Archives of Razi Institute, Vol. 62, No. 3, Summer (2007) 145-149

Stability and potency studies of anthrax vaccine (*Bacillus anthracis* 34F2 Sterne strain) in Iran

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Received 08 Dec 2006; accepted 11 May 2007

ABSTRACT

For evaluation the stability and potency of anthrax vaccine prepared in Razi Vaccine and Serum Research Institute in Iran, samples of different batches of vaccine were kept at 4-8 °C (refrigerator), 20-25 °C (room temperature) and 37 °C (incubator).The viable spores/ml of vaccines were determined using plate–counting method before and after holding at different temperatures monthly. Vaccine potency and duration of immunity conferred by the vaccine in animals were determined by challenging each group of vaccinated animals with virulent strain of *Bacillus anthracis* at 6 months intervals up to 24 months post vaccination. The results showed that the spores in vaccine remained viable at the recommended level for up to 3 years at 4 °C, 2 years at room temperature and 2 months at 37 °C. All the vaccinated animal groups resisted and survived against challenge 6, 12, 18 and 24 months post vaccination. So, it is concluded that the anthrax vaccine could be stable up to 3 years at 4-8 °C and the immunity conferred by vaccine in sheep and goats lasts for 24 months post vaccination.

Keywords: Anthrax vaccine, stability, potency, sheep, goats

INTRODUCTION

The current anthrax vaccine for use in most countries to protect animals against anthrax, is the vaccine prepared with live spores of a non-encapsulated but toxigenic variant of *Bacillus anthracis* 34F2 Sterne strain developed by Max Sterne in 1939 (Sterne & Ragnsen, 1939, Sterne 1939). He discovered a rough variant of virulent *B. anthracis* when grown in serum agar with increased CO2. This variant was incapable of forming a capsule and was subsequently found to have lost the pXO2 plasmid which codes for capsule formation (Sterne 1937). It has become the most widely used strain worldwide for animal anthrax vaccine production. This vaccine is intended for use in all susceptible domestic animals which are at risk of acquiring anthrax due to contact with the contaminated soil, forages, carcasses of infected animals, etc. (OIE Manual 2004, Turnbull *et al* 1998). Since the successful development of anthrax spore vaccine from a non-encapsulated, but toxigenic variant of *B. anthracis* in 50% glycerin saline solution in most countries (OIE Manual,

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2004, Misra 1991, WHO 1967, Sterne & Regnsen, 1939) and in merthiolate in Iran (Delpy & Mirchamsy 1949), the vaccine is being used widely in sheep, goats, cattle, horses, elephants, camels and buffalos. Some vaccine producers may use saponin (1:500) as an adjuvant in the vaccine (OIE Manual 2004, Misra 1991). The anthrax vaccine used in Iran contains 10-12×10^6 viable spores/ml suspended in physiological saline solution containing 1:20000 merthiolate w/v. The study presented here was undertaken to determine the stability of this vaccine prepared at Razi Vaccine and Serum Research Institute (RVSRI) in Iran holding at 4-8 °C (refrigerator), 20 - 25 °C (room temperature) and 37 °C (incubator), and also to determine the potency and duration of immunity afforded by vaccine in vaccinated animals.

MATERIALS AND METHODS

Anthrax spore vaccine. The anthrax spore vaccine (living) is prepared in Aerobic Bacterial Vaccine Research and Production Department of RVSRI, Iran according to (OIE Manual, 2004, Misra 1991, WHO 1967) instructions. It contains 10-12×10^6 viable spores of avirulent non-encapsulated *B. anthracis* strain (34F2 Sterne) per/ml in physiological saline solution containing 1:20000 merthiolate w/v. The vaccine was passed different quality control tests such as purity, safety and potency tests according to (OIE Manual2004, British Veterinary Pharmacopoeia 1998) in quality control department. Based on manufacturer's recommendations 0.5 ml of vaccine as a single dose for sheep and goats injected subcutaneously is protective at least for one year.

Stability tests. For determination of the stability of anthrax vaccine at different temperatures 15 vials from one filling lot were selected randomly, 5 vials were maintained at 4-8 °C (refrigeration) for long term stability, the 5 vials were maintained at room temperature and 5 vials at 37 °C for accelerated stability. The average number of viable spores/ml of vials maintained at different temperatures were determined at the end of each months by plate colony counting method in parallel with unstored vaccine and with the reference preparation. There should not be a drop in the number of culture spores bellow the prescribed limits required for immunization of animals (Misra 1991, British Veterinary Pharmacopoeia 1998).

Immunization trials. A total of 20 unvaccinated sheep and 20 unvaccinated goats of local breeds delivered from Animal Production Department of RVSRI. All animals were vaccinated subcutaneously each with one dose (0.5 ml) of the anthrax vaccine containing 10-12×10^6 viable spores/ml. Vaccinated animals were divided into 4 groups, each group containing 5 sheep and 5 goats. Eight unvaccinated animals (4 sheep and 4 goats) maintained as controls each received 0.5 ml physiological saline subcutaneously according to corresponding immunization protocols (OIE Manual 2004, British Veterinary Pharmacopoeia, 1998).

Virulent *B. anthracis* strain for challenge. Virulent strain of *B. anthracis* namely C2 strain with defined MLD (Minimum Lethal Dose, the minimum dose require to kill all the animals under experiment) for sheep and goats, maintained in this department in the lyophilized form in special ampoules was used as challenge strain. C2 strain is a virulent strain isolated in Iran. Each MLD of this strain contains 30000 viable spores. A challenge dose of 100 MLD (3×10^6 spores) in 1 ml physiological saline for each vaccinated animal and 10 MLD (3×10^5 spores) for each control animal administered subcutaneously.

Challenge tests. Each group of vaccinated sheep and goats were challenged at the end of six months intervals up to 24 month post vaccination with 100 MLD of the virulent *B. anthracis* C2 strain spores suspension in physiological saline solution according to the recommended instructions (OIE Manual, 2004, British Veterinary Pharmacopoeia

1998, Ivins 1994). One unvaccinated sheep and one unvaccinated goat as control animals also were challenged with 10 MLD of the same virulent strain along with vaccinated animals at each challenge test. All the animals observed for 10 days post challenge for death or survival and the death time was recorded.

Table 1. The number of viable spores in anthrax vaccine at different temperatures and different times.

Time	The number of viable spores /ml×10^6		
Month	4-8 ˚C	20-25 ˚C	37 ˚C
1	12.5	12	12.4
2	12.1	12.5	9.6
3	11.8	11.6	7.5
6	12.2	11.8	7.2
8	13.2	12.1	6.1
10	13.0	12.8	3.1
12	13.4	12.2	2.0
14	12.7	12.9	0
16	13.1	12.6	0
18	13.9	11.8	-------
20	13.2	12.5	-------
22	12.6	11.9	-------
24	12.1	11.7	-------
26	12	10.3	-------
28	11.4	9.2	-------
30	11.7	8.7	-------
32	11.0	8.6	-------
34	10.4	8.9	-------
36	9.3	8.7	-------
38	6.8	8.0	-------
40	6.7	7.0	-------
42	5.2	7.2	-------
44	4.7	5.0	-------
46	5.6	5.2	-------
48	4.1	3.7	-------

Confirmation the cause of death of animals. Ear blood smears were prepared from dead animals with caution. The smears were stained by MC Fadyeam staining method with polychrome methylene blue stain to observe the encapsulated *B. anthracis*. Bone marrow from dead animals were cultured on sheep blood agar medium plates with strict cautions and the plates were incubated at 37 °C for 24 hours to determine the cause of death of control animals.

RESULTS

The results of viable spores count in anthrax vaccine at different temperatures and different times up to 48 months are shown in Table 1. The results showed that the number of culturable spores in vaccine maintained at 37 °C was decreased from the second month of incubation (9.6×10^6 /ml).

Table 2. The results of challenge of vaccinated and control animals challenged with virulent strain of B. anthracis spores suspension (C2 strain).

Animals groups	Challenge time post vaccination (month)	Vaccinated groups			Control groups		
		Challenge Dose (MLD)	Survived/ Challenge	Percent of protection	Challenge Dose (MLD)	Survived/Challenge	Percent of protection
I	6	100	10/10	100	10	0/2	0
II	12	100	10/10	100	10	0/2	0
III	18	100	10/10	100	10	0/2	0
IV	24	100	10/10	100	10	0/2	0

After third month decreased to below the prescribed limits required for immunization of animals and finally became zero after one year. The number of viable spores in vaccine maintained at room temperature remained viable up to 2 years and decreased afterwards. The live spores in vaccine held in refrigerator remained viable up to 36 months at the prescribed limits required for immunization of animals. All the vaccinated animals survived after challenge with 100 MLD of virulent strain of *B. anthracis*, and all the unvaccinated control animals died 3-4 days after challenge with 10MLD of the same strain. The results of challenge trials are shown in Table 2. Stained smear from dead animals revealed numerous Gram positive bacilli which were surrounded by large pink capsule, typical characteristic of virulent *B. anthracis*. Cultures of bone marrow showed typical non-hemolytic and grind glass appearance of *B. anthracis* colonies on

sheep blood agar plates. The bacteria grown on blood agar confirmed by lack of motility, production of capsule in defibrinated horse blood within 6 hours. The results of challenge tests showed that the vaccine can confer immunity in animals which lasts at least for 24 months.

DISCUSSION

All vaccines are sensitive biological substances that progressively lose their potency (i.e. their ability to give protection against disease). This loss of potency is much faster when the vaccine is exposed to temperatures outside the recommended storage range. Thus, storage of vaccines at the correct recommended temperature conditions is vitally important in order that full vaccine potency is retained up to the moment of administration. It is recommended that, in each filling lot, number of all spores should be determined before and after holding at an appropriate temperature for an appropriate period in parallel with unstored vaccine and with the reference preparation. There should be no evidence of a fall in the number of culturable spores bellow the prescribed limits required for immunization of animals (OIE Manual 2004, Misra 1991). Various potency tests have been developed to measure consistency in the quality of bacterial vaccines during the manufacturing process. Potency tests measuring the biological activity of the immunogenic in a living system (bioassay) as in determining protection against challenge is recommended by different instruction protocols for anthrax vaccine (OIE Manual 2004, Habig 1993, Misra 1991). From the survival percentage (Table2), it seems that 100% of the vaccinated sheep and goats withstood the challenge infection with 100 MLD of virulent spores of *B. anthracis,* and 100% mortality was noted in all the control groups. The results of survival percentage in sheep and goats revealed that the potency of vaccine was satisfactory according to the recommended instructions. The anthrax spore vaccine (Living) used in Japan contains at least 5 million viable spores/ml (Minimum Requirements for Biological Products for animal use, Japan,1970) while the vaccine used in U.K. contains not less than 10 million spores/ml (British Veterinary Codex 1998) and the vaccine used in India contains 1 to 1.5 million viable spores/ml (Jaiswal & Mithal 1979), whereas the vaccine prepared in Iran contains 10-12 million spores/ml. British Veterinary Codex recommends the survival of 100% of sheep and goats inoculated with a potent vaccine containing not less than 10 million spores/ml on challenge with 100 MLD of virulent *B. anthracis*. The vaccine prepared in India was potent enough to protect 74% of guinea pigs and 84% of sheep and goats against challenge with 100 MLD of virulent spores (Jaiswal & Mittal, 1979). The vaccine prepared in Iran protected 100% of animals against challenge with 100 MLD of virulent spores up to at least 24 months post vaccination. The data in this study indicate that the anthrax vaccine is protective in sheep and goats against subcutaneous challenge with spores of virulent strain of *B. anthracis* up to 24 months post vaccination. The results have revealed that if the anthrax vaccine kept at appropriate temperature (4-8 °C) during storage and transportation it will be stable and could be used up to 3 years, whereas if cold chain is not available but the vaccine keep away from direct sunlight, in moderate temperature which the variation of temperature is not high and the temperature doesn't exceed 20 °C, it can be used up to 2 years. However we suggest that the anthrax vaccine until use should be kept at 4-8 °C in refrigerator.

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