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TRIAL FOR PREPARING A VACCINE GIVING EARLIER IMMUNE RESPONSE AGAINST HAEMORRHAGIC SEPTICAEMIA IN CALVES

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SUMMARY

Eight Haemorrhagic septicaemia (HS) vaccine formulations were evaluated to study their ability to elicit an early protective immunity in mice and calves. All HS vaccine formulations failed to protect mice against challenge with Pasteurella multocida virulent strain at 7th day post vaccination. Montanide ISA 25 vaccine was the best vaccine that could protect mice at 10th day post-immunization. Aluminum hydroxide gel and Saponin vaccine was the second vaccine that could protect mice at 15th day post-immunization. However, the other six formulations could protect mice between 21 and 35 day post-vaccination. The results of passive mouse protection test were in parallel with those of active mouse protection test.

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, septicaemic highly fatal disease, principally infecting cattle and water buffaloes (Carter and De Alwis, 1989). HS is considered economically to be the most important disease in Southeast Asia, Middle East, Central and South Africa (Verma and Jaiswal, 1998). Generally, in endemic countries the disease is commonly experienced in wet and humid weather (Sheikh et.al., 1996). In Egypt, the scourge of HS used to be seasonal, and in association with the annual rise and flood season of the River nile. These environmental conditions have been dismissed after the building of the high Dam. Egypt employs ìstamping outî strategy from more than forty years. The percentage of carriers, detected in Egyptian buffaloes and cattle populations, was ranging from 6-10 % (Fayed, 1973, Farid et.al., 1980 and Aboul Saoud, 1990).

Most strains isolated from carriers buffaloes and cattle populations are as virulent as those isolated from clinical cases of HS and are highly pathogenic both for mice and rabbits and this make chance of an outbreak of HS is still present in spite of continuous vaccination (Aboul Saoud, 1990). Animals of all ages are susceptible but, the most vulnerable age is 6 months to 2 years of age (Radostitis et.al., 2000). According to this contingency, in Egypt, an oil-adjuvant vaccine with mannite monooleate is produced to vaccinate calves under one year old, also to stand by as a contingency for emergency vaccination.

The rapid onset of the disease and its short course leave little opportunity for treatment. Also, administration of antiserum to animals exhibiting clinical signs as well as in-contact animals has been of questionable efficacy (Thomas, 1972). Thus, to prevent the spread of HS, a strategic emergency ring-vaccination would be preferable to limit and reduce the number of new clinical cases. Therefore, for effective HS control during outbreak, there is a need for a vaccine, which elicits a high antibody response and has earlier protection following vaccination.

The objective of the present study was to examine the efficiency of several HS vaccine formulations in eliciting an early protective immunity in mice and calves.

3. Materials and Methods:

3.1. P.multocida strain: P.multocida strain (type 6:B) a locally isolated strain from field cases of cattle with HS in Egypt (Geneidy and El-Affandy, 1963). This strain was used for preparation of *P.multocida* vaccine and for challenge of mice.

3.2. Vaccine preparation: Culture of *P.multocida* was prepared and standardized for vaccine production according to the method of De Alwis (1989). Stained smears of the culture were examined for purity. Formalin was added to the culture with a final concentration of 0.25%. After standing for 24 hours, the turbidity is standardized against a reference containing the equivalent dry weight/volume of 1.5 g per liter. Eight forms of the vaccine were formalized as follows:

a. Bacterin vaccine: It was a formalized culture of *P.multocida* only.

b. Alum precipitated vaccine: Hot potassium aluminum sulphate (Ubichem Limited) was added to the culture of P.multocida in percentage of 1%. After overnight storage with continuous agitation the vaccine was bottled for use as described by De Alwis, (1989).

c. Aluminum hydroxide gel vaccine (Honil Limited, London, UK): It was prepared by adding one volume of gel to three volumes of the antigen concentrate, (the best adsorption to P.multocida antigen) using a magnetic stirrer at approximately 300 rpm. according to Salt et.al., 1994.

d. **Aluminum hydroxide gel & Saponin vaccine**: It was prepared by adding one volume of the gel to three volumes of the antigen concentrate, and saponin (Ubichem, plc, UK) was added with 1 mg/ dose for each calve and 0.1 mg dose for each mouse, using a magnetic stirrer at approximately 300 rpm. as mentioned by Borja-Cabrera et.al., 2002.

e. **Oil adjuvant vaccine:** The oil phase consisted of 9 parts white paraffin oil and 1 part of spain 80 (Sigma). The oil was first sterilized and on cooling to 400c. The vaccine was made by placing the adjuvant into a blinder (Kalish, Montreal& Toronto Canada, Model 9020, HP 1.25). The bacterial suspension with 2% Tween 80 (Sigma)(V/V) was added slowly to the oil phase with percentage of 1:2. The mixture was blended well to obtain a stable emulsion. Emulsification continued for 30 minutes, after overnight storage, the mixture was re-emulsified then bottled and stored at room temperature for 2 weeks. A well made oil adjuvant vaccine is white in color and adheres evenly to clean glass (Stone et.al., 1978 and Geneidy et.al., 1967).

f. **The oil-in-water vaccine (Montanide ISA 25, Seppic, France)**: It was prepared by adding one volume of oil to three volumes of the antigen concentrate that was previously diluted in 0.06M sodium phosphate, 0.15M Nacl, pH 7.7, using a magnetic stirrer at approximately 300 rpm (Salt et.al., 1994).

g. **Montanide IMS 1313:** Was prepared by adding the adjuvant IMS 1313 (Seppic, France) to equal volume of the antigen concentrate, previously diluted in 0.06M sodium phosphate, 0.15M Nacl, pH 7.7, using a magnetic stirrer at approximately 300 rpm (Barnett et.al., 1998).

h. **The double-oil-emulsion vaccine (Montanide ISA 206)**: The ratio of the aqueous antigen to the oil adjuvant was 50:50. The mixture was stirred to form a water-in-oil-in-water blend. Initially, slow sheer mixing at 300 rpm for 5 min, followed by brief mixing cycle at the same speed after keeping at 40C for 24 h to give an extremely stable emulsion (Patil et.al., 2002).

3.3. Standardization of vaccine formulations: The aforementioned vaccine formulations were subjected to sterility and safety tests according to Cruickshank et.al., 1975 and De Alwis (1989) before being used in the vaccination programme.

3.4. Animals:

A. Mice: A healthy Swiss albino mice, each weighing 18-22 g, obtained from the mice farm at Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, were used for safety and potency test. For safety test, eight groups of mice (n = 10/group) were used, 1950 mice were used for potency test as shown in Table (1), and 96 mice were used for passive mouse protection test.

B. Calves: Twenty one Holstein-Friesian calves 3 months of age, with no history of HS or vaccination were used in the present study.

3.5. Vaccination schedule and potency test:

A. In mice: The active mouse protection test was carried out as described by Ose and Muenster (1968). The mice were divided into nine groups, designated A, B, C, D, E, F, G, H and I. Each group of mice from A-H was inoculated subcutaneously by one of the eight vaccine formulations. The mice of group I was kept as control. Each group was further divided into subgroups, 50 mice each, for receiving challenge inoculums at 7, 10, 15, 21, 28 and 35 days post vaccination. Challenge exposure of mice was done with a recently rabbit-passaged P.multocida grown for 18 hours at 37°C. Purity of the culture was assured by Gram, stain. On the day of challenge, groups of vaccinated and control (50 mice each) were divided into 10 group of 5, each group being challenged intraperitoneally with 0.1 ml of respective dilutions of a challenge inoculums in the range from -1 to -10 $\log_{10}$. Deaths were recorded for 7 days after challenge exposure. The median lethal dose (LD_{50}) was calculated for each group of mice, vaccinated and control groups based on the accumulated deaths on the 7th day using the Reed and Muench (1938) method. The (LD_{50}) of the vaccinated mice was compared to the (LD_{50}) of the non-vaccinated mice. A requirement of 2 logs of protection is necessary to qualify the prepared vaccine as an index of cattle protection (Ose and Muenster, 1968).

The groups of vaccinated mice that gave earlier protection in mice were excluded and the challenge test was repeated for the rest groups and so until determination of the time of protection was ended as shown in Table (1).

B. In Cattle: Calves were allotted into three groups. First and second groups each of 9 calves were injected subcutaneously each with 2 ml of the first and second vaccines that gave earlier immune response in mice (Montanide ISA 25 vaccine and Aluminum hydroxide gel & Saponin vaccine). The third group of 3 calves was kept as control. Blood samples were collected from the jugular vein of the calves just before they were vaccinated and at 7,10,15 days post vaccination. till prove protection by passive mouse protection test. 0.4ml of pooled se3ra from each group were injected into 8 mice subcutaneously and sera from each group were injected into 8 mice subcutaneously and after 24 hour each mouse was challenged with 100 LD_{50} of viruleny *P.multocida* as *described* by Sawada et all., (1985). An equal number of non treated for control mice were exposed similarly. The mice were observed for 7 days after exposure. Sera which protected any mice in the PMPT were designated positive according to De Alwis and Carter (1980).

RESULTS

The results as shown in Table (2) indicated that all the eight vaccines could not protect mice

against challenge at 7th day post-vaccination. The Montanide ISA 25 vaccine could protect mice against challenge with P.multocida at the 10th day post-vaccination followed by the Aluminum hydroxide gel & Saponin vaccine which can protect mice and calves against challenge at the 15th day post-vaccination. The other types of vaccines gave protection at different times which varied from 21 to 35 days post-vaccination. Table 2 indicated that both vaccine formulations that gave earlier protection could protect passively inoculated mice.

DISCUSSION

Emergency HS ring-vaccination could assist the damping down of the outbreak by reducing the release of HS into the immediate area around a disease focus and limit the number of new clinical cases (Salt et.al., 1994). Adjuvant have been used for many years to enhance protection against a higher challenge dose of virulent Organisms than bacterin alone and are able to elicit the formation of protective antibodies (Allison and Byars, 1986). Many newer adjuvant systems have incorporated synthetic components, this adjuvant elicited superior immune response and the rapidity of development of response was quicker Patil et.al., 2002). Mouse is used in replace of cattle and is the most feasible for routine batch testing (De Alwis, 1989).

The ability of single dose vaccination with the eight HS vaccine formulations to elicit early protective immunity in mice was evaluated in the

Table (1): No. of mice used for potency testing of different vaccines of *P.multocida.*

Type of vaccine	No. of mice used for challenge at different times post-vaccination					
	7 days	10 days	15 days	21 days	28 days	35 days
Formalized vaccine	50 mice	50 mice	50 mice	50 mice	-----	------
Alum precipitated vaccine	50 mice	50 mice	50 mice	50 mice	50 mice	-----
Aluminum hydroxide gel vaccine	50 mice	50 mice	50 mice	50 mice	-------	-----
Aluminum hydroxide gel & Saponin vaccine	50 mice	50 mice	50 mice	----	------	------
Oil adjuvant vaccine	50 mice	50 mice	50 mice	50 mice	50 mice	50 mice
Montanide ISA 25 vaccine	50 mice	50 mice	-----	------	------	-------
Montanide IMS 1313 vaccine	50 mice	50 mice	50 mice	50 mice	------	------
Montanide ISA 206 vaccine	50 mice	50 mice	50 mice	50 mice	50 mice	------
Control	50 mice	50 mice	50 mice	50 mice	50 mice	50 mice

Table (2): Comparative log protection post-challenge results of different HS vaccine formulations.

Date of challenge	Post challenge results	Mice vaccinated with different vaccines								
		Control	Formalized bacterin vaccine	Alum vaccine	Gel vaccine	Gel & Saponin vaccine	Oil adjuvant vaccine	Montanide ISA 25 vaccine	Montanide IMS 1313 vaccine	Montanide ISA 206 vaccine
At 7th day post vaccination	LD50 after challenge inoculation	$10^{-8.5}$	$10^{-8.37}$	$10^{-8.25}$	$10^{-7.78}$	$10^{-7.57}$	$10^{-8.46}$	$10^{-8.25}$	$10^{-8.54}$	$10^{-8.16}$
	Log protection	------	*0.13	*0.25	*0.72	*0.93	*0.04	*0.25	*0.04	*0.34
At 10th day post vaccination	LD50 after challenge inoculation	$10^{-8.78}$	$10^{-7.84}$	$10^{-8.12}$	$10^{-8.12}$	$10^{-7.46}$	$10^{-8.37}$	$10^{-6.5}$	$10^{-7.84}$	$10^{-8.16}$
	Log protection	------	*0.94	*0.66	*0.66	*1.32	*0.41	**2.28	**0.94	**0.62
At 15th day post vaccination	LD50 after challenge inoculation	$10^{-8.19}$	$10^{-6.5}$	$10^{-7.5}$	$10^{-6.47}$	$10^{-6.05}$	$10^{-7.62}$	------	$10^{-6.5}$	$10^{-7.62}$
	Log protection	------	*1.69	*0.69	*1.72	**2.14	*0.57	-------	*1.69	*0.57
At 21th day post vaccination	LD50 after challenge inoculation	$10^{-7.62}$	$10^{-5.37}$	$10^{-6.25}$	$10^{-5.62}$	------	$10^{-6.62}$	------	$10^{-5.25}$	$10^{-6.37}$
	Log protection	------	**2.25	*1.37	*2.0	-----	*1.0	-----	**2.37	*1.25
At 28th day post vaccination	LD50 after challenge inoculation	$10^{-7.84}$	------	$10^{-5.37}$	-------	-------	$10^{-6.25}$	------	------	$10^{-5.81}$
	Log protection	------	------	**2.47	------	------	*1.59	-------	-------	**2.03
At35th day post vaccination	LD50 after challenge inoculation	$10^{-8.25}$	-------	------	------	------	$10^{-5.62}$	-------	-------	-------
	Log protection	-----	-----	-----	------	------	**2.63	-------	-------	-------

* Not protective post challenge results.
** Protective post challenge results.

Table (3) :Mouse protection test of sera of calves vaccinated with Montanide ISA 25 vaccine and Aluminum hydroxide gel & Saponin vaccine

Time post vaccinatrion	Non vaccinated control		Sera of calves vaccinated with											
			Montanide ISA 25 vaccine						Gel & Saponin vaccine					
	Survived mice	Died mice	Survived mice	Died mice					Survived mice	Died mice				
	After 7 days	After 7 days	After 7 days	After 24 days	After 48 days	After 72 days	After 96 days	After 120 days	After 7 days	After 24 days	After 48 days	After 72 days	After 96 days	After 120 days
Pre vaccination	0	8	0	5	3	0	0	0	0	7	1	0	0	0
At 7th day post-vaccination	0	8	0	1	5	2	0	0	0	3	2	1	1	1
At 10th day post-vaccination	0	8	4	0	0	4	0	0	0	2	2	1	2	1
At 15th day post-	0	8	5	0	1	0	0	2	6	0	0	0	0	2

present investigation. It is noticed from the results in Table (2) that all HS vaccine formulations could protect mice against challenge with P.multocida within 35 days post vaccination. All HS vaccine formulations failed to protect mice against challenge at 7th day post vaccination. Montanide ISA 25 vaccine was the best vaccine that could protect mice at 10th day post-immunization. These results come in agreement with the previous results of Barnett et.al. (1998), who found that Montanide ISA 25 gave strong protection following vaccination surpass the other adjuvant. Also, such results in the present work disagreed with the results of Salt et.al., (1994), who found that the Montanide ISA 25 vaccine with Foot and Mouth Disease (FMD) antigen could be effective in preventing disease within four days post-vaccination. This difference can be attributed to the difference in the antigen, and may be also due to difference in immunity to each antigen, may be due to that immunity with FMD antigen differs from HS antigen and the immunity with HS antigen is delayed due to the development of humoral immunity, which may have been responsible for protection.

Also, the present results indicated that Aluminum hydroxide gel and Saponin vaccine was the sec-

ond vaccine that could protect mice at 15th day post-immunization. The formalized vaccine, Aluminum hydroxide gel vaccine and Montanide IMS 1313 vaccine could protect mice against challenge with HS at the 21st day post-vaccination. From the results of the present study, it could be seen that the formalized vaccine couldn't provide earlier protective immunity than the adjuvanted vaccines; however, the absorption of the aqueous vaccines is better than the adjuvant ones. In this concern, Reddy et.al., (1996), stated that serum samples collected from the calves vaccinated with Al-gel vaccine showed peak antibody titers on 21 days post vaccination. It is of interest to note that the Alum precipitated vaccine and the Montanide ISA 206 vaccine could protect mice at 28th day post-vaccination. On the other hand, such results in the present work disagreed with the results of Cox et.al., 1999), who concluded that both Aluminum hydroxide gel and Saponin vaccine and Montanide ISA 206 vaccine with FMD antigen provide a rapid and protective immunity in sheep as early as three days following vaccination and Patil et.al., 2002), who found that the Montanide ISA 206 vaccine with FMD antigen elicited superior immune response at any given period than aluminum hydroxide gel vaccine and the rapidity of development of response was quicker. Lastly, the oil adjuvant vaccine could protect mice at 35th day post-vaccination.

The passive mouse protection test (PMPT) has been described as satisfactory for measuring immunity in vaccinated animals Chadrasekaran et.al., 1994). Survival even of one mouse in the test group identifies an immune serum provided that all of an equal number of control mice die (De Alwis and Carter, 1980). Meanwhile, Gupta and Sareen (1976) and Nagy and Penn (1976), Concluded from their studies, that there is a good correlation between results of passive mouse protection on the serum of immunized cattle and the degree of protection against challenge of cattle and buffalo.

In the present study, as shown in Table (3) none of the pre-vaccination serum samples from all vaccinated or control calves showed the presence of antibody before vaccination. This means that they were neither previously exposed to *P.multocida* infection nor received P.multocida vaccine before being used in this experiment. Inoculation of Montanide ISA 25 vaccine didn't passively protect mice against HS challenge at 7th day post-vaccination. At the 10th day post-vaccination calves developed detectable mouse protective antibody which passively protected mice against challenge.

Inoculation of Aluminum hydroxide gel and Saponin vaccine didn't passively protect mice against challenge at 7th and 10th day post-vaccination. At the 15th day post-vaccination calves developed detectable mouse protective antibody which

passively protected mice against challenge. Comparing the results of active mouse protection test with that of the passive mouse protection test, it appears that both tests gave almost the same results which indicated the presence of , high correlation between them.

In conclusion, it could be suggested that Montanide ISA 25 vaccine shown to be efficacious in eliciting early protective immunity in mice and calves as early as 10 days following vaccination and could be recommended as an emergency vaccine for controlling HS infection in cattle and buffaloes during outbreaks as a ring vaccination in area around the outbreak.

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