Influence of using of saponin-carbopol mixture adjuvant on the potency of freeze-dried Bovine Ephemeral Fever virus vaccine

By

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المستخلص:

تأثير استخدام المحفز المناعي خليط الصابونين والكربابول على فاعلية لقاح فيروس مرض حمى الثلاث ايام المجفد

تهدف هذه الدراسة إلى استبيان تأثير الكاربابول كمساعد للقاح حمى الثلاثة أيام الذى يتم تثبيطه وقت التحصين باستخدام الصابونين حيث تم تحضير الكربابول بثلاث تركيزات مختلفة (٠,١-٠,٢-٠,٥٪) مع مادة الصابونين بنسبة ثابتة (٠,٢ميكروجرام/مل) كمذيب خليط للقاح٠ وقد تبين أن مادة الكاربابول آمنة عند حقنها فى العجول ضمن اختبار السلامة لهذه المادة كما تم تحصين ثلاث مجموعات العجول البقرى باللقاح بعد اذابته بالمذيب المركب من المادتين معا بالنسب المذكورة كل على حده والمجموعة الرابعة باللقاح بعد اذابته بمادة الصابونين فقط بالاضافة الى مجموعة تركت دون تحصين كضابط للتجربة٠ هذا وقد تم تتبع المستويات المناعية فى الحيوانات المحصنة على مدارفترات متعاقبة باستخدام اختبار المصل المتعادل واختبار الاليزا حيث أظهرت نتائج هذه الاختبارات ان استخدام مذيب الكربابول بتركيز(٠,٢أو ٠,٥٪) مع مادة الصابونين (٠,٢ميكروجرام/مل) للقاح حمى الثلاث ايام المجفد قد اعطت أعلى مستويات مناعية (٣,١٢- ٣,١٤ لو٢ تتابعا) ، والكثافة الضوئية (٣,٣٩٩٥- ٣,٥٠٢٦ تتابعا)عنها فى حالة استخدام الصابونين وحده (٢,٦٨ لو٢) والكثافة الضوئية (٢,٩٤٣٢) ولذا يوصى بأستخدام الصابونين مضافا إليه مادة الكربابول كمحفز مناعى للقاح حمى الثلاث ايام المجفد٠

ABSTRACT:

Identification of optimal complex adjuvant and bovine ephemeral fever (BEF) antigen combination to elicit potent, protective, and long lasting immunity has been a major challenge for improvement of the vaccines potency against bovine ephemeral fever virus. In this study, three different concentrations (0.1, 0.2 and 0.5%) of a cross-linked polyanionic carbopol combined with a constant concentration (0.2 µg /ml) of saponin were used as solvents for the freeze dried BEF vaccine. The immunogenicity of these formulae was evaluated through application of serum neutralization test (SNT) and enzyme linked immunosorbent assay (ELISA) on serum samples obtained from different vaccinated groups of cattle in comparison with those obtained from cattle vaccinated with the BEF vaccine reconstituted in saponin diluent only. The results showed that the usage of (0.2% or 0.5%) of carbopol with (0.2 µg /ml) of saponin as a diluent for the freeze dried BEF vaccine induced the highest levels (3.12 and 3.14 Log_2 respectively) of specific bovine ephemeral fever antibodies with long lasting immunity in vaccinated cattle. Thus, it could be recommended to use this diluent (carpobol plus saponin) as adjuvant to bovine ephemeral fever virus vaccine.

INTRODUCTION

Bovine ephemeral fever (BEF) virus is an economically important arbo-viral disease. The disease is caused by bovine ephemeral fever virus, a member of the genus Ephemerovirus in the family Rhabdoviridae **(1)**, that affects cattle and water buffalo, and is widespread in tropical and subtropical areas, BEF characterized by biphasic fever, anorexia, lameness and recumbency **(2)** In most cases, the main impact is on productivity, it can result in decreased milk yield, loss of condition and reproductive losses, and recovery can be prolonged in some animals **(3)**. Since the exact vector of BEF

has not been identified, prevention efforts are mainly aimed at efficient vaccination of susceptible animals **(4)**.

The goal of vaccination is to stimulate a strong, protective and long-lasting immune response to the administered antigen. For the achievement of these objectives; potent adjuvant and novel vaccine strategies are required to make the vaccine sufficiently immunogenic to initiate a potent immune response **(5&6)**.

Vaccines can be made more efficacious by including an appropriate adjuvant in the composition. Adjuvants are used to accomplish two objectives; they slow the release of antigens from the injection site, and they stimulate the immune system.

The use of a polymer of acrylic acid cross-linked with various polyol compounds as an adjuvant is described in U.S. Such polymers are commercially available under the trademark "Carbopol" It is believed that Carbopol behaves in a manner similar to other gels such as collagen and aluminum hydroxide; the active agent is adsorbed on the polymer and the combination is retained at the injection site. The polymer is only slowly dispersed and the active agent is retained at the site for prolonged slow release **(7)**.

Saponins have been widely used as adjuvants for many years and have been included in several veterinary vaccines. On the other hand, the adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production **(8)**. It induces a strong adjuvant effect to T-dependent as well as T-independent antigens. Also it induces production of cytokines such as interleukins and interferon that might mediate their immune stimulant effects **(9& 10)**.

The goal of the present study was to exploit the polyanionic and cross-linked nature Carbopol for a “controlled release” of the BEF antigen. It is also wished to determine if,

upon combination with Carbopol, Saponin might elicit improved antibody responses in comparison to responses generated using Saponin only.

MATERIAL AND METHODS

1-BEF virus strain:

It was isolated from Toukh Tambasha, Monofia Governorate, Egypt during an outbreak in summer 2000 **(11&12)**. This isolate designed as Abbasia virus strain (BEF/AVS/2000) was supplied by the department of Pet Animal Vaccine Research (DPAVR), Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt and used as the seed for vaccine preparation after its adaptation to tissue culture.

2-BEF vaccine:

The vaccine was prepared in freeze-dried form **(13)** and supplied by the department of Pet Animal Vaccine Research, Veterinary Serum and Vaccine Research Institute Abbasia, Cairo.

3- Cell culture:

Baby hamster kidney cell line (BHK-21) was used for virus and titration by SNT **(14)**. It was supplied by the Pet Animal Vaccine Research Dept. Veterinary Serum and Vaccine Research Institute; Abbasia, Cairo.

4- Preparation of adjuvants:

4.1- **Saponin:**

It was supplied under the cat. No.16109; lot. 71500 by Sigma-Aldrish Labochemikalien Gm6H; Germany. It was prepared as watery solution in a concentration of 0.2 μg /ml in phosphate buffered saline (PBS) **(15)**, and was used as a diluent to inactivate the live attenuate BEF virus vaccine at the time of vaccination and injected directly after reconstitution.

4.2- **Carbopol:**

It was supplied by Lubrizol as powder. It was dissolved in hot water to prepare three different concentrations (0.2, 0.4 and 1%) of aqueous stock solutions **(7)**. The prepared solutions were subjected to heat sterilization by autoclaving at 121°C for 20 min, then storaged at 4°C until further use.

4.3- Preparation of complexes mixture (saponin-carbopol) adjuvant:

A 1:1 (v/v) of different concentrations of carbopol solution as mentioned with a solution of 0.2 µg/ml saponin was mixed to be obtained three concentrations (0.1, 0.2 and 0.5%) of complexes mixture finally, and then neutralization with 20% Sodium hydroxide to permit them to be readily to dissolve the freeze-dried vaccine.

5-Quality control of the prepared adjuvants:

Sterility, safety and potency were evaluated for the prepared adjuvants **(16)**.

6-Animals:

Eighteen healthy, non-vaccinated beef calves, approximately 12 to 18 months of age were tested and confirmed to be negative to BEF antibodies as screened by SNT. They were divided into six groups, each group contained three animals. Each one of the first three groups was vaccinated with two doses of freeze-dried BEF vaccine reconstituted in one of the different preparations of complexes adjuvant as follow:(0.1% of carpobol plus 0.2 µg /ml of saponin) for 1st group, (0.2% of carpobol plus 0.2 µg /ml of saponin) for 2nd group and (0.5% of carpobol plus 0.2 µg /ml of saponin) for 3rd group. In all cases the used inoculated a dose was 2ml with two weeks interval. The fourth group was vaccinated with two doses of the freeze-dried vaccine reconstituted in saponin of a conc. (0.2 µg /ml) alone. The fifth group was kept as unvaccinated control group. The sixth group was used to test the safety of carbopol where each animal was

inoculated with (0.5%/animal) and kept under daily clinical observation for 10 days post inoculation for detection of any abnormalities. Also daily rectal temperature could be recorded and daily clinical examination could be detected. All animals were kept under hygienic measures receiving balanced ration and adequate water and subjected to daily clinical examinations.

7- Sampling:

Serum samples were collected from all animals from the first week post vaccination till the 40^{th} week for evaluation of the induced neutralizing BEF antibodies.

8-Serum neutralization test (SNT):

It was performed using the micro-technique to detect neutralizing antibodies against BEF virus. The antibody titer was estimated as the final serum dilution Log_2 **(17)**.

9- Enzyme Linked Immunosorbent Assay (Indirect ELISA):

BEF antigen was prepared from infected BHK_{21} cells and concentrated by PEG (6000) MOL. Wet **(18)**, then, used in ELISA to estimate the specific antibodies of BEF virus **(19)**.

RESULTS AND DISCUSSION

Bovine ephemeral fever is an infectious but preventable disease. Primary vaccination in calves followed by regular boosts with quality vaccine usually provides satisfactory protection particularly in countries where the disease is endemic. Appropriate adjuvant selection may be essential to optimize the potency and to tailor the immune response of quality vaccines.

So, this study deals with comparative evaluation of humeral immune response of cattle vaccinated with BEF vaccines with two types of adjuvant “saponin with different concentrations of carbopol". The freeze-dried BEF vaccine showed that they are sterile and safe. Carbopol-based adjuvant suspensions have been used in veterinary vaccines since the 1970’s. They have been demonstrated to be safe and effective when used in all animal species **(7)**. The result of sterility test

applied on the prepared adjuvants showed that they are sterile as they are free from aerobic and anaerobic bacteria and fungi**.**

From the results shown in **table (1)**, it is clear that the first three groups of calves vaccinated with two doses of freeze-dried BEF vaccine reconstituted in complexes solvent, the BEF serum neutralizing antibody titers exhibited protective neutralizing BEF antibody titer (1.2 Log_2) **(13)** started from 2nd wpv (1.3,1.54 and 1.43)and reached the highest titer at 12th WPV (2.75, 3.12) in the 1st and 2nd groups respectively, while in 3rd group (3.14) at 16th wpv than persisted in protective titer till 32nd wpv (1.21) in the 1st group and at 40th wpv (1.40 and 1.42) in the 2ndand 3rd groups respectively. The vaccinated calves in the fourth group showed that protective neutralizing serum antibody titer (1.42) started from 3rd wpv, reached highest titer at 12th wpv (2.68) and persisted in protective titer till 28th wpv (1.37).

On the other hand, the ELISA results in the **table (2)** showed that positive serum antibody level (O.D) started from 2nd WPV (1.6031), reached highest level (O.D) (3.0487) at 12th WPV in 1st group and at 16th wpv in both 2nd and 3rd groups (3.3426 and 3.5026 respectively), persisted positive till 40th WPV. While the fourth group, showed that positive serum antibody level (O.D) started from 2nd WPV (1.4236), reached highest level (2.9432) at 12th WPV, and then dropped after 32th wpv. These results were shown in comparison to that of the calves kept as non-vaccinated (fifth group) that gave negative results.

All vaccinated groups showed protective level of antibodies near the second and third week post vaccination as saponin based adjuvants have the ability to stimulate 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 **(8)**. Saponins induce a strong

adjuvant effect to T-dependent as well as T-independent antigens. Saponin not only has stimulatory effects on the components of specific immunity, but also presents some non-specific immune reactions such as inflammation **(20&21)** and monocyte proliferation **(22&23)**. Observation of a rise in antibody titer following immunization with the dried freezed vaccine dissolve in complexes adjuvant "saponin plus Carbopol" as in the first three groups of calves and longer lasting immunity than in the fourth group. This observation is attributed to a combined effect of direct B-cell activation and antigen delivery by the anionic polymer coupled with saponin ability to enhance antibody responsiveness **(24)**. Carbopol administration triggered rapid and robust leukocyte recruitment, pro-inflammatory cytokine secretion and antigen capture largely by inflammatory monocytes. This indicates that Carbopol has immuno-stimulatory effects in addition to direct B-cell activation, possibly serving as an antigen delivery system **(25&26)**. The immune-modulating activities of polyanions were first described over 30 years ago **(27&28)** and more recently, polyacrylicacid polymers termed carbomers have been evaluated as adjuvants in veterinary vaccines **(29&30**). These carbomers are not harmful in mammals and are more effective than antigen alone. Carbopols have been combined with other adjuvant formulations such as MF59 to yield additive or potentially synergistic adaptive immune responses **(31&32)**.

Concerning the concentration of carbopol used, it was found the highest antibody level obtained with the concentration of 0.5 then 0.2 %, where the concentration of 0.1% showed less result compared with the higher concentration. This result agrees with the disclosure in U.S. Pat. No. 3, 178,350, mixtures of Carbopol 934 with various virus vaccines, resulted in greatly enhanced antibody production in the host when the Carbopols were in the range of 0.25 to 0.50% of the mixture **(7)**.

Considering the neutralizing antibody responses, it was clear that BEF adjuvanted with 0.5% Carbopol plus 0.2 µg /ml saponin elicited better humoral response than adjuvanted with saponin alone.

Table (1): Mean BEF serum neutralizing antibody titers calves vaccinated with BEF vaccine with different adjuvants

Animal Groups	Mean serum neutralizing antibody titer (Log_2)/WPV*														
	0	1st	2nd	3rd	4th	6th	8th	12nd	16th	20th	24th	28th	32nd	36th	40th
First	0	0.86	1.30	1.56	1.94	2.34	2.54	2.75	2.68	2.40	2.10	1.61	1.21	1.1	0.9
Second	0	1.10	1.54	1.92	2.38	2.80	2.80	3.12	3.00	2.87	2.74	2.51	2.21	1.84	1.40
Third	0	0.9	1.43	1.79	2.32	2.67	2.97	3.10	3.14	2.95	2.72	2.57	2.21	1.85	1.42
Fourth	0	0.75	1.16	1.42	1.76	2.14	2.56	2.68	2.43	1.94	1.72	1.37	1.12	0.93	0.81
Fifth	0	0	0	0	0	0	0	0	0	0		0	0	0	0

WPV*: week post vaccination

Protective serum neutralizing antibody titer=1.2$\log_2$ **(13)**.

1st group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.1% of carpobol plus 0.2 µg /ml of saponin).

2nd group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.2% of carpobol plus 0.2 µg /ml of saponin).

3rd group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.5% of carpobol plus 0.2 µg /ml of saponin).

4th group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.2 µg /ml of saponin) alone.

5th group: was kept as unvaccinated control

Table (2): BEF- ELISA optical density (OD) in calves vaccinated with of BEF vaccine with different adjuvants.

Animal groups	Mean BEF-ELISA optical density /weeks										
	0	1st	2nd	3rd	4th	6th	8th	12nd	16th	20th	24th
First	0.220	1.1621	1.6031	1.8613	2.2422	2.8426	2.9938	3.0487	2.9756	2.5924	2.1662
Second	0.1966	1.4412	1.8231	2.2723	2.7252	3.3216	3.3995	3.4795	3.3426	3.2147	3.0836
Third	0.2035	1.2356	1.7628	2.1269	2.6594	3.0145	3.3995	3.4928	3.5026	3.3269	3.0426
Fourth	0.2021	1.0165	1.4236	1.6854	2.0233	2.4569	2.8236	2.9432	2.6957	2.2154	1.9824
Fifth	0.1945	0.2033	0.1902	0.1951	0.2242	0.2163	0.1961	0.1945	0.2032	0.1988	0.2241

WPV*: week post vaccination

Positive ELISA-OD is over one.
1st group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.1% of carpobol plus 0.2 µg /ml of saponin).
2nd group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.2% of carpobol plus 0.2 µg /ml of saponin).
3rd group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.5% of carpobol plus 0.2 µg /ml of saponin).
4th group: Vaccinated with BEF vaccine reconstituted in the adjuvant (0.2 µg /ml of saponin) alone.
5th group: was kept as unvaccinated control

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