Isolation of Anthrax Spores from Soil in Endemic Regions of Isfahan, Iran

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Summary
To isolate and detect anthrax spores from soil in different regions of Isfahan, Iran a total of 60 environmental specimens were collected during 2003. Bacterial endospores were extracted via flotation in distilled water and were cultured on blood agar and selective PLET media. *Bacillus anthracis* was identified using bacteriological and biological tests. Viable *Bacillus anthracis* spores were isolated from 9 (15%) soil samples of the 60 collected specimens in which 6 (66%) of isolates were encapsulated. The isolated bacteria and their virulence were confirmed with polymerase chain reaction (PCR) using specific primers. Its recommend that because of the existence of highly virulent strain of *Bacillus anthracis* in this region, a review on implementation of control programs such as regular vaccination of all susceptible livestock and surveillance of the disease in animals and human in such endemic areas is required.

Key words**:** *Bacillus anthracis*, anthrax, soil, Iran

Introduction

Bacillus anthracis (*B.anthracis*) is the causative agent of anthrax, an infectious, often fatal disease of animals and man. *B.anthracis* is a large encapsulated, Gram–positive and spore-forming rod. Anthrax spore is resistant to heat and chemical disinfectants and can germinate in soil at pH6.5 at proper temperature (Hirsh & Zee 1999, Quinn *et al* 1994, OIE manual 2000). So, *B.anthracis* is found in areas where

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anthrax spores are located. Anthrax is transmitted to grazing animals through ingestion of spores via contaminated feed and water. The incidence of infection may increase as a result of drought or overgrazing when there is a greater likelihood of animals breathing or ingesting spore contaminated dust (Watson & Kier 1994). Other routes of transmission by biting flies or contact with contaminated faces have been described (Sjostedt *et al* 1997, OIE manual 2000). Soil is contaminated with the spores from the carcasses of infected animals (Manchee *et al* 1981, Watson & Keir 1994, Dragon *et al* 2001, Hunter *et al* 1989). Another source of spores in soil may be contaminated effluents from plants working with animal products such as leather tanneries (OIE manual 2000, Sjostedt *et al.* 1997). 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 (Watson & Keir 1994, Titball *et al* 1991).

From many years ago vaccination of the animals is the only way for prevention and control of the disease in Iran. But during the last years different epizootics of anthrax were recorded mainly in central parts of Iran. The bacteriological and molecular study described here was initiated in order to determine the soil contamination in Isfahan province of Iran, which can be used for planning the prevention and controlling programs of the disease in future.

Materials and Methods

Environmental sampling. During 2003 soil specimens were collected randomly from wallows and open meadows and also from carcass disposal sites and livestock habitats in different anthrax endemic regions of Isfahan province in central part of Iran. During collecting specimens, the areas were considered contaminated and appropriate safety precautions were taken. The collected environmental specimens were composed of a heterogenous mixture, which included soil, animal faces from carcass disposal sites and livestock habitats, vegetation, etc. Approximately 500g of

the top soil to a maximal depth of 20cm were collected using a scoop and carefully transferred to labeled plastic bags. A total of 60 specimens from different regions of Semirum, Daran, Faridan and Shahreza areas were send to the laboratory in Razi Vaccine & Serum Research Institute (RVSRI), Karaj.

Spore extraction and bacteria identification. Bacterial endospore extraction and screening was carried out under careful precautions in the anthrax diagnostic laboratory at RVSRI according to OIE manual (2000). Briefly, an overnight-incubated soil suspension was filtrated through 0.45µm and the deposit was suspended in sterile PBS. The aliquot was heated at 65°C to destroy vegetative cells and activate the spores. Then the suspension was centrifuged and the resuspended pellet was streaked onto duplicate plates of PLET agar and blood agar media. One set of cultured plates were incubated at 37°C and the other at 40°C, both aerobically. Colonies emerging at the end of 24–48h incubation were examined for morphological and cultural features of *B.anthracis*.

Biochemical and biological tests. The colonies identified as *B.anthracis* were selected and further biochemical tests were conducted according to conventional bacteriological methods (Hirsh & Zee 1999, Quinn *et al* 1994). In order to confirm the lethality of the isolates inoculum comprised of a saline suspension containing different numbers of spores injected subcutaneously to mice, guinea pig, rabbit, rat, sheep, and goat.

Polymerase chain reaction (PCR). The DNA was extracted by three times freezing and thawing of the inactivated bacterial cultures in liquid nitrogen. Then the samples were vortexed vigorously and centrifuged at 12000*g* for 1min. The supernatant were taken and used as DNA sample. The oligonucleotide primers Bac F (5′-AAT GAT AGC TCC TAC ATT TGG AG-3′) and Bac R (5′-TTA ATT CAC TTG CAA CTG ATG GG-3′) for amplification of a 330bp *B.anthracis* specific fragment, PA F (5′-CGA AAA GGT TAC AGG ACG G-3′) and PA R (5′-CAA GTT CTT TCC CCT GCT A-3′) for detection of toxin gene at a 152bp fragment

and, Cap F (5′-GTA CCT GGT TAT TTA GCA CTC-3′) and Cap R (5′-ATC TCA AAT GGC ATA ACA GG-3′) for amplification of a 209bp capsule gene fragment were used. The PCR reaction was carried out in 0.2µl microtube with a final volume of 25µl. The reaction solution contained 4mM $MgCl_2$, 200µM of each dATP, dCTP, dGTP and dTTP, 0.4µM of each primer, 2.5µM of 10×reaction buffer and 3µl of DNA template. The cycling parameters were an initial denaturation at 94°C for 5min, fallowed by 35 cycle of denaturation at 94°C for 50sec, annealing at 50°C for 50sec, and extension at 72°C for 30sec. The final cycle was fallowed by an extension at 72°C for 1min. The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with 0.5µg/ml ethidium bromide.

Results

Out of the total 60 environmental soil specimens collected and screened with blood agar and PLET media, 9 specimens (15%) exhibited domed, circular, white colonies 4-8 mm in diameter that were morphologically similar to *B.anthracis*. 6 isolates were from Semirum and 3 isolates were from Shahreza. No positive sample was isolated from Daran and Faridan. The isolates were characterized biochemically and they were identified *B.anthracis* base on the reaction patterns. Giemsa–stained smear prepared from bicarbonate culture, and polychrome methylene blue–stained smear prepared from horse blood culture displayed rods with thick, enveloping, purple capsule around the bacteria compatible with *B.anthracis* (Figure 1). Six of the positive samples developed heavily mucoid on the bicarbonate plates. Giemsa-stained smear displayed rods with thick, enveloping, purple capsules. All of these 6 isolates were confirmed to be encapsulated *B.anthracis* by PCR.

Isolates of *B.anthracis* from the soil were lethal to mice, guinea pig, rabbit, sheep, and goat. The inoculated animals died within 24-72h post-inoculation but rat was resistant and did not die after inoculation of very high doses (Table 1). Different organs of the dead animals were examined bacteriologically and the presence of the

microorganism was confirmed. M'Fadyean-stained smear prepared from blood and different organs of the dead animals revealed large numbers of encapsulated bacilli.

Figure 1. *Bacillus anthracis isolated from soil sample is shown as square ended blue bacilli in short chains surrounded by pink capsules in a mouse liver smear (polychrome methylene blue stain)*

Table 1. *Route of inoculation, death time and relative resistance of animals inoculated with isolated Bacillus anthracis*

Animal	Route of inoculation	Death time (h) post-inoculation	Relative resistance
Mouse	Intraperitoneally	24-48	Very susceptible
Guinea pig	Subcutaneously	24-48	Very susceptible
Rabbit	Subcutaneously	48-72	Susceptible
Rat	Intraperitoneally	No death	Resistant
Sheep	Subcutaneously	48-72	Very susceptible
Goat	Subcutaneously	48-72	Very susceptible

Amplification of expected species-specific DNA band (152bp) by PCR confirmed the conventional bacteriological methods for identification of *B.anthracis*. The relative fragment of capsular gene (209bp) was amplified among all *B.anthracis* isolates which were identified as encapsulated by polychrome staining method. The capsular fragment was not amplified in vaccine *B.anthracis* strain (34F2 Sterne) and 3 soil isolates which did not contain capsule as was investigated by staining method. PA primers were amplified the expected fragment of 330bp in all *B.anthracis* isolates (Figure 2).

Figure 2. *PCR results: Lane A: 50bp DNA ladder, lane B: vaccine strain (non-encapsulated), lanes C, D and E: soil isolates of Bacillus anthracis (encapsulated)*

Discussion

Anthrax is still one of the most serious infectious disease in animals and man in Iran because of its wide ranging distribution. There have been no systematic studies of this pathogen in Iran, although controlling the disease through vaccination has been carried out for many years. For planning the prevention and control programs, it is necessary to know the epidemiology of the disease. In recent years, there have been some outbreaks of anthrax in some regions of Isfahan province in central part of Iran. Therefore, we decided to study the presence of the *B.anthracis* spore in the soil of these places. In this study a total of 60 soil specimens from Semirum, Daran, Faridan and Shahreza have been examined and viable *B.anthracis* spores isolated from 9 (15%) samples. Inoculation of laboratory animals confirmed the identity and revealed that the isolates were very virulent.

The deposition of anthrax bacteria in soil has been examined in details in several investigations. After the deliberate contamination of the Gruinard Island during the Second World War, sampling was regularly performed during 40 years the spores were, almost without exception, isolated from the top 6cm of soil (Manchee *et al* 1981). Similar studies were carried out in northern Canada and south Sudan (Dragon

et al 2001, Ramachandran *et al* 1988). Alkaline soil containing adequate nitrogen, calcium, and organic matter is required in conjunction with extreme weather changes, such as a drought followed by heavy rains. When these conditions are met, the organisms are thought to undergo a vegetative cycle in soil and then resporolate. This process could generate sufficiently high soil concentrations of anthrax spores to cause disease in grazing animals, producing the occasional outbreaks separated by long disease-free intervals that have been observed (Kauffman 1990, Van Ness 1972). Recurrent anthrax outbreak has been associated with calcareous, neutral or slightly alkaline soils rich in organic matter, which encourage survival of spores (Van Ness, 1971, Hugh Jones & Hussaini, 1974 and 1975). In this experiment pH of the samples had range from 7.3 to 8.7. Therefore, slightly alkaline pH as well as calcareous nature of the soil in these areas is suitable conditions for survival of spores. Animals may acquire the disease through inhalation of aerosolized spores during wallowing, or may ingest lethal levels of spores while grazing. Studies of the ecology of anthrax reveal a relationship among bacteria, environment, and host. They also indicate that anthrax is an infectious disease that its agent may multiply outside the affected host. Epidemiological evidence also indicates that suitable soils maintain an organism-spore–organism cycle for years without infecting livestock (Van Ness 1971).

PCR assay by using *B.anthracis*, capsule, and protective antigen specific primers confirmed the biochemical and biological findings. Amplifying the selected fragments indicated the applicability of PCR in the identification of the microorganism from soil as well as clinical samples. The current methods being used for identification of *B.anthracis* are time-consuming and do not specific, and-above-all- there is the risk of transmission of anthrax in the laboratory. The PCR assay is most reliable, rapid and minimizes the risks of transmission of anthrax in the laboratory (Sjostedt *et al* 1997). Our results indicate that PCR can use as an alternative or additional test for identification and confirmation of *B.anthracis* and

presence of capsule and protective antigen. According to isolation of *B.anthracis*, which was very virulent specially for sheep and goat, a review on implementation of control programs such as vaccination of all susceptible animals and surveillance of the disease in animals and human in such endemic regions is required. In addition, similar studies are suggested for better understanding of anthrax situation in other problematic parts of the country.

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