Determination of anthrax foci through isolation of *Bacillus anthracis* form soil samples of different regions of Iran

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ABSTRACT

To isolate and detect anthrax spores form soil in different regions of Iran in order to find the anthrax foci, a total of 668 environmental specimens were collected during 2003-2004. Bacterial endospores were extracted from soil specimens via flotation in distilled water, incubation at room temperature, filtration, heat shock and culture on blood agar and selective PLET media. *Bacillus anthracis* was identified using conventional bacteriological, biochemical and biological tests. Viable *B. anthracis* spores were isolated from 61 (9.1%) soil samples of the 668 collected specimens in which 21 (34.4%) isolates were encapsulated with different virulence from very high virulence to low virulence and the others (40 isolates) were unencapsulated. The isolated bacteria and their virulence were confirmed with Polymerase Chain Reaction (PCR) using B. anthracis, *cap* and *pa* specific primers. They also confirmed by inoculation into laboratory animals. Isolation of *B. anthracis* from soil in this study is reported for the first time in Iran. The encapsulated virulent bacteria were isolated from soil specimens of *Isfahan, Khuzestan, West Azarbaijan, Khorasan, Charmohal Bakhtiari and Gazvin* provinces. It is recommended that due to existence of highly virulent strains of *B. anthracis* in these regions, a review of implementation of control programs such as regular vaccination of all susceptible livestocks and surveillance of the disease in animals and human in such endemic areas is required.

Keywords: *Bacillus anthracis*, Soil, Iran, Anthrax spore

INTRODUCTION

Anthrax is primarily a disease of herbivores, although all mammals, including humans, and at least some avian species can contact it. The disease has worldwide distribution and is a zoonosis. Mortality can be very high, specially in herbivores. The etiological agent is the spore-forming, Gram-positive, rod-shaped, non-motile, *Bacillus anthracis*, the only obligate pathogen within the genus Bacillus.

Sporulation only occurs when the vegetative form is exposing to the atmosphere and conditions are unfavorable for the continued multiplication of the vegetative form. Anthrax spores are resistant to heat and chemical disinfectants (Hirsh & Zee 1999, Quinn *et al* 1994, OIE Manual 2004). To prevent environment contamination, it is usual not to perform a necropsy on the carcass of suspected or confirmed cases. If the carcass is opened by necropsy or scavengers, the vegetative form of *B. anthracis* is released from the acidic environment of

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decay and produce spores that create foci of contamination (OIE Manual 2004).

B. anthracis shed by infected animals at death is found in or on products form such animals, or in soil contaminated by them, as resistant spores that may persist for years in soil (Wilson & Russel 1964, Manchee *et al* 1981, Dragon *et al* 2001, Van Ness 1971).

The ability of these spores to remain viable for many years in animal products, soil and industrial environments is an important factor in the epidemiology of anthrax and explains the predominant occurrence of the disease in herbivores (Wilson & Russel 1964, Watson & Keir 1994, Van Ness 1971, Titball *et al* 1991).

Anthrax spores are transmitted to animals through ingestion of contaminated water, hay or grazing in areas which have previously experienced anthrax. 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 & Keir 1994).

B. anthracis requires very specific conditions for growing in soil. 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 re-sporulate. This process could generate sufficiently high soil concentration of anthrax spores to cause disease in grazing animals, producing the occasional outbreak separated by long disease–free intervals that have been observed (Kauffman 1990, Van Ness 1972).

Investigations of anthrax in Iran have been limited to field observation during active epizootics and have been focused mainly on the host. The environmental sources and movement of *B. anthracis* between outbreaks is unknown. Currently from many years ago vaccination of the animals is the only way for prevention and control of the

disease in Iran. The bacteriological study described here was initiated in order to determining the spore contamination of soil in different parts of Iran which can be used for planning the prevention and control of the disease and its surveillance in the country.

MATERIALS AND METHODS

Environmental sample collection. During years 2003-2004 several trips were undertaken to different parts of Iran to collect specimens from soil. Soil specimens were collected randomly from wallows and meadows in different regions. Soil specimens were also collected from carcass disposal sites, and sheep and goats habitats in these areas. During collecting specimens, the areas were considered contaminated and appropriate safety precautions were taken. The collected environmental specimens were composed of a heterogeneous mixture which included soil, animal faces, vegetation, etc. Samples were collected using a scoop. Approximately 500 grams of the top soil to a maximal depth of 20 cm were collected and carefully transferred to labeled plastic bags. A total of 668 specimens from different regions of the country at different times were collected and sent to the laboratory in Razi Vaccine and Serum Research Institute (RVSRI). Number of collected specimens from different regions is shown in Table 1.

Spore extraction and B. *anthracis* identification. Bacterial endospore extraction and screening was carried out under careful precautions in the anthrax diagnostic laboratory at RVSRI under class A laboratory laminar flow hood, according to OIE manual (2004) with some modifications. Briefly, an overnight incubated soil suspension in distilled water was filtered through 0.45 μm filter and the deposit on the filter was suspended in sterile PBS. The aliquot was heated at 65 °C for 20 minutes to destroy vegetative cells and activate the spores. Then the suspension was centrifuged and the pellet reconstituted in about 2 ml of sterile PBS.

Table 1. Province distribution of collected soil specimens during 2003-2004.

Provine	Number of specimens	Regions
Isfahan	88	Semirum, Shahreza, Daran, Ferydoon shahr, Najafabad
Fars	101	Marvdasht, Darab, Bavanat, Neyriz Daryoon, Firoozabad
Hormozgan	48	Hajiabad, Bbandarabbas
West Azarbayjan	25	Urumieh, Salmas
Tehran	25	Varamin,
Mazandaran	28	Sari
Gazvin	25	Gazvin
Kohkilovieh & Booyerahmad	36	Yasooj, Dehdasht
khorasan	42	Gonabad, Sabzevar, Kashmar, Esfarayen
Kermanshah	20	Paveh, Islamabad
Hamedan	16	Hamedan, Malayer
Kordestan	26	Gorveh, Bane, Maryvan, Bijar
Yazd	22	Mehriz, Taft
Lorestan	28	Aligoodarz, Chogalvandi
Markazi	65	Arak,khomain, Delijan, Shazand,Farahon, Ashtian,
Chahr Mohal-e-Bakhtiari	21	Sharkord, Lordegan, Broojen
Kerman	24	Kerman, Joopar
Khuzestan	28	Dasht-e-azadegan
Total	**668**	

The resuspended pellet was streaked on the duplicate of PLET (*Polymyxin, Lysozyme, Ethylene diamine tetraacetic acid*, *Thallium acetate*) and blood agar media. Colonies emerging at the end of 24-48 hours incubation were examined for morphological and cultural features of *B. anthracis*. The identity of colonies isolated with a morphology similar to *B. anthracis* on the PLET plates, was confirmed by lack of haemolysis on blood agar plates, a zone of inhibition of growth around 10 units penicillin discs and the ability to produce capsule in sterile defibrinated horse blood and development of mucoid, encapsulated colonies on bicarbonate agar as determined by Gimsa–stain or polychrome methylene blue stain (Carman *et al* 1985, OIE Manual 2004, Ramachandran *et al* 1988, Kinsely 1966, Dragon & Rennie, 2001).

Biochemical 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).

Biological tests. In order to confirm the lethality of the isolates of *B. anthracis*, inoculum comprised of a saline suspension containing different numbers of spores injected subcutaneously into mice, guinea pig, rabbit, rat, sheep and goat (Table 2).

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 1 min. The supernatant was 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 330 bp *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 152 bp 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 209 bp capsule gene fragment were used. The PCR reaction was carried out in 0.2 ml microtube with a final volume of 25 µl according to Cheun *et al* 2003 and Sjostedt *et al* 1997.

RESULTS

Out of the total 668 environmental soil specimens collected from different places and screened with PLET and blood agar media, only 61 (9.1%) isolates exhibited domed circular white colonies, 4-8mm in diameter without hemolysis on blood agar, that were morphologically similar to *B. anthracis*. The highest percentage of specimens with *B. anthracis*-like colonies were from Khorasan province while the lowest percentage was recovered from Lorestan province (Table2). *B. anthracis*-like colonies were not

recovered from Hamedan, Hormozgan,Yazd, Tehran, Mazandaran, Kermanshah, Kordestan and Kerman provinces. The isolates were characterized biochmically 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*. Despite the selectivity of PLET medium, contamination of the plates was observed. The contaminants in plates including *B. anthracis*-like colonies that were later discounted through confirmatory tests were high. These colonies were mostly *B. cereus, B. circulans, B. megaterium, B. subtilis and B. sphearicus.* The rate of recovery of *B. anthracis* from the specimens was inversely related to the degree of heat inactivation. No organisms were recovered when suspension exposed to 70-80 ºC. The experience showed that heat treatment at 65 ºC for 20 min and then culture on PLET and blood agar and incubating for 24 hours at 37 ºC is the best method. *B. anthracis*-like colonies from PLET were picked and tested with blood agar plates and penicillin discs. Sixty one (9.1%) specimens yielded isolates that, like *B. anthracis* were non-hemolytic and were inhibited to some degree by penicillin. Six isolates from Isfahan province, 2 from Fars, 5 from Khorasan, 2 from Kohkilovieh, 1 from Khuzestan, 2 from West Azarbayejan, 1 from Chaharmohal-e-bakhtiari and 2 from Ghazvin developed heavy mucoid colonies on the bicarbonate plates that were consistent with *B. anthracis*. Giemsa-stained smears prepared from the bicarbonate cultures and polychrome methylene blue-stained smear prepared from defibrinated horse blood culture displayed rods with thick, enveloping purple capsules around the bacteria compatible with *B. anthracis*. All of these 21 isolates were later confirmed to be encapsulated by PCR. These 21 isolates of *B. anthracis* were lethal to mice, guinea pig, rabbit, sheep and goat with different virulence and lethality. The inoculated animals died within 24-72 hours post-inoculation (Table 3).

Rat was resistant and did not die after inoculations even in high doses. 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. The sensitivity of each animal inoculated, to each one of these isolates was different. The MLD Values (Minimum Lethal Dose required to kill all the exposed population by parenteral inoculation) of one isolate (for example) were as 30 spores for mouse, 70 for guinea pig, 1000 for sheep and goat and 7×10^{4} For rabbit (Table 3). Amplification of expected species-specific DNA band (152 bp) by PCR confirmed the conventional bacteriological methods for identification of *B. anthracis*. The relative fragment of capsular gene (209 bp) was amplified among all *B.anthracis* isolates which were identified as encapsulated by polychrome methylene blue staining method. The capsular fragment was not amplified in vaccine strain (*34 F2 Sterne*) and 40 soil isolates which did not contain capsule as was investigated by staining method. Protective Antigen (PA) primers were amplified the expected fragment of 330 bp in all 61 *B. anthracis* isolates (Figure 1).

Table 2. Number of *B. anthracis* isolates and contaminants with *B. anthracis*-like properties recovered from environmental specimens from anthrax endemic regions in different parts of Iran.

Province	F	Is	M	Kho	K	Khu	L	WA	CH	G	Total
Speciemen Collected	101	88	65	42	36	28	28	35	21	30	668
B. anthracis-like Colonies on PLET	12 (11.9%)	10 (11.4%)	12(18.5%)	8(19%)	2(5.7%)	3(1o.7%)	1(3.6%)	6(17.1%)	2(9.5%)	5(16.6%)	61(9.1%)
Non –haemolytic Penicillin sensitive	2 (1.2%)	10 (11.4%)	12(18.5%)	8(19%)	2(5.7%)	3(1o.7%)	1(3.6%)	6(17.1%)	2(9.5%)	5(16.6%)	61(9.1%)
Mucoid colonies on Bicarbonate agar	2 (1.2%)	6 (6.8%)	0(0%)	8(19%)	2(5.7%)	1(3.8%)	0(0%)	2(5.7%)	1(4.7%)	2(6.7%)	24(3.6%)
Formation of capsule in horse serum	2 (1.2%)	6 (6.8%)	0(0%)	5(11.9%)	2(5.7%)	1(3.8%)	0(0%)	2(5.7%)	1(4.7%)	2(6.7%)	21(3.1%)
Encapsulated bacilli in dead animals	2 (1.2%)	6 (6.8%)	0(0%)	5(11.9%)	2(5.7%)	1(3.8%)	0(0%)	2(5.7%)	1(4.7%)	2(6.7%)	21(3.1%)
Lethal for animals	2 (1.2%)	6 (6.8%)	0(0%)	5(11.9%)	2(5.7%)	1(3.8%)	0(0%)	2(5.7%)	1(4.7%)	2(6.7%)	21(3.1%)
PCR positive	2(1.2%)	6(6.8%)	0(0%)	5(11.9%)	2(5.7%)	1(3.8%)	0(0%)	2(5.7%)	1(4.7%)	2(6.7%)	21(3.1%)

F= *Fars,* IS= *Isfahan,* M= *Markazi,* Kho= *Khorasan,* K= *Kohkylovieh,* Khu= *Khuzestan,* L= *Lorestan*, WA= *West Azarbayejan*, CH= *Choharmohal -e-bakhtiari,* G= *Gazvin*.

Table 3. sensitivity of animals to one of the isolates of *B. anthracis* isolated from soil.

| Animals	Inoculation route	Number of spores	Death time	Sensitivity
Mouse	Intraperitoneally	30	24-48	Very sensitive
Guinea pig	Subcutaneouslly	70	24-48	Very sensitive
Rabbit	Subcutaneouslly	70000	48-96	Sensitive
Rat	Intraperitoneally	2.1×10^6	_____	Resistant
Sheep	Subcutaneouslly	1000	48-72	Very sensitive
Goat	Subcutaneouslly	1000	48-72	Very sensitive

DISCUSSION

The deposition of anthrax bacteria in soil has been examined in details in several investigations. After the deliberate contamination of *Gruinard Island* during the Second World War, sampling was regularly performed during 40 years the spores were isolated from the top 6cm of soil (Manchee *et al* 1981). Dragon *et al* (2001) in a similar study in northern Canada demonstrated that from 588 environmental specimens, 11 samples (1.9%) were shown to contain viable *B. anthracis* spores. Ramachandran *et al* (1988) in their study in South Sudan isolated *B. anthracis* from 2 of 5 soil samples. Alkaline soil containing adequate nitrogen calcium and organic matter in conjunction with extreme weather changes such as a drought followed by heavy rains are necessary for perpetuating anthrax. When these conditions are met the organisms are thought to undergo a vegetative cycle in soil and then re-sporulate. This process could generate sufficiently high soil concentration of *B. anthracis* 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). The disease in man is still prevalent in southern Europe and various countries in Asia, Africa, the Middle East and former USSR (Watson & Keir 1994).Though anthrax has not been eradicated, the development of effective animal and human vaccines has reduced its importance for humans and animals in developed countries over the last century. In Iran anthrax is still one of the most serious diseases in animals and man. There have been no systemic studies of this disease In Iran, although controlling it through vaccination has been carried out for many years. For planning the

prevention and control programs, it is necessary to known the epidemiology of the disease, so we decided to identify the anthrax foci in different regions of the country.

In this study a total of 668 Soil specimens from different regions of Iran have been examined. Viable *B. anthracis* spores isolated from 61 (9.1 %) specimens. All 61 isolates were directly associated with distributed areas of carcass disposal sites, livestock production places and nomads roads. Only 21 specimens from 61 isolates (3.1 % of all specimens) were able to produce capsule and were lethal for animals with different virulence and lethality. The number and percentage of isolated virulent and avirulent isolates are shown in Table 2 and their distributions in different regions of Iran are shown in Figure 1. 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 & Husseini 1974 and 1975). In this experiment *pH* of the samples had range from 7.3–8.7. Therefore, slightly alkaline *pH* as well as calcareous nature of soil in these areas in Iran is suitable conditions for survival of Anthrax 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).

Different studies have shown that the virulence of B. *anthracis* can differ among isolates or strains. Some variation in virulence can be related to the presence or absence of the plasmids. Isolates lacking either pXO1 or pXO2 plasmids are considered either avirulent or significantly attenuated (Welkos 1991, Coker *et al* 2003).
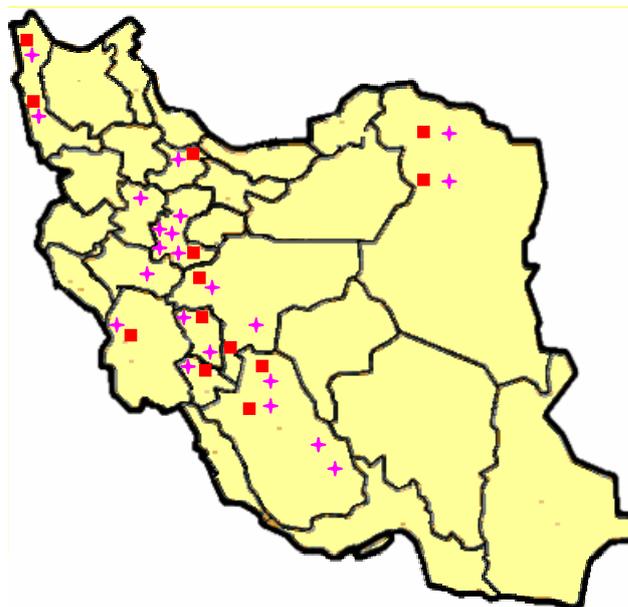
Figure 1. The anthrax foci in different regions of Iran.
■ The regions which live encapsulated B. anthracis have been isolated from their soil specimens.
✚ The regions which live un-encapsulated B. anthracis have been isolated from their soil specimens.

In animals the infectious dose is highly strain dependent, and this is likely to be true in human as well. The capsule and the toxin are encoded by genes on two separate plasmids. Loss of one or both of these plasmids, with consequent loss of ability to produce capsule and/or toxin, leads to loss of virulence. Naturally occurring mutants lacking one or both of these plasmids are providing to be not uncommon in the environment. Turnbull *et al* (1992) speculate that these may be modified variants or virulent counterparts, and thus indicative of virulent strains elsewhere in the system, either in the present or at some time previously (Watson & Keir 1994, Cocker *et al* 2003, Turnbull *et al* 1992).

PCR assay by using *B. anthracis*, capsule, and protective antigen specific primers confirmed the biochemical and biological finding. Amplifying the selected fragments indicated the applicability of PCR in identification of the microorganism from soil as well as clinical samples. The current method being used for identification of *B. anthrasic* are time-

consuming and don't 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 risk of transmission of anthrax in the laboratory (Sjostedt *et al* 1997, Cheun *et al* 2003). 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.

It is possible that the sandy soil was unsuitable for holding spores, and any persent, are quickly removed via water action. The acidity of the soil may have reduced the longterm viability of spores formed in the soil (Dragon and Rennie 1995). It is possible that the absence of existence of spores in some regions could be due to the nature of sandy soil or low *pH* or very hot weather such as in Hormozghan, Yazd and Kerman. The spores of *B. anthracis* did not isolate from soil specimens from these areas. Therefore, soil contamination is very important for perpetuating anthrax in farm animals and man in endemic regions. Since the disease will be most prevalent in summer and autumn especially in drought years in endemic regions when pastures are poor and the animals during grazing have closer contact with soil. So special attention should pay to complete vaccination of all susceptible animals in these regions.

It was originally believed that these soil conditions influenced vegetative anthrax bacilli and allowed for cycles of germination, growth and re-sporulation resulting in an overall increase in spore concentration (Van Ness 1971). However, vegetative *B. anthracis* have very specific nutrient and physiological requirements survive outside a host. Instead, the specific soil factors linked to endemic areas may reflect environment condition that aid in maintaining anthrax spores in soil and prolonging their viability (Dragon & Rennie 1995, Titball *et al* 1991). While sporadic outbreaks have shown that concentration of anthrax spores high enough to cause disease in animals are obviously present some where

in the endemic regions, it is unknown if the level of spores found here represent a sufficient dosage to cause disease. At least for contracting anthrax, a minimum lethal dose exist, although the magnitude of this dose depends very heavily on the strain of *B. anthracis* and the state of health of the host. Further complicating of the matter is the possibility of seasonal transient stresses, such as the estrus cycle and hot weather which may compromise animal immunologially, thereby reducing the infectious dosage required (Grainer and Saunders, 1989).

According to our finding in this study, some isolates of *B. anthracis* which are isolated from different parts are highly virulent, specially, for sheep and goats, so 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 problematic parts of the country. The anthrax spore detection system described here and isolation of virulent *B. anthracis* in this study could be used to survey the disease, and aid government in improving its response plans to the disease.

References

Cheun, H.I., Makino S.I., Watari, M., Erdenebaatar, J., Kawamoto, K. and Uchida, I. (2003). Rapid and effective detection of anthrax spores in soil by PCR. *Journal of Applied Microbiology* 95: 728-733.

Coker, P.R., Smith, K.L., Fellows, P.F., Rybachuck, G., koosoulas, K.G. and Hugh-Jones, M.E. (2003). *Bacillus anthracis* virulence in guinea pigs vaccinated with anthrax vaccine adsorbed is linked to plasmid quantities and clonality. *Journal of Clinical Microbiology* 41(3): 1212-1218.

Dragon, D.C. and Rennie, R.D. (2001). Evalution of spore extraction and spore purification method for selective recovery of viable *Bacillus anthracis* spore. *Letters in Applied Microbiology* 33: 100-106.

Dragon, D.C. and Rennie, R.P. (1995). The ecology of anthrax spores. Thaug but not invincible, *Canadian Veterinary Journal* 36: 295-301.

Dragon, D.C., Renine, R.P. and Elkin, B.T. (2001). Detection of anthrax spores in endemic regions of northern Canada. *Journal of Applied Microbiology* 91: 432-441.

Grainer, R.S. and Saunders, J.R. (1989). Aspects of the epidemiology of anthrax in Wood Buffalo National park and environs. *Canadian Veterinary Journal* 30: 953-956.

Hirsh, D.C. and Zee, Y.C. (1999). The jenus Bacillus. In: L.Ernst, B. Berstain, D.C. Hirsh (Eds.), *Veterinary Microbiology.* Pp: 246-249. Blackwell Science Inc., USA.

Hugh-Jones, M.E., and Hussaini, S.N. (1975). Anthrax in England and Wales. 1963-72. *Veterinary Records* 79: 256-261.

Hunter, L., Corbett, W. and Grindem, C. (1989). Zoonosis updated: anthrax. *Journal of American Veterinary Association* 8: 1028-1031.

Kauffman, A.F. (1990). Observation on the occurrence of anthrax as related to soil type and rainfall. Proceeding of the international workshop on anthrax. Salisbury *Medical Bulletin (Suppl.)* 68: 16-17.

Kinsly, R.F. (1966). Selective medium for *Bacillus anthracis*. *Journal of Bacteriology* 92: 784-786.

Manchee , R., Broster, M., Melling, J., Henstridge, R. and Stagg, A. (1981). Bacillus anthracis on Gruinard Island. *Natore* 294: 254-255.

Mikesell, P., Ivins. B.E., Ristroph, J.D., Vodkin, M.H., Drier, T. and Leppla, S. (2002). Plasmids, Pasteur and anthrax, *ASM news* 49: 320-322.

OIE (2004). *Manual of standards for diagnostic tests and vaccines* (5th edn.). Chapter 2.2.1, Pp: 283-294. OIE, France.

Quinn, P.J., Carter, M.E., Markey, B. and Carter, G.R. (1994). Bacillius species, In: *Clinical Veterinary Microbiology*. Pp: 178-183. Mosby, Wolfe Publishing, London.

Ramachandran, S., EI-jack, M.A., Williams, T. and Azril, R.(1988). Anthrax in taing (Damalisus Lunatus Heuglin, (1963) and transhumant cattle in South Sudan. *Indian Veterinary Journal* 65: 1074-1079.

Sjostedt, A., Eriksson, u., Ramisse, V. and Garrigue, H. (1997). Detection of *bacillus anthracis* spores in soil by PCR. *FEMS Microbiology Ecology* 23: 159-168.

Titball, R.W., Turnbull, P.C.B. and Huston, P.A. (1991). The monitoring and detection pf *Bacillus anthracis* in environment. *Journal of Applied Bacteriology* (Suppl.) 70: 9s-18s.

Turnbull, P.C.B., Huston, R.A., Ward, M.J. (1992). *Bacillus anthracis* but not always anthrax. *Journal of Applied Bacteriology* 72: 21-28.

Van Ness, G.B. (1971). Ecology of anthrax, *Science* 172: 1303-1307.

Watson, A. and Keir, D. (1994). Information on which to base assessments of risk from environments contaminated with anthrax spores. *Epidemiology and Infection* 113: 479-490.

Welkos, S.L. (1991). Plasmid–associated virulence factors of non-toxigenic (PXO1⁻) *Bacillus anthracis*. *Microbiology and Pathology* 10: 183-198.

Wilson, J. and Russel, K. (1964). Isolation of *Bacillus anthracis* from soil stored 60 years. *Journal of Bacteriology* 87: 237-238.