Evaluation the Efficacy of Anthrax Vaccine against Challenge with a Highly Virulent Strain of *Bacillus Anthracis* Isolated from Soil in Sheep, Goats and Guinea Pigs in Iran

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Summary

Protection of animals immunized against Bacillus anthracis is usually demonstrated by challenging with an appropriate dose of a strain of *Bacillus anthracis* that is lethal to unvaccinated animals inoculated at the same time. In this study the protective efficacy in anthrax vaccine ($34F_2$ sterne strain spore) was evaluated in sheep, goats and guinea pigs challenged with subcutaneous inoculation of a highly virulent *Bacillus anthracis* spores recently isolated from soil in Iran. This strain has been shown that it is lethal for sheep, goats and guinea pigs in very low dose. It was found that the vaccine provides highly protection (100%) after challenging the immunized animals against this strain in sheep, goats and guinea pigs. So, indicates the superior protective efficacy of the vaccine. The authors recommend that this strain can be used instead of other strain which has been used previously for challenging the immunized animals, and also guinea pig is a good substitution for evaluating anthrax vaccine.

***Key words*:** Efficacy, Anthrax vaccine, Challenge, Sheep, Goat, Guinea pig

Introduction

Bacillus anthracis is the causative agent of anthrax, an infectious often fatal disease of domestic and wild animals, and man. *Bacillus anthracis* is a large encapsulated

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Gram-positive, non- motile, spore- forming rod, and 1-1.5µm by 4-10µm with square ends. It can be cultivated in ordinary nutrient media under aerobic and anaerobic conditions. *Bacillus anthracis* shed by infected animals at death is found in or on products from such animals, or in soil contaminated by them, as resistant spores that may persist for years in soil (OIE Manual 2000, Hirsh & Zee 1999).

The ability of these spores to remain viable for many years in animal products, soil and industrial environment is an important factor in the epidemiology of anthrax and explains the predominant occurrence of the disease in herbivores. Anthrax spores are transmitted to animals through ingestion of contaminated water, hay or grazing in areas, which have previously experienced anthrax (OIE Manual 2000, Watson & Keir 1994).

In Iran and most countries of the world prevention and control of anthrax in domestic animals have been done by using live anthrax spore vaccines (Delpy & Mirchamsy 1947). The anthrax vaccine in Iran, Australia, Great Britain, China, Colombia, Czech Republic, Ethiopia, France, Hungary and many other countries, is made using 34F2 Sterne strain of *Bacillus anthracis*, but in Italy, Pasteur II strain, in Romania 1190R-Stamatin strain and in Russia strain 55 are using for anthrax vaccine production (Turnbull 1988). Due to existence of the anthrax and its occasional outbreaks in some parts of the country, the rate of vaccination of animals has been increased annually. There are 70 millions of sheep and goats, and 5 millions of cattle and buffalos in Iran. So the kind of vaccine and its immunogenicity for prevention and control of the disease in such population of animals is very important. In recent years it has been tried to cover all the animal population under anthrax vaccination programs. The vaccine is used annually and protects the animals against the anthrax for at least one year. For evaluation the immunogenicity of vaccine, the challenge of vaccinated animals with an appropriate dose of a suitable strain of *Bacillus anthracis* has been recommended (OIE Manual 2000, British Veterinary Pharmacopoeia 1998).

Evaluation of immunogenicity of anthrax vaccine in Iran is done with a strain of virulent *Bacillus anthracis* named C_2 strain. It has been observed that this strain is not highly virulent and also is not lethal for mouse. In this study we decided to evaluate the immunogenicity of the anthrax vaccine produced in Razi Vaccine and Serum Research Institute (RVSRI) against challenge with a newly isolated strain of *Bacillus anthracis*, which has been isolated from soil in Iran recently (Moazeni Jula *et al* 2004). This strain is highly virulent and is lethal for mouse, guinea pig, rabbit, sheep and goat (its virulence is about 1000 times more than the virulence of C_2 strain for sheep and goat). After determination of Minimum Lethal Dose (MLD), minimum dose required to kill all the exposed population, for this strain in guinea pigs, sheep and goats (Moazeni Jula *et al* 2004), the immunogenicity of the vaccine in these animals has been evaluated against challenge with this strain. So, this paper describes experiments designed to evaluate the protection afforded by the Sterne spores vaccine ($34F_2$ Sterne strain of *Bacillus anthracis*) against challenge with the isolate of *B. anthracis* recently isolated from soil in Iran.

Materials and Methods

Animals. Ten healthy guinea pigs each weighing 300-500 grams at the beginning of immunization were delivered from laboratory animals production department in RVSRI and used for evaluation of anthrax vaccine against isolated field strain of *Bacillus anthracis*. Ten healthy animals were also used as control group.

Ten healthy sheep each weighing 40-45 kg and 10 healthy goats each weighing 25-30 kg at the beginning of immunization were delivered from laboratory animals production department in RVSRI and used for challenging against isolated field strain of *Bacillus anthracis.* Three healthy animals were also used as control group.

Vaccine. Anthrax spore vaccine (living, for veterinary use) used for immunization of animals was produced in Aerobic Bacterial Animal Vaccines Production Department in RVSRI according to OIE and WHO instructions. This vaccine is produced using an uncapsulated strain of *Bacillus anthracis* namely $34F_2$ Sterne strain. Each dose of vaccine for sheep and goat (0.5 ml) contains 5×10^6 to 6×10^6 viable spores, and for large animals (2 ml) contains $10\text{-}12\times10^6$ spores/ml viable spores. According to the manufacturer's recommendations, 0.5 ml for sheep and goats and 2 ml for large animals (cattle and horse) as a single dose is protective at least for one year.

Challenge test. A toxigenic encapsulated virulent strain of *Bacillus anthracis* isolated from soil in Semirum test area of Isfahan province of Iran in 2004 (Moazeni Jula *et al* 2004) was used for challenging the vaccinated animals along with unvaccinated control groups.

Immunization of animals. Each group of animals (guinea pigs, sheep and goats) were immunized subcutaneously at day 0 with one dose (0.5ml) of anthrax spore vaccine. Each animal in control groups received physiological saline according to the corresponding immunization protocol (OIE 2000, British Veterinary Pharmacopoeia 1998). All the animals observed for 21 days.

Preparation of challenge dose. Spore suspension used for challenging of animals were obtained from cultures of newly isolated strain of *Bacillus anthracis* grown on sheep blood agar plates at 37ºC. After complete sporolation, the spores were harvested using sterile physiological saline solution (pH=7.3) and glass beads. Then ten fold serial dilutions ranging from 10^{-1} to 10^{-9} dilutions were done in physiological saline solution. The number of viable spores in each dilution was determined via plate colony counting method. Five animals per each dilution were inoculated subcutaneously with each dilution of the spores (1 ml for each animal). The experiments were done twice. The last dilution, which killed all the population of

animals, was determined as MLD for that specific animal species. MLD for sheep and goat were 1000 spores and for guinea pig were 70 spores.

Challenging of the vaccinated animals. According to the recommended instructions, 100 MLD (1×10^5 spores/ml) for each immunized sheep and goat, and 10 MLD (1×10^4 spores/ml) for each non-immunized control sheep and goat administered subcutaneously 21 days after immunization. 100 MLD (7×10^3 spores/ml) for each immunized guinea pigs, and 10 MLD (7×10^2 spores/ml) for each non-immunized control guinea pigs administered subcutaneously 21 days after vaccination (OIE Manual 2000, British Veterinary Pharmacopoeia 1998). All animals were monitored for 10 days post challenge for death or survival and the death time of animals was recorded.

Heart blood and edema smear from dead guinea pigs and ear blood smear from dead sheep and goats were made with caution. The smears were stained by Mc Fadyean staining method with polychrome methylene blue stain (OIE Manual 2000) to observed the encapsulated *Bacillus anthracis.* Heart blood from dead guinea pigs and bone marrow from dead sheep and goats were cultured on sheep blood agar medium plates with strict cautions and the plates were incubated at 37ºC for 24 hours to determine the cause of death of animals.

Results

All the vaccinated sheep, goats and guinea pigs survived after challenge with 100 MLD of the newly isolated strain of *Bacillus anthracis*, and all the control unvaccinated sheep and goats died after challenge with 10 MLD of the same strain within 3-4 days post challenge. The control unvaccinated guinea pigs died after 1-2 days post challenge (Table 1). Stained heart blood smears from guinea pigs revealed numerous gram-positive bacilli. These bacilli were surrounded by large pink capsule, which is typical characteristic of virulent *Bacillus anthracis*.

Cultures of heart blood from dead guinea pigs and bone marrow from dead sheep and goats showed typical non-hemolytic and grind glass appearance colonies of *Bacillus anthracis* on sheep blood agar plates. The bacteria grown on blood agar confirmed by lack of motility and production of capsule after culturing in sterile defibrinated horse blood within 6 hours.

Table 1. *Results of challenging animals with newly isolated strain of Bacillus anthracis.*

Group	Animals	Challenge dose (MLD)*	No. of animals challenged	No. of animals Survived
Vaccinated	Sheep	100	10	10
	Goats	100	10	10
	Guinea pigs	100	10	10
Control	Sheep	10	3	0
	Goats	10	3	0
	Guinea pigs	10	10	0

* *1 MLD for sheep and goats= 1000 spores, 1 MLD for guinea pigs= 70 spores*

Discussion

Protection of animals immunized against *Bacillus anthracis* is usually demonstrated by challenging with an appropriate dose of a strain of *Bacillus anthracis* that is lethal to unvaccinated animals inoculated at the same time (OIE Manual 2000, British Veterinary Pharmacopoeia 1998). Several strains of *Bacillus anthracis* have been reported previously to cause fatal infection in immunized guinea pigs. Little and Knudson (1986) have immunized guinea pigs with either a protective antigen vaccine or a live Sterne strain spore vaccine and challenged with virulent *Bacillus anthracis* strain isolated from various host species from the United States and foreign sources. They identified that 9 of the 27 challenge isolates were vaccine resistant.

They showed there was a great discrepancy that might be explained by the different isolates used for challenge (Little & Knudson 1986).

Fellows *et al* (2001) tested the efficacy of a human anthrax vaccine in guinea pig, rabbit and rhesus Macaques against spore challenge by *Bacillus anthracis* isolates of diverse geographical origin. Initially groups of vaccinated guinea pigs challenged intramuscularly with spores from 33 isolates of *Bacillus anthracis*, survival of the vaccinated groups varied from 6 to 100%. Then vaccinated white rabbits were challenged with spores from 6 of the isolates that were highly virulent in vaccinated guinea pigs. The vaccine completely protected the rabbits from 4 of the isolates. Subsequently 2 of these isolates were then used to challenge vaccinated rhesus Macaques. The vaccine protected 80 and 100% of the animals from these 2 isolates. They demonstrated that although the vaccine confers variable protection against different *Bacillus anthracis* isolates in guinea pigs it is highly protective against these same isolates in both rabbits and rhesus Macaques (Fellows *et al*. 2001).

In a similar study Ivins *et al* (1994) tested the efficacy of a standard human vaccine in guinea pigs challenged with spores from two virulent strains of *Bacillus anthracis*, Vollum 1B and Ames. They showed that protection against challenge with the Vollum 1B strain was better than with the Ames strain (Ivins et al 1994). In Iran, the immunized animals vaccinated with $34F_2$ Sterne strain of *Bacillus anthracis* usually challenge with a virulent strain of *Bacillus anthracis* namely C_2 strain. The MLD of this C_2 strain for sheep and goat is usually 3×10^5 viable spores (present reports in RVSRI). In This study we used a strain of *Bacillus anthracis* recently isolated from soil (Moazeni Jula *et al* 2004) for challenging the immunized sheep, goats and guinea pigs in comparison to C_2 strain to determine the rate of protection of vaccinated animals against this newly isolated strain of *Bacillus anthracis*. The MLD of this strain as mentioned before 1×10^3 viable spores which is 300 times less than the MLD of C_2 strain, (means its virulence for sheep and goat is 300 times more than C_2 strain). The present study was undertaken to clarify either the immunized animals

vaccinated with $34F_2$ Sterne strain spore vaccine can resist against challenge with the newly isolate strain of *Bacillus anthracis* or not. Although various antigen preparations appear to provide a substantial degree of protection when immunized animals are challenged with the standard Vollum strain, early studies by Auerbach and Wright (1955), and Ward *et al* (1965) demonstrated that certain *Bacillus anthracis* isolates were able to override this immunizing in guinea pigs. Although guinea pigs were immunized effectively against a *Vollum* challenge, they were not protected against challenge with some other isolates of *Bacillus anthracis.*

Vaccination of guinea pigs with Sterne strain spore vaccine appears to provide broad protection against challenge with various anthrax isolates. The dose of spores administered, the strain of avirulent spores used, and the presence of adjuvant are all important factors influencing the ability of a spore vaccine to protect against challenge (Little & Knudson 1986, Sterne 1939). Different animal species vary greatly in their resistant to infection by anthrax or by different strains of *Bacillus anthracis* (Ivins *et al* 1994, Welkson & Freindlander 1988). While differences have been observed in the virulence of various isolates of *Bacillus anthracis*, the basis of vaccine resistance in strains has not been elucidated Welkson *et al* (1993) have demonstrated that only a portion of these virulence differences can be attributed to plasmid-related effects.

In evaluation of anthrax vaccine, it is important to test protection afforded by the vaccine by using a wide variety of challenge isolates. Therefore, since in this study the vaccinated animals showed protection against this highly virulent strain. It can be concluded that the vaccine has high immunogenicity for animals and can produce high immunity in animals against other highly virulent strains. So it indicates the superior protective efficacy of the vaccine.

For evaluation of the anthrax vaccine we recommend that it is better to use this new isolated strain of *Bacillus anthracis* for challenging the immunized animals to be sure

that all the vaccinated animals can protect against any other strain of *Bacillus anthracis* that may be confronted too.

Due to highly virulence of this strain and resistance of immunized animals against challenging with it, we recommended this strain to be used in challenge tests.

According to the results of this experiment that showed vaccinated guinea pigs were highly protected against challenge with the newly isolated strain, therefore we recommend that guinea pig can be good substitution for sheep and goat in evaluating anthrax vaccine. In this case the evaluation of the vaccine will be safer, feasible, cheaper and easier and the dangerous of spreading of bacteria can be completely under control. Evaluation of protection afforded by immunization against anthrax has been made by either survival test or measurement of the serological titer to the antigen used as an immunogen. Little and Knudson (1986) indicated that although a high antibody titer was obtained after immunization, as demonstrated by immunization with Sterne strain spores or protective antigen (PA) vaccine plus lethal factor (LF), it did not reflect the level of expected protection. This was demonstrated after challenge with a vaccine-resistant isolate. Ward *et al* (1965) also recorded death of guinea pigs with high antibody titer which were challenged with a vaccine-resistant isolate (littlle and Knudson 1986). Since this vaccine is a live immunogen, and the newly isolated strain is very highly virulent, safety factors must be considered before their use and also in handling the challenged and dead animals.

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