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# Investigation into Bacillus anthracis Spore in Soil and Analysis of Environmental Parameters Related to Repeated Anthrax Outbreak in Sirajganj, Bangladesh


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*Short Communication*

# Investigation into *Bacillus anthracis* Spore in Soil and Analysis of Environmental Parameters Related to Repeated Anthrax Outbreak in Sirajganj, Bangladesh

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## *Abstract*

The study was conducted for the isolation and detection of *Bacillus anthracis* spores from soil collected from Sirajganj district (a north-western district of Bangladesh), and to assess the parameters that may relate to the repeated anthrax outbreak. A total of 48 soil samples were collected from the study area during January to November 2012. Endospores were extracted from soil and the *Bacillus anthracis* was identified using conventional bacteriological, biochemical and sensitivity test against Penicillin-G. The viable *B. anthracis* spores could be detected from 14 (29.17%) soil samples. Moisture content, pH, calcium and organic carbon contents of the soils were measured and the values of the endospore positive samples ranged from 6.31-28.37%, 5.17-7.22, 484.35-1372.35 ppm and 0.15-2.35%, respectively. All the endospore positive soil samples were of loamy type, while none of the clay type soil was found to be positive for *B. anthracis*, suggesting the influence of soil type on the occurrence of anthrax endospore in studied area. The mean pH of anthrax positive soil was weakly acidic (6.38±0.15), indicating that a suitable pH range for anthrax spore was present in the soil of Sirajganj. During the disease outbreak period (May and June) the average temperature of this area was $32^0$C and the average rainfall was 158 mm and 90 mm, respectively. Although the temperature variation had no significant influence on the occurrence of anthrax spore, rainfall was found to be significant.

**Keywords:** anthrax, *Bacillus anthracis* spore, Bangladesh, ecology

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# บทคัดย่อ

## การสำรวจสปอร์ของ *Bacillus anthracis* ในดิน และการวิเคราะห์ภาวะสภาพแวดล้อมที่สัมพันธ์กับการระบาดของโรคแอนแทรกซ์ใน Sirajganj ประเทศบังคลาเทศ

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ทำการแยกและการตรวจหาสปอร์ของ *Bacillus anthracis* จากดินที่เก็บจากอำเภอ Sirajganj (เขตทางตะวันตกเฉียงเหนือของประเทศบังคลาเทศ) และประเมินพารามิเตอร์ที่อาจเกี่ยวข้องกับการระบาดของโรคแอนแทรกซ์ซ้ำ ตัวอย่างดินทั้งหมด 48 ตัวอย่างถูกเก็บรวบรวมจากพื้นที่การศึกษาในช่วงเดือนมกราคม ถึง พฤศจิกายน ปี ค.ศ. 2012 นำมาสกัด พบสปอร์จากดินและระบุว่าเป็น *Bacillus anthracis* โดยใช้การตรวจสอบแบคทีเรีย ชีวเคมี และความไวต่อ Penicillin G ตรวจพบสปอร์ของ *B. anthracis* ที่มีชีวิตจำนวน 14 ตัวอย่าง (ร้อยละ 29.17) ทำการวัดค่าความชื้น ความเป็นกรดด่าง ปริมาณแคลเซียมและอินทรีย์คาร์บอนของดิน รวมไปถึงปริมาณตัวอย่างที่ตรวจพบสปอร์ พบว่ามีค่าเท่ากับ 6.31-28.37% 5.17-7.22 484.35-1,372.35 ppm และ 0.15-2.35% ตามลำดับ ตัวอย่างดินที่พบว่ามีสปอร์ปนเปื้อนเป็นดินร่วนทั้งหมด ในขณะที่ไม่พบสปอร์ของ *B. anthracis* ปนเปื้อนในดินเหนียว พบว่าเป็นในเชิงบวกสำหรับ *B. anthracis* ชี้ให้เห็นถึงอิทธิพลของชนิดของดินต่อการเกิดสปอร์แอนแทรกซ์ในท้องที่ที่ทำการศึกษา ค่าความเป็นกรดด่างของดินที่ตรวจพบสปอร์แอนแทรกซ์เป็นดินที่มีความเป็นกรดอ่อน (6.38±0.15) ชี้ให้เห็นว่าในช่วงความเป็นกรดด่างที่เหมาะสมต่อการเจริญของสปอร์แอนแทรกซ์มีอยู่ในดินที่ Sirajganj ในช่วงระยะเวลาที่เกิดการระบาดของโรค (เดือนพฤษภาคมและมิถุนายน) อุณหภูมิเฉลี่ยของพื้นที่นี้คือ $32^0$ซ. และปริมาณน้ำฝนเฉลี่ยคือ 158 มม. และ 90 มม. ตามลำดับ แม้ว่าการเปลี่ยนแปลงของอุณหภูมิไม่ได้มีอิทธิพลสำคัญ แต่พบว่าปริมาณน้ำฝนมีความสำคัญต่อการเกิดของสปอร์แอนแทรกซ์

**คำสำคัญ:** แอนแทรกซ์ สปอร์ *Bacillus anthracis* บังคลาเทศ สภาพแวดล้อม

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## *Introduction*

Anthrax (popularly known as - *Torka*, *Duckmina*, *Duckshal*, *Dhash* or *Dharash* in Bangladesh) is an acute disease caused by a soil-borne, spore forming bacterium, *Bacillus anthracis*. When the vegetative form of the bacterium is exposed to the atmosphere and conditions are unfavorable for the continued multiplication, it forms spore which is resistant to heat and chemical disinfectants (Hirsh and Zee, 1999; OIE, 2004), and this dormant stage may persist for years in soil as viable (Dragon et al., 2001). These viable spores, acting as an important factor in the epidemiology, are transmitted to herbivorous animal through ingestion of soil contaminated feed and water (Titball et al., 1991). Several environmental parameters like geographical location, soil type, ambient temperature, rainfall, relative humidity etc. are potential associates for the survival of bacterial spore and maintaining the ecological conditions of repeated outbreak of anthrax (Dragon and Rennie, 1995).

As a common problem, the disease naturally occurs around the globe. Within last 10 years, the disease has been reported in the USA (Mongoh et al., 2008), Australia (Durrheim et al., 2009), Sweden (Lewerin et al., 2010), Italy (Fasanella et al., 2010) and many places in Europe at various frequencies. However, the disease is especially found in tropical and sub-tropical countries (Biswas et al., 2011). In many African and Asian countries, especially in countries having poor vaccination coverage among susceptible livestock, anthrax outbreak occurs periodically in animals, and subsequently transmits to human (WHO, 2008). Until 2009, the disease was periodically reported in animals and human in Bangladesh (Ahmed et al., 2010). However, in recent years, the disease has occurred repeatedly exerting panic to farmers; the outbreaks speculate that it is no longer sporadic rather than enzootic in Bangladesh (Ahmed et al., 2010; Fasanella et al., 2013). In

Bangladesh, the outbreak is mostly prevalent in Sirajganj (a north-western district located about 141 km far from the capital city) which is considered as one of the top most Cattle belt areas (Ahmed et al., 2010; Biswas et al., 2011). Investigation into anthrax in Bangladesh was limited to field observation during active epizootics focusing mainly on the host leaving the environmental factors untouched. To date, there is no report that describes the soil and weather related factors responsible for the repeated anthrax outbreak in Bangladesh. Therefore, the present study was undertaken to investigate the association of anthrax spore in soils in Sirajganj district, and to evaluate the environmental parameters that might have positive influence on the survival of the bacterial spores in the soil.

## *Materials and Methods*

***Site selection and sample collection:*** The study was conducted in 3 *Upazillas* (sub-district) of Sirajganj (a north-western district of Bangladesh) (Figure 1) namely Shahzadpur, Belkuchi and Ullapara over a period of January to November 2012. A total of 48 soil samples were collected randomly from anthrax reported areas. The place of sample collection in the study area was selected based on suspected carcass disposal or burial sites, comparatively low-lying area, livestock habitats and livestock pasturing sites. Approximately 400-gm of surface soil from a maximum depth of one-foot was collected in double layered plastic bags and transported to laboratory as early as possible.

***Isolation and identification of Bacillus anthracis:*** Isolation and identification of the bacteria were carried out at the Bacteriology and Molecular Microbiology Laboratory of the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU). Physical and chemical parameters of the collected soils were examined at the Department of Soil Science, BAU. The isolation and identification of *B. anthracis* from the soil samples was performed according to the procedures described in the *Manual for Laboratory Diagnosis of Anthrax* (WHO, 2003) and *OIE*

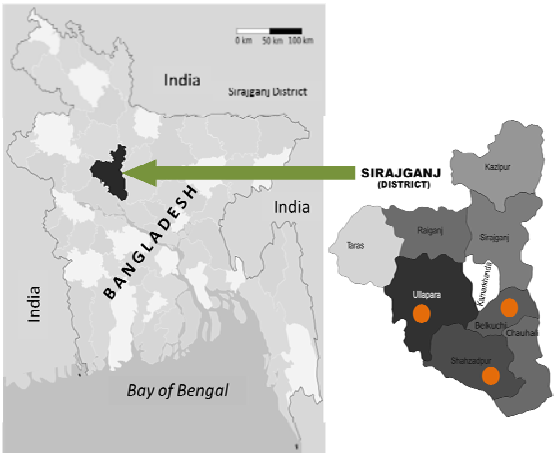


**Figure 1** Map of Shirajganj dirstrict showing the outbreak areas. Shahzadpur, Ullapara and Belkuchi *upazillas* (red circled) belong to the specified Sirajganj district.

*Terrestrial Manual 2008* (OIE, 2008). In brief, one gram of soil sample was blended in 10 ml of sterile distilled water and placed in a water bath at 62.5±0.5$^0$C for 30-60 min. The heat will destroy all non-spore-forming bacteria. 10-fold dilution to $10^{-2}$ or $10^{-3}$ was then prepared. From each dilution, 250-300 µl was plated on to Polymyxin B - Lysozyme - EDTA - Thallous acetate agar (PLET agar, Sigma-Aldrich, Switzerland) and incubated at 37$^0$C for 40-48 hours. The PLET agar is a selective medium for *B. anthracis* that inhibits all Gram-negative and most Gram-positive bacteria including *B. cereus* (Dragon and Rennie, 2001). For confirmatory identification, the colonies were grown on Blood agar, Nutrient agar and Gelatin stab agar to observe the characteristic morphology (WHO, 2008). Microscopic examination was done after staining the bacteria by Gram's Method and MacFadyean reaction (WHO, 2008). In addition, the bacteria were subjected to biochemical tests and antibiotic susceptibility test against Penicillin-G (10 IU/disc; Oxoid, UK) (Dragon et al., 2005; WHO, 2008).

***Physical and chemical analysis of soil:*** Soil type (*e.g,* sandy, loamy or clay) was determined by gross examination. Soil moisture content was determined by Gravimetric method (Wagner et al., 1999). Soil pH was determined by glass electrode pH meter as described by Eckert and Thomas Sims (1995). Soil calcium content was determined according to the outlines of Wolf and Beegle (1995). Organic carbon was determined by wet-oxidation method (Grewal et al., 1991).

***Collection of the data regarding weather parameters (temperature and rainfall):*** Weather related data of the study was collected from the website of Accuweather (http://www.accuweather.com).

***Data analysis:*** Statistical analysis was performed using Statistical Package for Social Science (SPSS) commercial software packages (version 17). Frequency tables and cross tables were produced to present study findings. One-way ANOVA was used to see association among soil parameters followed by Dunkan's Multiple Range Test (DMRT). Cramer's V test was used to measure possible association between different subareas for presence of *B. anthracis* spore in the soil. A $p$ value of $< 0.05$ was considered significant in all analysis.

## *Results and Discussion*

Among the 48 soil samples, 14 (29.17%) were found to be positive for the presence of spore of *B. anthracis* in selective PLET agar medium. The frequency of positive samples among the study areas is mentioned in Table 1. The bacteria formed rough, creamy-white, 2-3 cm in diameter and tacky colonies on PLET agar, non-hemolytic and grey colonies on blood agar, Medusa headed colony on Nutrient agar, inverted fir tree like growth in Gelatin stab culture, and cotton wool like growth in Nutrient broth. All the positive isolates were susceptible to Penicillin-G and liquefied gelatin slowly. The isolates were found positive for catalase, Voges-Proskauer (VP) and Methyl-Red (MR) and negative for indole test. The bacteria fermented Dextrose, Sucrose and Maltose

producing only acid and did not ferment Lactose and Mannitol. Gram-positive bacilli arranging single, pair or chain were observed under microscope. Blue-black bacilli were seen with pink amorphous capsule under microscope (100X objectives) with immersion oil through staining with 1% Polychrome Methylene Blue.

Different parameters of the soil samples like soil type, moisture, pH, calcium content and organic carbon, and presence of spore in the collected samples are summarized in the Table 2. Among the 48 soil samples, 77.08 and 22.92% were loamy and clay type, respectively. All the positive soils (29.17%) were loamy type (Table 1). The association between pH and calcium contents were highly significant ($p < 0.05$), but moisture content and organic carbon content did not vary significantly ($p > 0.05$) among the places (Table 2). The mean moisture content of the positive soil samples was 16.69±2.06%, whereas the pH, calcium content and organic carbon contents were 6.38±0.15, 831.77±62.16 ppm and 0.86±0.17%, respectively (Table 2). The pH level differed significantly ($p < 0.05$) between anthrax spore positive and negative soils, whereas moisture content, Ca level and organic content did not vary significantly ($p > 0.05$) (Table 2). The association between the subareas and the presence or absence of spore in the soil was not significant (Table 3). The monthly average temperature in Sirajganj district ranged from 19-32$^0$C; the highest average temperature (32$^0$C) was recorded in May-June, and the lowest average temperature (19$^0$C) was recorded in December and January, respectively. The monthly average precipitation of rain in Sirajganj district ranged from 0-310 mm; the highest average rainfall (310 mm) was recorded in July, and the lowest average rainfall (0 mm) was recorded in March.

The endospores of *B. anthracis* are highly resistant to environmental stress and in favorable conditions the spores may exist as dormant for decades between epidemics. Sirajganj, a north-western district, is considered as one of the important milk pocket areas in Bangladesh. In recent years, the outbreak of anthrax has repeatedly occurred in this area. Through this study, different factors including survival of *B. anthracis* spore in soil and various environmental parameters were investigated to reveal the possible causes of the repeated outbreak. Here, out of 48 soil samples, 14 (29.17%) were found to be positive for the presence of *B. anthracis* endospore. Shahzadpur, Ullapara and Belkuchi are the most prevalent *upazillas* (sub-district) in Sirajganj, accordingly soil samples were collected from these areas. The highest prevalence of soil association with the spore was found in Shahzadpur (50%), however, the association between different areas for the presence of spores was not significant. In 2010, an investigation into the association between anthrax spore and the soil of Shirajganj district was done, in which soil and turbinate bone samples were collected only from the suspected places located within the anthrax affected farm compound (Fasanella et al., 2013). However, in our study, we focused on the versatile places such as the low-lying areas, livestock habitats, livestock pasturing sites and suspected burial site of the dead animal in open environment. Thus, the findings of our study widen the existing report on the occurrence of anthrax spore in the Shirajganj district. There are several reports on the examination of anthrax bacteria in soil in the world. After the deliberate contamination of the Gruinard Island during the World War II, sampling was done regularly over 40 years and the spores were, almost without exception, isolated from the top 6 cm of soil (Manchee et al., 1981). Similar results also found in northern Canada, south Sudan, and Isfahan in Iran (Dragon et al., 2001; Moazeni-Jula et al., 2004). In a study, Moazeni-Jula et al. (2004) could isolate 9 (15%) isolates of the bacterium from 60 soil specimens. Dragon et al. (2005) described the sensitivity of isolation of *B. anthracis* from soil using PLET medium. However, none of the negative samples could be declared to be free from anthrax spore. To overcome this constraint in confirmatory identification, we performed sugar fermentation, biochemical and antibiotic sensitivity tests.

**Table 1** Prevalence of *B. anthracis* spore in soil samples

| Sample sources | Soil samples examined (n = 48) | | Positive samples (%) | Soil type of positive sample |
|---|---|---|---|---|
| | Loamy type | Clay type | | |
| Shahzadpur, Sirajganj | 12 | - | 6 (50.00) | All loamy |
| Belkuchi, Sirajganj | 13 | 5 | 4 (22.22) | All loamy |
| Ullapara, Sirajganj | 12 | 6 | 4 (22.22) | All loamy |
| Total | 37 (77.08%) | 11 (22.92%) | 14 (29.17%) | |

**Table 2** Chemical parameters of soil samples of different study areas

| Parameters | Subareas | | | | Overall | | |
|---|---|---|---|---|---|---|---|
| | Shahzadpur, Sirajganj | Belkuchi, Sirajganj | Ullapara, Sirajganj | *p* value | *B. ahtracis* positive | *B. anthracis* negative | *p* value |
| Moisture (%) | 11.57±1.06 | 17.77±2.37 | 17.04 ± 2.21 | 0.135$^{NS}$ | 16.69±2.06 | 15.64±1.60 | 0.711$^{NS}$ |
| pH | 6.41±0.15 | 5.64±0.11 | 6.34±0.09 | 0.000$^{S}$ | 6.38±0.15 | 5.96±0.09 | 0.016$^{S}$ |
| Ca (ppm) | 671.06±35.75 | 782.60±47.92 | 1137.83±70.40 | 0.000$^{S}$ | 831.77±62.16 | 911.05±55.93 | 0.414$^{NS}$ |
| Organic Carbon (%) | 0.93±0.21 | 0.82±0.14 | 1.12±0.11 | 0.305$^{NS}$ | 0.86±0.17 | 1.00±0.10 | 0.440$^{NS}$ |

NS: Non-significant ($p > 0.05$), S: Significant ($p < 0.05$)

The moisture content of soil mainly depends on its type, which may influence in the long time persistency of anthrax spore. In this study, 100% of the positive soils were loamy type. Although the influence of loamy type soil on anthrax outbreak is not clearly known, it is thought that loamy type soil increases anthrax outbreak (Fox et al., 1977). The mean moisture content of the anthrax positive soil specimens was 16.69±2.06, which was higher than that of the negative samples ($p > 0.05$) suggesting that a moisture range between 6.31-28.37% might be favorable for the viability of *B. anthracis* spore in the soil.

The soil pH controls the availability of many nutrients in soil (Pabian and Brittingham, 2012). Alkaline soil containing high nitrogen, Ca, and organic matter gives favorable condition to the spore for growing in soil (Dragon and Rennie, 1995; Jula et al., 2007; Hugh-Jones and Blackburn, 2009). Besides, an alternative hypothesis has been tested by Dey et al. (2012) who found that a kind of moist soil amoeba (*Acanthamoeba castellanii*) might take part in germination and intracellular multiplication of *B. anthracis* spores. The weakly acidic soil may provide good condition to the spore (Artenstein et al., 2004). In our study, the mean pH of the anthrax positive soil was slightly acidic (6.38±0.15) and differed ($p < 0.05$) from the anthrax negative samples (5.96±0.09). In contrast, Moazeni-Jula et al. (2004) found that a slight alkaline pH range (7.2-8.7) was suitable. The soil that is rich in organic matter and calcium promotes the survival of resilient *B. anthracis* spores (Dey et al., 2012). We found that the calcium and organic carbon contents in the anthrax positive specimens were lower compared to the negative samples, but this difference was not statistically significant ($p > 0.05$).

In addition to adequate Ca, nitrogen and organic matter in the soil, anthrax outbreak requires favorable seasonal changes such as warm weather followed by heavy rain (Moazeni-Jula et al., 2004; Dey et al., 2012). The bacteria are thought to undergo a vegetative cycle when the above conditions are fulfilled. By this process, anthrax spores could be concentrated in top soil to cause disease in grazing animals, occurring outbreak separated by disease-free intervals. In May-June 2012, anthrax outbreak occurred in Shahzadpur, Ullapara and Belkuchi *upazillas* (IEDCR, 2012). At that time period, the highest monthly average temperature in these areas was $32^0$C indicating that a moderate high temperature provides a microenvironment that promotes cycling of anthrax spore. All the studied areas were low-lying areas, thus enhancing the possibility of having anthrax spore (Hugh-Jones and Blackburn, 2009). During March 2012, the average rainfall was 0 mm with an average warm temperature ($29^0$C), whereas a heavy rainfall occurred in the following months, suggesting that a favorable condition for the germination and accumulation of anthrax spore (Hugh-Jones and Blackburn, 2009).

**Table 3** Association between subareas and *B. anthracis* status

| Subareas | *B. anthracis* status | |
|---|---|---|
| | Positive | Negative |
| Shahzadpur, Sirajganj | 6 | 6 |
| Belkuchi, Sirajganj | 4 | 14 |
| Ullapara, Sirajganj | 4 | 14 |
| **Creamer's V** | 0.265 ($p = 0.186$*) | |

*There is no significant association between subareas and presence of *B. anthracis* spores in soil ($p > 0.05$)

## Conclusion

The repeated anthrax outbreak in livestock (mostly cattle) and subsequent infection to human has been considered as a nationwide alarming issue in Bangladesh. The study revealed, for the first time in Bangladesh, certain ecological factors that might be responsible for survival of anthrax spore in soil, *e.g.* soil type, Ca content, organic carbon content and soil pH.

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