PREPARATION OF A THERMOSTABLE BOVINE EPHEMERAL FEVER VIRUS VACCINE INACTIVATED ON THE TIME OF USE

SHENDY, M. B; A. T. EL-DAKHLY and M. M. YOUSSEF

Veterinary Serum and Vaccine Research Institute. Abbasia. Cairo. P.O. Box: 131

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Abstract

The herein study was devoted to overcome the obstacles dealing with the utilization of the live attenuated Bovine Ephemeral Fever (BEF) vaccine viz, the cold chain and the undesirable post-vaccinal reaction. Methylglucoside-sucrose (MS) was proved to be an excellent stabilizer for BEF thermostable vaccine. The permissible titer of the lyophilized MS-BEF thermostable vaccine was achieved even after 6 months at 25°C, 3 months at 37°C and 20 days at 45°C. The study revealed that freeze-dried MS-BEF thermostable vaccine was reconstituted with saponine (0.2µg/dose) as inactivator just at the time of inoculation. Such investigations were carried out on 4 groups of calves each was consisted of three animals. The first group animals were inoculated with the MS-BEF thermostable vaccine kept at 25°C for 6 months. The second groups were inoculated with the fore-mentioned vaccine kept at 37°C for 3 months. The third group animals were vaccinated with BEF vaccine kept at 45 for 20 days. (The vaccine titer was $10^{5.5}$ $TCID_{50}$/ml in the three above mentioned groups), whereas the fourth group was kept as non-vaccinated normal controls. The three animal groups received booster dose at two weeks post-first vaccination. It has been deduced that such vaccine effectively induced high mean BEF neutralizing antibody titers post- boostering vaccination and protected cattle for one year. Thus, MS stabilizer was recommended for preparation of thermostable live BEF vaccine inactivated at the spot with saponine.

Keywords: Bovine Ephemeral Fever Virus Vaccine; Freeze-dryer; Stabilizer; Thermostability.

INTRODUCION

Bovine ephemeral fever (BEF) is a vector-borne disease of cattle caused by the ephemerovirus of the family Rhabdoviridae. Bovine ephemeral fever (BEF) is an economically important viral disease of cattle and buffalo which occurs mostly in tropical and subtropical climates in Africa, Asia, the Middle East and Australia (Walker, 2005). Despite of the extensive use of inactivated vaccines for the prevention of BEF, a controlled study of their field effectiveness has never been performed. Both live attenuated and inactivated vaccines are available for field use, with the former shown to induce longer lasting immunity than inactivated vaccines (Vanselow et. al., 1995, Aziz-

Boaron et. al, 2013). Attenuated vaccines are considered less safe as possible risks include adverse clinical signs and reversion to virulence, especially due to relatively high mutation rate of RNA viruses (Della-porta and Snowdon, 1977). In general, live attenuated veterinary vaccines suffer a lot from serious deterioration during vaccination campaigns due to the difficulty in maintaining cold-chain during the storage and transport of vaccine, which inevitably results in loss of vaccine potency in tropical and subtropical environments and this, is one of the major constraints in control of the viral diseases. Thermo-stable vaccines are considered more suitable under tropical field situations where the viability of the virus vaccine will be vulnerable. Most live attenuated vaccines are highly thermo-labile and require maintenance of cold chain from vaccine production till delivery. Methylglucoside is a monosaccharide derived from glucose. It can be prepared in the laboratory by the acid-catalyzed reaction of glucose with methanol (Helferich, and Sch· fer, 1996). This MS stabilizer was used for as thermostability of rabies vaccine was successfully formulated into stable, dry foam using lyophilization process. Freeze-dried Live attenuated rabies virus vaccine was stable for 23 months at 22°C and 2 months at 37°C. Stability decreased as temperature increased, yet lyophilized rabies vaccine was remained stable for at least 3 hours at 80°C (Todd et al., 2015). The current used BEF vaccine is a thermo-labile vaccines as it is stabilized by lactalbumin hydrolysate–sucrose and then lyophilized. So MS stabilizer was used in lyophilization process in the current study to determine the thermostability and potency of this vaccine at different temperatures storage shelf life.

MATERIALS AND METHODS

1. Virus propagation:

A Locally isolated bovine ephemeral fever virus (BEF/AVS/2000) was propagated in BHK-21 monolayer cell culture with a titer of $10^{6.5}$ $TCID_{50}$/ ml, and used for prepared vaccine; virus titration and serum neutralization.It was propagated and titrated in Dept. of Pet Animal Vaccine Research, Veterinary Serum and Vaccine Research Institute (VSVRI). Cairo. Egypt.

2.Vaccine stabilizer:

2.1. Methylglucoside-Sucrose (MS):

Methyl α-glucopranoside ($C_7H_{14}O_6$), white crystals. ≥ 99. 6%

(HPLC area %), was supplied by Sigma-aldrich.

3. Preparation of Freeze-dried bovine ephemeral fever vaccine:

BHK cells were infected with bovine ephemeral fever virus (BEF/AVS/2000) at a multiplicity of infection (MOI) of 0.1. BEF virus supernatant was mixed with equal volume of methylglucoside-sucrose (MS) stabilizer Bronshtein, (2005). After 48 hours of lyophilization, these vials were placed at 25, 37 and 45°C in dry incubator for 12 months. Viability of these vials was detected at different times as in table (1). Virus titers were estimated according to Cunningham (1973).

4. Evaluation of prepared live freeze-dried BEF stabilized with MS vaccines:

The prepared 1ive BEF vaccine was subjected to examination of its sterility, safety and the potency as the following:

4.1. Sterility test:

Samples from each prepared lyophilized BEF vaccine formulae were cultured on Tryptose Phosphate, Thioglcolate broth, Sabouraud's agar and PPLO media according to (Parveen, et. at., 2011).

4.2. Safety test:

Three calves were injected with 10 doses of prepared BEF vaccines, S/C in different sites and observed for 15 days for development of any clinical signs or local reaction according to Manal (2005).

4.3. Potency test:

Humoral immune response of calves vaccinated with prepared BEF vaccine was evaluated using serum neutralization test (SNT) as described by **Ferreira (1976).**

4.3.1.Experimental animals:

Fifteen apparently healthy local breed calves (6-8months old) weighting about 250-300Kg weight, were proved to be seronegative to bovine ephemeral fever virus and antibody. These calves were divided into 4 groups, (3 animals per each). The vial was reconstituted at time of using with 20 ml of saponine (concentration of 0.2µg/dose) as inactivator, three groups were inoculated subcutaneously with the detected times of viability, in a dose of 2ml and boostering after two weeks. The last group was kept as unvaccinated control as in **table (2)**.

5. Serological assay:

Blood was collected from the vaccinated and non-vaccinated calves weekly for 4 weeks post vaccination and then every 4 weeks till the end of the experiment. Sera were tested for anti- BEF antibodies by means of a serum neutralization test (Rossiter, et. al., 1985). The mean SN titer was expressed as the $\log_{10}$ of the final serum dilution which protected culture cells in 50% of the wells.

6. Statistical analysis:

Data were expressed as mean "F "single factor calculated. One way ANOVA was used to compare between groups. P-value of less than 0.05 was considered statistically significant (Snedecor and Cochren, 1986).

RESULTS AND DISCUSSION

Table 1. Viability of bovine ephemeral fever virus vaccine with MS stabilizer after lyophilization and Storage at different temperatures.

Temperature	Bovine ephemeral fever virus titer (log_{10}/ TCID/ml)													
(°C)	Initial	1h	3 h	5 h	10 h	5 D	20 D	1M	3M	5M	6M	9M	10M	12M
25°C	6.30	6.30	6.30	6.30	6.28	6.21	6.20	5.98	5.7	5.5	5.5	5.0	5.0	4.8
37°C	6.30	6.30	6.25	6.25	6.24	6.12	5.60	5.52	5.5	4.5	4.4	4.0	4.0	3.70
45°C	6.30	6.27	6.00	5.90	5.65	5.53	5.50	4.50	3.2	3.0	2.1	1.1	0.00	0.00

MS: Methylglucoside-Sucrose.

H: hours, D: days, M: months.

N.B: Protective level of bovine ephemeral fever virus titer at least $10^{5.5}$ $TCID_{50/ml}$.

Table 2. Mean BEF serum neutralizing antibody titers in vaccinated and non-vaccinated calves with thermostable BEF vaccine.

Animal Groups	Mean BEF serum neutralizing antibody titer^{o}												
	1WPV ♦♦	2WPV	↑	1WPB ♦♦♦	2WP	3WP	1MPB ♦♦	2M	3M	5M	7M	9M	12M
1*	5.6	8.8	2nd dose	18.4	19.2	28.8	41.6	54.4	60.8	67.2	70.4	76.8	73.2
2	5.6	6.4	↓	14.4	17.6	18.4	32.8	32.2	35.2	41.6	54.4	53.6	56.00
3	4.6	6.4	(booster)	10.4	9.6	11.2	16.00	20.8	32.0	32.8	44.4	48.8	50.6
4	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00	0.00

1st gp.**:** vaccinated with the thermostable BEF vaccine at 25°C (6 months).

2nd gp.**:** vaccinated with the thermostable BEF vaccine at 37°C (3 months).

3rd gp.**:** vaccinated with the thermostable BEF vaccine at 45°C (20 days).

oMean BEF serum neutralizing antibody titer=the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 $TCID_{50}$ of BEF virus.

♦♦WPV= week post vaccination. ♦♦♦WPB= week post booster. ♦♦ MPB= month post booster.

N.B: the protective antibody titer of inactivated BEF vaccine is 32.

*Differences with P≤ 0.05 were considered statistically significant.

Vaccination – so far - is still the best way for combating and even eradication of infectious diseases. However, Most of the conventional live attenuated vaccines are heat labile. This may represent a major problem especially in hot localities. So, cold chain is absolutely necessary (OIE, 2016). The data of the first experiment revealed that utilization of the Methylglucoside-Sucrose (MS) as a stabilizer for Bovine Ephemeral Fever virus attenuated vaccine were very satisfactory. The starting titer of BEF vaccine suspension was 6.5 $\log_{10}$ $TCID_{50}$ / ml that was subjected to lyophilization (Freeze-drying) after mixing with MS stabilizer (1:1). The vaccine permissible titer was maintained at 25℃ for 6 months, 3 months at 37℃ and 20 days at 45℃ denoting that stabilization of BEF vaccine with MS stabilizer maintained its titer as being thermostable vaccine as shown in table (1). The same motions were previously ascertained by Mariner, et al., (1990) and Zhai, et al., (2004) who stressed on the fact that freeze – dried MS stabilized vaccine sustained its potency and validity for relatively longer time than the traditional vaccines. They declared that sugars stabilize membranes and proteins by hydrogen bonding to the polar residues of the biomolecules, working as a water substitute, in addition the concentrated sugar solution that lowers the nucleation temperature of the water of the virus membrane, consequently preventing formation of ice crystals within both the virus as well the ambient medium. Our results also came in full agreement with Thachamvally et al., (2011), who demonstrated that the PPR vaccine in lyophilized form was usually stable at room temperature (25℃) for 24-26 days. While Diallo et al., (2007) reported that PPR virus vaccine stabilized with Lactalbumin Hydrolysate-Sucrose (LS) attained its stability for 14 days at 45℃ with minimal loss in its potency. Our studies proved that reconstitution of BEF–MS lyophilized vaccine using saponine (as inactivator and as an adjuvant) conferred calves with neutralizing antibodies sustained for a whole year. The saponine reconstituted BEF vaccine gave 100% protection to the vaccinated calves. The vaccine was absolutely safe, without undesirable post-vaccinal reaction. There was no post–vaccinal prolonged pyrexia or inflammation at the site of inoculation and the vaccine was well tolerated when injected subcutaneously without noticeable toxicity to the animals. The same observations were previously emphasized by Allison and Byars, (1992) who declared that saponine is one of the phyto-chemical adjuvant which possesses immune stimulating activity; it has the ability to potentiate the immune response for a year. Moreover, it is safe, no deleterious effects on the animals. Whereas these observations were recorded also by Phuong, et al., (1999) and Castrucci, et al., (1993). Humoral immune response to the

prepared BEF vaccine using serum neutralization test (SNT) after single dose application. The mean specific BEF neutralized antibody titers were detectable by the 1st week post vaccination in vaccinated animal groups, where reached antibody titers (41.6 and 32.8) were recorded by the 1st month in groups-1 and 2 respectively, and 32.0 in group-3 at 3rd month post boostring as shown in table (2). These results agreed with who found that protective serum BEF neutralizing antibody titer is: 32 according to (Wang, et al., 2001). By statistical analysis for the obtained results by using ANOVA test – single factor according to Snedecor and Cochren (1986), it was revealed that there was a significant difference (at $P \leq 0.05$) between group-1 (vaccinated with BEF vaccine at 25°C) and other groups. Thus, it was clear that prepared live attenuated BEF vaccine with a novel stabilizer is more stable, viable and effective at 25°C, 37°C and 45°C, respectively.

In conclusion, the results of present study indicated that freeze-dried live BEF vaccine adjuvanted and inactivated with saponine on the time of use and formulated by MS stabilizer is highly thermo-stable and provoked protective immunity without inducing any adverse actions.

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تحضير لقاح مقاوم للحرارة لحمى الثلاث أيام المثبط وقت الاستخدام

محمد بريك شندى ، أشرف الداخلى طه ، محمد محمود يوسف

معهد بحوث الامصال واللقاحات البيطرية- العباسية- القاهرة
ص.ب:١٣١

كرست هذه الدراسة للتغلب على المعوقات المتعلقة بأستخدام لقاح حمى الثلاث أيام الحى المستضعف المجفد لقدرة اللقاح على الثبات الحرارى والاثار غير المرغوبة بعد التحصين ،وتعتبر مادة مثيل جليكوسيد وسكروز كمثبت قوى مقاوم للحرارة لأنتاج لقاح حمى الثلاث أيام المجفد، حيث ان المعايرة الوقائية للقاح بأستخدام هذا المثبت حقق ثبات حرارى حتى بعد ستة أشهر عند درجة حرارة٢٥ °م ، ثلاثة أشهر عند ٣٧° م وعشرون يوما عند ٤٥° م والذى يتم تثبيطه بمحلول الصابونيين(بتركيز٠,٠٢ميكروجرام/جرعه) وقت الحقن٠ أجريت هذه الدراسة على أربع مجموعات من ألابقار (٣أبقار، مجموعه) ، المجموعة الاولى حقنت باللقاح حمى الثلاث أيام المقاوم للحراره عند٢٥ °م لمدة ٦ أشهر، المجموعة الثانية حقنت بذات اللقاح عند ٣٧° م لمدة ٣ أشهر والمجموعة الثالثة حصنت باللقاح المحفوظ عند ٤٥° م لمدة عشرون يوما، حيث ان معايرة اللقاح المحفوظ عند الثلاث درجات الحراره المذكوره كانت ٥٠٥ جرعه وقائية مل ، تم حقن المجموعات الثلاثة بجرعة ثانية بعد مرورأسبوعين من التحصين بالجرعة الاولى ، بينما المجموعة الرابعة تركت بدون حقن كضابط للتجربة٠ أكدت النتائج الى قدرة هذا المثبت للحفاظ على حيوية الفيروس لمدة أطول بالاضافه الى قدرة اللقاح على إنتاج أجسام مضادة لفيروس حمى الثلاث أيام بمعيار كاف لحماية الابقار من الاصابة بالمرض لمدة عام لذا يوصى بأستخدام مثبت مثيل جليكوسيد وسكروز لتحضير لقاح حمى الثلاث أيام الحى المجفد ذو الثبات الحرارى المثبط بمحلول الصابونيين٠