inactivated EIV suspension was adjuvanted with carbomer 0.25% (50% carbomer 0.5% to 50% antigen).

Formula (3), inactivated EIV suspension was adjuvanted with carbomer 0.5% (50% carbomer 1% to 50% antigen)

The pH was adjusted to 7-7.5. The prepared EI vaccine was dispensed in vials, each contained one ml representing the horse dose and contained not less than 10 $\log_2$ HA unites/0.05ml (Kucera and Bechenhaur, 1977). All vaccine vials were caped and kept at 4˚C.

Quality control testing of the prepared vaccine

Sterility test of the prepared vaccine

Both the finished products and the virus-inactivated fluid were tested. To rule out bacterial, fungal, and mycoplasma contaminations, samples from them were grown on several medium (Nutrient agar, Sabouraud agar, Thioglycolate broth (Oxford, England)) (OIE, 2019b).

Safety

Two horses were given one millilitre of each of the manufactured vaccine formulations I/M, and each horse received two doses separated by a month. After the second dose, all horses were monitored for two weeks while maintaining appropriate hygiene, and body temperatures were taken.

Potency in guinea pigs

Four sets of five seronegative guinea pigs (G. pigs) each had a total of twenty G. pigs.
Formula (1) was used to administer the inactivated EI vaccine by subcutaneous injection (S/C) to Group (A).
Formula (2) was used to administer 1 ml of the inactivated EI vaccine to Group (B).
Formula (3) was used to administer 1 ml of the inactivated EI vaccine to Group (C).
Group (D) was maintained as a control group under identical test settings.

Serum samples were taken from all groups twenty-one days after the vaccination. Following potassium periodate treatment, these samples were evaluated for HI antibody titer using the HI test (OIE, 2019b).

Stability of EI vaccine

Vials of each formula of the inactivated EI vaccine were kept at 4ºC for 27 months to evaluate the stability of the produced vaccines. Five guinea pigs were used each time to test the potency of the samples, which were obtained at intervals of 0, 12, 24, and 27 months. Five G. pigs were kept in the control group under the identical conditions as the experiment but were not vaccinated.

All sera samples were analyzed for EI- HI antibody titer utilising the HI test following potassium periodate treatment, per the instructions in (OIE, 2019b).

Immunogenicity In horses

Six (2-4 years old) local breed horses with low HI antibody titers (4 against EIV) or seronegative status were separated into three groups.
Formula (1), an inactivated EI vaccine, was administered to Group (A) during the vaccination.
Formula (2), an inactivated EHV-1 vaccine, was administered to Group (B) during the vaccination.

As a control, Group (C) was maintained under identical circumstances to the tests.

Two doses of the manufactured vaccinations were administered to each group, spaced one month apart. Each dose was administered by injection (I/M) into the lower neck third (Wilson, 1999). These horses were closely monitored to observe any regional or global reactions.

Sample collection

All horses had their serum samples taken at various intervals, including two weeks after the first dose and booster injection, then monthly for seven months after the booster injection.

Serological testing

Following potassium periodate treatment, the immunological response was assessed using the HI test, as per (OIE, 2019b).

RESULTS

Potency in G. Pigs

As shown in Table 1, G. pigs were inoculated with three formulae of vaccine; formula (1) in Group (A) that was inoculated with inactivated EIV adjuvanted with combination of saponin 1 mg/horse dose and 20% Alhydragel and the results revealed that the mean EI-HI antibody titer were 9.2 $\log_2$ and Group B that was inoculated with formula (2) inactivated EIV adjuvanted with carbomer 0.25% (50% carbomer 0.5% to 50% antigen), the mean EI-HI antibody titer was 10.4 $\log_2$. While, Group (C) that was inoculated with formula (3) inactivated EIV adjuvanted with carbomer 0.5% (50% carbomer 1% to 50% antigen), the mean EI-HI antibody titer was 10.6 $\log_2$. Regarding to the statistical analysis performed using ANOVA Test between the three groups, it was found that there was a significant difference ($P<0.05$) between the three groups and on applying the Student's T-Test between the groups (A x B, A x C, B x C), it was found that the significant difference was found in ANOVA test is attributed to the significant difference (at $P<0.05$) between Group (A) and Group (B), also between Group (A) and Group (C), while there was no significant difference ($P>0.05$) between Group (B) and Group (C).

Table 1. EI- HI antibodies titer in sera of Guinea pigs inoculated with the prepared vaccine formulae.

Guinea pigs No.	EI-HI antibodies titer in Guinea pig sera three WPI		
	Group (A)	Group (B)	Group (C)
1	8	10	11
2	9	10	11
3	9	10	11
4	10	11	10
5	10	11	10
Mean	9.2	10.4	10.6

WPI: weeks post inoculation.

Evaluation of the Stability of the prepared vaccines kept at 4ºC tested in groups of G. pigs

Table 2 shows the immune responses in G. pigs inoculated with the prepared vaccine formula kept for different intervals time (Zero, 12, 24, and 27 months) at 4°C. The mean EI-HI antibodies titer produced by the two vaccine formulas (1, 2) was 9.2 $\log_2$, 10.6 $\log_2$ at zero time) respectively and after 24 months of incubation at 4°C stabled at (9 $\log_2$, 10.4 $\log_2$) respectively, then decreased to (8 $\log_2$) at 27 months for formula 1 and (9.2 $\log_2$) at 27 months for formula B cleared that the two formula were potent and stable for 2 years at 4°C.

Focusing on the statistical analysis performed using Student's T-Test between group (A) and group (B) in Table 2, it was found that there were significant differences ($P<0.05$) between the both groups, where Group (B) (using formula 2) gave significant results when compared with group (A) (using formula 1).

Immunogenicity In horses

The antibody titer against EIV was evaluated using HI test (Table 3). EI-HI mean antibody titer in sera of horse groups A and B inoculated with both vaccine Formulas (1 and 2) were (7 $\log_2$-8 $\log_2$), respectively at two-week post inoculation (WPI), which

increased to (8.5 log_2-9 log_2), respectively 2 weeks post booster dose. Antibodies reached their maximum titer at 3 months post vaccination in group A (10 log_2) and 3 to 4 months post vaccination in group B (10.5 log_2), then began to decline gradually in all horses till 7 months post vaccination (6.5 log_2, 8 log_2 respectively) but remain within the protective level 64 (6 log_2).

Table 2. Stability of the prepared vaccines kept at4oC tested in groups of guinea pigs.

Vaccine Formulae	Guinea pigs Group	Time of storage	Mean HI antibodies titer
Formula (1)	1	Zero time	9.2
	2	12 months	9.2
	3	24 months	9
	4	27 months	8
Formula (2)	5	Zero time	10.6
	6	12 months	10.6
	7	24 months	10.4
	8	27 months	9.2
Control	9	Non inoculated	-ve

Formula 1: Inactivated EIV adjuvanted with combination of saponin 1 mg/horse dose and 20% Alhydragel.
Formula 2: Inactivated EIV adjuvanted with carbomer 0.25% (50% carbomer 0.5% to 50% antigen).

Focusing on the statistical analysis performed using Student's T-Test between group (A) and group (B) in Table 3, it was found that there were significant differences ($P<0.05$) between both groups, where Group (B) (using formula 2) gave significant results when compared with group (A) (using formula 1).

DISCUSSION

Proper vaccination against EIV is necessary to minimize and prevent spreading of the disease. The first and still most widely used type of vaccine for many years was whole-inactivated EIV vaccines (Paillot, 2014). There are many types of natural and synthetic adjuvants which can improve the efficacy of animal vaccines, such as aluminum compounds, emulsions, saponins, and carbomer. All over the world, these ingredients have already been used in approved products.

Several factors, including effectiveness in the target animal species, induction of a quick and durable protective immunity, animal safety, and cost effectiveness, should be considered when choosing and developing adjuvants for animal vaccines. One of the requirements for meeting the above criteria is the presence of an appropriate adjuvant or their combinations (Burakova *et al*., 2018).

The goal of the current study was to evaluate inactivated vaccinations against the equine influenza virus that contain carbomer adjuvant to those that contain a combination of saponin and aluminum hydroxide gel as adjuvant. In this experiment, binary ethylenimine (BEI) was used to inactivate EIV with HA titers of 10 log_2 and 0.05 ml and infectivity titers of 10 log_{10} and 0.1 ml, respectively (Eman, 2005). This outcome was consistent with that of Kucera and Beckenhaur (1977), who made an inactivated EI (A/equi-2) vaccination using a field strain that had 9 to 10 log_2 hemagglutinating units. The virus's HA titer remained unchanged after inactivation. When injected into SPF-ECE 9–11 days old, it exhibited no haemagglutinin activity (OIE, 2019b).

Regarding the sterility of the vaccine, samples from the finished products as well as the viral fluids before and after the inactivation process were taken, and they were found to be sterile and devoid of any contaminants (OIE, 2019a). The produced vaccination formulations were administered to horses, and no local or systemic reactions were noted, confirming vaccine safety as advised by (OIE, 2019b).

The collected data showed the effectiveness of several vaccination formulations in G. pigs. Serum samples taken 3 weeks after inoculation (WPI), the mean HI antibody titers for groups (A), (B), and (C) were 9.2, 10.4, and 10.6 log_2, respectively. It was more protective against EIV 64 (6 log_2) than what was stated by the European Pharmacopoeia (2005) and the OIE (2019) (both sources), while the control group (D) produced unfavorable outcomes.

From the obtained results, groups B and C which adjuvanted with carpomer (0.25% and 0.5%) respectively had nearly the same results with slightly higher HI antibodies comparable to group A which adjuvanted with combination of aluminum hydroxide gel and saponin. There were no significant differences between the antibody responses stimulated by vaccines in groups B and C. So, we used formula 2 with carpomer concentration 0.25% in testing immunogenicity in horses.

These obtained results come in accordance with Eman *et al*. (2009); Soliman *et al*. (2011) and OIE (2019b) who tested the potency of EI vaccine in guinea pigs as preliminary study to complete the study in target host (horses).

Stability of the prepared vaccine formulae that kept at 4 ºC for 27 months were tested in groups of G. pigs. The mean HI antibodies titers in vaccine formulae 1 & 2 were 9 & 10.4 Log_2 and 8 & 9.2 Log_2 at 24 months and 27 months; respectively. While at Zero time were 9.2 & 10.6. It was proved that both formulae were potent and stable at 4ºC for two years.

Seroconversion of both vaccine formulae (1 & 2) in horses with low pre-vaccination HI antibody titer. The mean EI-HI antibody titers expressed in log_2 in sera of horses' groups A & B at two-weeks post vaccination with formula 1 & 2 were (7, 8),

Table 3. EI –HI antibody titers in horses vaccinated with inactivated EI vaccines.

Time of sampling	EI –HI antibody titers in sera of horses							
	Group (A)			Group (B)			Group (C)	
	*H1	H2	Mean	H3	H4	Mean	H5	H6
Prevaccination	0	0	0	0	0	0	0	0
**2 WPV	7	7	7	8	8	8	0	0
***(b) 4WPV	6	6	6	7	7	7	0	0
6WPV	9	8	8.5	9	9	9	0	0
****2 MPV	9	9	9	10	9	9.5	0	0
3MPV	10	10	10	11	10	10.5	0	0
4MPV	9	10	9.5	10	11	10.5	0	0
5MPV	9	9	9	10	10	10	0	0
6 MPV	8	8	8	9	9	9	0	0
7 MPV	7	6	6.5	8	8	8	0	0

*H: Horse; **WPV: week post vaccination; ***(b): booster dose; ****MPV: month post vaccination.

respectively. Then it decreases but remains within the protective level until 4 weeks (6, 7), respectively. By booster dose at the 4th week post inoculation, a higher level of HI antibodies was obtained 2 weeks post booster dose (8.5, 9.0), respectively. Antibodies reached their maximum titer at 3 months post vaccination in group A (10) and 3 to 4 months post vaccination in group B (10.5). Then it began to decline gradually in all horses till 7 months post vaccination (6.5, 8); respectively, but remain within the protective level 6 $\log_2$ (64) as reported (OIE, 2019b).

The results of the current study agree with the previous results of Mumford *et al.* (1994), as they stated that Equine influenza vaccines contained inactivated whole virus and carbomer adjuvant stimulated higher levels and longer lasting antibody to haemagglutinin in ponies than vaccines of equivalent antigenic content containing aluminum phosphate adjuvants. Also, Safaa *et al*. (2021) used carbomer as adjuvant for improvement the immunogenicity of Equine Herpes virus-1 vaccine.

CONCLUSION

El vaccine adjuvanted with Carpomer induced higher antibody titer as well as longer lasting immune response in horses comparable to those induced by combination of aluminum hydroxide gel & saponin.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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