

Research Article



Prevalence of *Bacillus anthracis* Spores in Soil of District Badin

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Abstract | Anthrax is an ignored zoonotic disease of all livestock animals that is prevailing in developing countries. The etiological agent of anthrax is a rod-shaped bacteria named *Bacillus anthracis* (*B. anthracis*) which is spread by endospores that could remain viable in the environment for decades. The current study investigated the prevalence of *B. anthracis* spores in soil of district Badin in relation to soil characteristics. A total of 100 samples were collected from different talukas (20 from each taluka) such as Golarchi, Tando Bago, Talhar, Badin and Matli of district Badin. Soil samples were randomly collected from areas those were selected based on suspected carcass disposal or burial sites, comparatively low-lying area and livestock pasturing sites. An overall prevalence of 20% in soil samples of district Badin were recorded. The highest prevalence of *B. anthracis* spores was recorded in soil samples of Talhar, Golarchi and Tando Bago (25%), that followed by Badin (15%), and Matli (10%). Among 100 soil samples, 46 were found as silty loam type, 44 silty clay loam type, while remaining 10 were recorded as clay loam type. The highest prevalence of anthrax spores were observed in clay loam type soil (70%), that followed by silty clay loam (18.18%) and silty loam type soil (10.86%). The chemical properties of soil samples of district Badin were also recorded during the current study and results showed an overall of 4.21, 8.47, 0.54 and 7.04 as EC (electrical conductivity) (dS/m), pH, organic matter (OM%) and calcium carbonate (CaCO₃%) respectively. The correlation among chemical properties of soil samples and prevalence of *B. anthracis* were also evaluated. The results indicated a positive correlation between OM% and prevalence of *B. anthracis* (0.0276) and calcium contents of soil and prevalence of *B. anthracis* (0.1775). While, a negative correlation was observed among soil pH and prevalence of *B. anthracis* and EC and prevalence of *B. anthracis*. These results indicated that soil of all areas of district Badin are heavily contaminated by *B. anthracis* spores. The findings of current study suggested a realistic implementation plan for the prevention/control of anthrax in all areas of district Badin, Sindh.

Keywords | Anthrax, *Bacillus anthracis*; Badin, Chemical properties, Physical properties, Soil

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INTRODUCTION

Bacillus anthracis is the etiological agent of anthrax, a serious and worldwide disseminated zoonotic disease affecting a wide array of wild and domestic animals, consistently also humans. The disease may cause high mortality, especially in herbivores. *B. anthracis* is a spore-forming, Gram positive, non-motile, rod-shaped, and obligate pathogen of genus Bacillus. Spores formation only happens

when the bacterium is exposed to extreme environmental conditions that are totally opposed for the continued multiplication of the vegetative form. *B. anthracis* spores are highly resilient to chemical disinfectants and environmental conditions including high temperature (Dey et al., 2012; OIE, 2008), thus could be persist in soil for decades, and considered very difficult to eliminate from the environment. Therefore, epidemiological surveys of different geographical locations for determination of disease

prevalence and circumstances that favour *B. anthracis* persistence in soil are the most important areas of anthrax research (Dragon et al., 2001).

It has been suggested that the ecology and epidemiology of anthrax is largely influenced by environmental factors that probably effect the spore concentrations at various sites and regions (Ganz et al., 2014). Though, the ecobiology of *B. anthracis* entails more research to completely illuminate the mechanism responsible for transmission and spread of organism in various geographical locations. It will progress effective means for eradication of the disease from contaminated areas, and cut the risk of future epidemics (Dey et al., 2012).

The qualitative and quantitative estimation of microbes in a certain portion of soil are determined by a multifaceted interaction of variable quantities of moisture, temperature, sunlight, nutrients, redox potential and soil pH. Microorganisms may be native or enter the soil indirectly through manure application, animal deposits, or contaminated sewage water (Mongoh et al., 2008). Factors like, predation, infection and competition by other microorganisms (viruses, bacteria, fungi, protozoa and archaea) further preferred the major species, mainly in diverse rhizosphere (nutrient rich soil zone adjacent a plant root). It has hypothesized that strategies that bacteria use to exist in soil competition may, in turn, offer the competence to infect individuals (Cheun et al., 2003).

Keeping in understanding the above scenario, the current study was planned to explore the prevalence of *B. anthracis* spores contamination in soil of different areas of district Badin. It further identified the association of anthrax risk with various soil types.

MATERIALS AND METHODS

SAFETY MEASURES

Among infectious diseases, anthrax is a disease itemized in the World Organization for Animal Health (OIE) Terrestrial Animal Health Code and mandatory to report to the OIE (OIE Terrestrial Animal Health Code). According to this manual, while dealing with anthrax bacteria appropriate safety precautions must be strictly followed and laboratory work must be done with a minimum health hazard to the environment (biocontainment) and staff (biosafety) (OIE, 2008).

COLLECTION OF SOIL SAMPLES

A total of 100 samples were collected for this study from five different talukas (n= 20 from each talukas) such as Gollarchi, Tando Ghulam Ali, Tando Bago, Talhar and Matli of district Badin, Sindh, Pakistan. The place of sample col-

lection in the study area were selected based on suspected carcass disposal or burial sites, comparatively low-lying area, livestock habitats and livestock pasturing sites. Approximately 400gm of superficial soil from a maximum depth of one-foot were collected in double layered plastic bags and were transported in cooling box to the Vaccine Production Unit (VPU) Tandojam in Anthrax Spore section for further evaluation process.

PROCESSING OF SOIL SAMPLES FOR PHYSICAL AND CHEMICAL ANALYSIS

The physical analysis of soil samples for soil type (clay, silt and loam) were physically analyzed through methods as suggested by FAO, (1974). Whereas, for chemical analysis, the soil samples were investigated through the methods suggested by Eckert and Sims (1995) for pH, whereas, electrical conductivity (EC dS/m) was calculated through the method as suggested by Richards (1954). Calcium carbonate (CaCO₃%) were observed by method developed by Wolf and Beegle (1995), while, organic Matter (OM%) were determined in the soil samples through oxidation-reduction titration with ferrous sulphate as suggested by Walkley, (1947).

PROCESSING OF SOIL SAMPLES FOR MICROBIOLOGICAL ANALYSIS

Bacterial spore extraction of soil samples was carried out under careful precautions at Vaccine Production Unit (VPU) Tandojam in Anthrax Spore Section according to OIE manual (2008). Extraction of spores was done using 10 g of soil sample that was suspended overnight in 100 ml of sterile distilled water. Supernatants were sieved through 0.45µm pore cellulose nitrate, and deposits were suspended in 5 ml sterilized phosphate-buffered solution (PBS, pH: 6.6 ± 0.2), aliquoted and heated at temperature regimen of 70°C for 20 min in water bath. Sample suspension was centrifuged at 4000 rpm for 20 minutes then it was pelleted and re-suspended in 2ml PBS. Then the aliquot was plated onto heart infusion (BHI) agar, nutrient agar, MacConKey's agar, Muller Hinton agar, egg yolk agar and blood agar. Cultured plates were incubated at 37 degree Celsius, in an aerobic incubator. Colonies evolving at the end of 24–48h incubation period were studied for cultural characteristics and morphological features defined to *B. anthracis*. Confirmation of *B. anthracis* was done on the basis of biochemical properties and antibiotic penicillin-G (10 IU/disc; Difco, Labarotries Inc) susceptibility test as described previously (Rajput et al., 2017a).

PROCESSING AND ANALYSIS OF DATA

All the resulted data were entered a computer database and analyzed using analysis of variance (ANOVA) with statistical analytical system software (SAS, 2009). Correlation analysis was also performed to determine the relationship

among chemical properties of soil and prevalence of *B. anthracis* spores.

and Tando Bago (25%) followed by Taluka Badin (15%) and Taluka Matli (10%), respectively (Table 2).

RESULTS

CHEMICAL PROPERTIES OF SOIL SAMPLES COLLECTED FROM DISTRICT BADIN

The chemical properties of soil samples collected from district Badin were evaluated and the results reported in Table 1. The lowest EC was calculated in the soil samples of Taluka Matli (3.72 dS/m) and highest was calculated in the soil samples of Taluka Talhar (4.55 dS/m). The overall mean EC (dS/m) of soil samples of district Badin was found 4.21. The lower pH value was observed in the soil samples of Taluka Talhar (8.34) and higher value was found for the soil samples of Taluka Matli and Golarchi (8.52). The overall pH of soil of district Badin was found 8.46. The higher OM% was calculated in the soil samples of Taluka Tando Bago (0.57) and lower OM% was observed in Taluka Talhar (0.48). The overall OM% of district Badin was 0.536. The higher CaCO₃% was found in the soil sample of Taluka Matli (7.8%) and lower was found in Taluka Talhar (6.65%). The overall CaCO₃% was recorded in soil sample of district Badin was 7.04. The highest number of *B. anthracis* positive soil samples were recorded for Taluka Talhar, Golarchi and Tando Bago (05), followed by Taluka Badin (03), while lowest was found in Taluka Matli (02).

Table 1: Chemical Properties of Soil Samples Collected from different Talukas of district Badin

Taluka Names	EC dS/m	pH	OM %	CaCO ₃ %	Positive samples for <i>B. anthracis</i> *
Talhar	4.55	8.34	0.48	6.65	05
Matli	3.72	8.52	0.54	7.8	02
Badin	4.30	8.48	0.53	6.75	03
Golarchi	4.24	8.52	0.56	7.1	05
Tando Bago	4.24	8.47	0.57	6.9	05
Overall	4.21	8.466	0.536	7.04	20

* out of 20 (n= 20 for each Taluka)

EC: electrical conductivity (dS/m; deciSiemens per meter); OM: organic matter (%); CaCO₃: calcium carbonate (%)

PREVALENCE OF *B. ANTHRACIS* SPORES IN DISTRICT BADIN

As shown in Table 2, among 100 soil samples of district Badin, 46 were silty loam type, 10 were clay loam type and 44 were silty clay loam type in physical nature. The higher prevalence of *B. anthracis* spores was observed in clay loam type soil samples (7/10; 70%) followed by silty loam type soil (8/44; 18.18%) and silty clay loam type soil samples (5/46; 10.86%) respectively. The higher *B. anthracis* spores were found in the soil samples of Taluka Talhar, Golarchi

CORRELATION AMONG CHEMICAL PROPERTIES OF SOIL SAMPLE AND PREVALENCE OF *B. ANTHRACIS* SPORES IN DISTRICT BADIN

The correlation among chemical properties of soil samples and prevalence of *B. anthracis* were evaluated and presented in Table 3. The results indicated that positive and lower correlation was observed among OM% and prevalence of *B. anthracis* (0.0276) and CaCO₃% and prevalence of *B. anthracis* (0.1775). While negative correlation was observed among soil pH and prevalence of *B. anthracis* and EC and prevalence of *B. anthracis*.

DISCUSSION

Although great advances in understanding the biology and ecology of *B. anthracis*, anthrax sustained to cause significant losses to livestock industry throughout the globe. Spread of *B. anthracis* is environmentally facilitated and such ecological communications in the environment probably effect its spread to consequent animal hosts that swallow spores during grazing (Ganz et al., 2014). However, although outbreaks are more or less infrequently occurring today, especially in developing countries, anthrax is still of relevance and in order to control outbreaks it is of major importance to gain further understanding of the ecology of *B. anthracis* (Dragon et al., 2001). Epidemiological studies have declared that in the higher and middle latitudes of Europe, *B. anthracis* infection in animals is now either absent or present only in very intermittent level, however it is found comparatively common in countries adjacent the Mediterranean Sea.

In Pakistan, *B. anthracis* infection is still one of the important infectious diseases of man and livestock animals because of its extensive dispersal. In some parts of the country, the disease is sporadic during the rainy season (Shabbir et al., 2015). Unfortunately, no efficient studies of this disease in Pakistan have carried out yet, although controlling the disease through immunization has been adopted for many years. The strategic plans for effective anthrax control and its prevention must need precise epidemiology of the disease. In current decade, some epidemics of anthrax have been reported by the veterinarians in some regions of district Badin, Sindh (unpublished data). Therefore, we decided to carry out this epidemiological study to record the presence of the *B. anthracis* spores in soil of district Badin.

In current investigation a total of 100 soil samples from different talukas of district Badin were examined and among those 20 were found positive for *B. anthracis* spores. Another study reported 15% prevalence of anthrax spores

Table 2: Prevalence of *B. anthracis* spores in different types of soil samples at district Badin

Soil Type	No. of Samples Examined						No. of Positive Samples						Prevalence in Soil
	Talhar	Matli	Badin	Golarchi	Tando Bago	Total	Talhar	Matli	Badin	Golarchi	Tando Bago	Total	
Silty Loam	12	10	09	07	08	46	01	00	00	02	02	5	10.86%
Clay Loam	05	03	01	01	00	10	03	02	01	01	00	7	70%
Silty Clay Loam	03	07	10	12	12	44	01	00	02	02	03	8	18.18%
Total	20	20	20	20	20	100	05	02	03	05	05	20	20%
Prevalence in different Talukas							25%	10%	15%	25%	25%	100%	

Table 3: Correlation among chemical properties of soil samples and prevalence of *B. anthracis* spores in district Badin

Particulars	EC dS/m	pH	OM %	CACO ₃ %	Prevalence%
EC dS/m	1				
pH	-0.3457	1			
OM%	-0.4893	0.6478	1		
CACO ₃ %	-0.4190	0.4134	0.4841	1	
Prevalence%	-0.1565	-0.1286	0.0276	0.1775	1

EC: electrical conductivity (dS/m; deciSiemens per meter); OM: organic matter (%); CaCO₃: calcium carbonate (%); prevalence (%)

in Isfahan, Iran using similar culture technique (Jula et al., 2004), while a Canadian study reported 1.9% prevalence of anthrax spores in soil samples of Northern Canada (Zaidan et al., 2015). A study investigating the soil-borne zoonotic pathogens in Pakistan through real time PCR reported the 37.90% prevalence of *B. anthracis* in Lahore (Shabbir et al., 2015), whereas, our earlier work demonstrated the 40% prevalence of anthrax spores in soil (Rajput et al., 2017a) and 28% in hair/wool samples of small ruminants (Rajput et al., 2017b) in endemic areas of Tharparker. These variations in prevalence rate of anthrax spores could be justified because of geographical differences (Mshelia et al., 2016), ecological factors including soil pH, presence of certain cations in soil, particularly calcium, and oxygen contents of soil (Hammerstrom et al., 2011).

Our study has further indicated significant differences in the percentage prevalence of *B. anthracis* spores in different talukas of district Badin. The highest number of *B. anthracis* positive soil samples were recorded for Taluka Talhar, Golarchi and Tando Bago (05), followed by Taluka Badin (03), while lowest was found in Taluka Matli (02). Studies have suggested a positive correlation between the animal market chain and/or highly populated animal areas with the geographical spread of diseases.

Environmental factors, such as humidity and high ambient temperature could provide a milieu for spread and survival of *B. anthracis* spores (Kracalik et al., 2013). District Badin of Sindh province have temperature as high as 40°C and relative humidity almost 75% during most times of the year (Leghari et al., 2000), which favoured the high incidence of anthrax spores in the present study area. The current study area (Badin district) is a drought affected area of Sindh province, having least human development index (HDI), where scarcity of both water and food are quite common (Leghari et al., 2000). The factors including nutritional deficiencies suppress the development of animal's immunity, thus play a vital role in the onset of infection (Rakib et al., 2016; Mondal and Yamage 2014).

The moisture content of soil mainly depends on its physical properties and type, which may influence the spore viability and persistency in soil. The soil of district Badin was found loamy type and highest prevalence (70%) was recorded in clay loam soil. These results are in accordance with a previous study from Bangladesh that reported that all positive soil samples were of loamy type (Ahsan et al., 2013).

The soil pH directly related with the ratio of many soil nutrients (Pabian and Brittingham, 2012). Alkaline soil containing high Ca, nitrogen, and organic matter that provide favorable conditions to the spore growth and/or survival in soil (Jula et al., 2007; Hugh-Jones and Blackburn, 2009). It is also well recognized that a slight alkaline pH (7.2-8.7) and soil that rich in calcium and organic matter promotes the survival of resilient *B. anthracis* spores (Moazeni-Jula et al., 2004; Dey et al., 2012). These aforementioned facts were in agreement to our current findings as we found the positive correlation between organic matter and prevalence of *B. anthracis* and CaCO₃% and prevalence of *B. anthracis*, however we observed a negative correlation between soil pH and prevalence of *B. anthracis*. Our result regarding negative correlation of pH and spores

occurrence needs further investigation, as it is not inconsistent with the studies of Dey et al. (2012).

CONCLUSIONS

From the current report, it is concluded that *B. anthracis* spores were prevailing (20%) in soil samples of all talukas of district Badin. A comparatively higher prevalence was recorded in Taluka Talhar, Golarchi and Tando Bago (25%), that followed by Badin (15%), and Matli (10%). When positive soil samples were analyzed for physical properties, it was noted that the highest prevalence of anthrax spores were observed in clay loam type soil (70%), that followed by silty clay loam (18.18%) and silty loam type soil (10.86%). Furthermore, a positive correlation was recorded between organic matter and prevalence of *B. anthracis* spores and calcium contents of soil and prevalence of *B. anthracis* spores.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS CONTRIBUTION

ASM and AAK conceived the experimental design. ASM done experiments. KRB and IAS helped in experiments and paper writing. AAK and SHA helped in statistical analysis and proof reading.

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