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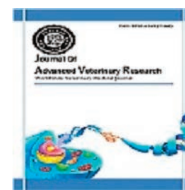

The Rich Mapping: Be a Supplementary Approach for Anthrax Control at Community Level

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ABSTRACT

Anthrax is one of the most important diseases that can be transmitted from cattle to humans, especially in countries with a high density of cattle. Cattle are usually infected by the ingestion of *Bacillus anthracis* spores with food ingredients. For that reason, the adequacy of anthrax spores in the soil is considered as one of the causes of anthrax in cattle. The present study is based on the identification of anthrax spores in the soil and the development of a map based on the presence of spores, so that the people of the community can take a practical step in this regard in selecting the place for their cattle to graze. For this purpose, soil samples were collected from the studied areas. After extraction of soil DNA with DNA extraction kits, all samples were tested using a qualitative detection method. For a rapid and reliable qualitative detection system for Pathogenic *B. anthracis* spores isolated from soil samples, the specific primer of two plasmids, pXO1 (gene Pag), pXO2 (genes Cap), and S-layer locus (gene Sap) were used. The presence of spores was found in 7 out of 50 samples. The results of a qualitative detection method of *Bacillus anthracis* spores in the pastureland inserted as per their GPS coordinates. Based on the presence of pathogenic anthrax spore in a specific location, a map was then prepared and displayed in the community. So that the cattle owners can decide where their cattle will graze and be careful to prevent spore contact of their animal. Among the various effective anthrax prevention measures, rich mapping according to the presence of spores can be helpful in community level that will protect people from anthrax disease in the future.

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Introduction

Anthrax is one of the zoonotic diseases that can be transmitted from infected domesticated animals to human (Fasanella *et al.*, 2010; Mableson *et al.*, 2014;). This disease has been seen in different levels in different countries of the world since ancient times (Vieira *et al.*, 2017). In Bangladesh, it is considered as endemic disease affecting mainly ruminants with sporadic zoonotic occurrences in humans (Ahmed *et al.*, 2010; Chakraborty *et al.*, 2012). The disease is caused by a bacterium called *Bacillus anthracis*, which is capable of producing spores. These spores survive for a long time in adverse weather and wait for the right environment (Dragon *et al.*, 2001). In its natural forms, the disease is initiated by the introduction of spores into the body usually via contaminated feedstuff during grazing in the spore contaminated grazing area (Turnbull and Organization, 1998; Moazeni Jula *et al.*, 2004). Cattle usually get the disease by swallowing a sufficient quantity of anthrax spores while grazing on pasture contaminated (made impure) with anthrax spores. According to the British biological warfare work in the early 1940s, it can be known that lethal infection by the oral route in sheep, horses, and cattle was $5x10^8$ spores (Carter and Pearson, 1999). Upon ingestion of the spores, the infection may occur through the intact mucous membrane, through defects in the epithelium around erupting teeth, or scratches from tough, fibrous food materials. The organisms are resistant to phagocytosis, in part due to the presence of the poly-o-glutamic acid capsule, and proliferate in regional draining lymph nodes, subsequently passing via the lymphatic vessels into the bloodstream; septicemia, with a massive invasion of all body tissues, follows. *B. anthracis* produces a lethal toxin that causes edema and tissue damage, causing sudden death resulting from shock and acute renal failure and terminal anoxia (Welkos *et al.*, 1989). Death of the host and contact of infected tissues with air results in a return to the spore form of the bacterium otherwise vegetative *B. anthracis* in unopened carcasses are destroyed by putrefactive bacteria (Manjunathareddy *et al.*, 2015). Thus, the spore – vegetative cell – spore cycle is essential for the pathogenic lifestyle of this developmental bacterium (Ndiva Mongoh *et al.*, 2008). Vegetative bacilli are not believed to be the disease contagion for any form of anthrax (Liu *et al.*, 2004). Bangladesh soil conditions (soil type, moisture, pH, calcium, and organic carbon content), together with the ubiquitous environment like flood,

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rainfall, animal population density, carcass disposal method, represent an ideal situation for the spread of anthrax in the animal because of spore's survival abilities (Ahsan *et al*., 2013; Sarker *et al*., 2020). During this time, they are a potential source of infection for grazing livestock that generally do not represent a direct risk of infection for people. Ecological studies of a repeated anthrax outbreak in the Sirajganj area, a research team of Bangladesh Agricultural University has seen that the presence of pathogenic spores in the soil is one of the reasons that triggered repeated anthrax outbreak in Bangladesh (Ahsan *et al*., 2013; Fasanella *et al*., 2013; Ahsan *et al*., 2015). Through their studies, the presence of pathogenic anthrax spores in the soil and its rapid detection methods have been established (Nazir *et al*,, 2015). If we identify those areas whose soils and pastures are contaminated with the spores and restrict those areas for grazing of cattle, it will be reducing the chances of animals picking up anthrax spores. Since the animal-to-animal transmission is rare and human only get the disease when comes in contact with a dead or infected animal, avoiding infected pastures can be one of the ways to prevent anthrax in both animal and human. Therefore, the present study was undertaken as one of the integrated approaches for an anthrax free model area development in Bangladesh. The study was based on the presence of spores in the soil through applied effective and rapid PCR based detection method after that a map model was created for areas contaminated with spore (risky zone). So that this map can be useful for understanding and provide necessary scientific evidence-based information for that community to control anthrax disease.

Materials and methods

Site selection and sample collection

The study was conducted in the Jamtail village of Kamarkhandha upazila of Sirajganj district, north-western Bangladesh, located in between 24°18' and 24°27' north latitudes and in between 89°35' and 89°42' east longitudes where "An integrated approach to establish an anthrax-free model area in Bangladesh" project was running from July 2018 to June 2020. This research was funded by Ministry of Education (MoE), Government of the people's republic of Bangladesh, Project No: 2018/501/MoE. A total 50 soil samples with their GPS coordinates were collected in double layered plastic bags form several locations of the model area and their neighboring pasture lands. Approximately 5 g of surface soil from a maximum depth of one foot was taken. After that it was transported to the laboratory as early as possible. The laboratory works were done in the Bacteriological and Molecular Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. For ensuring the highest safety of this study Bio-safety cabinet (class-2) was used.

Detection of Bacillus anthracis spores in soil samples

Kit based DNA extraction protocol and PCR based qualitative detection method was used. Using TaqMan PCR, specific primers and probes were used for the identification of pathogenic *B. anthracis* strains from sap gene on S-layer locus, pag gene and cap gene on two plasmids, pXO1 and pXO2.

Soil DNA extraction

FavorPrepTM soil DNA Isolation Mini Kit of FAVORGEN Biotech Corporation, Taiwan were used for soil DNA extraction.

PCR amplification

For rapid and reliable qualitative detection system for pathogenic *B. anthracis* spores isolated from soil samples, specific primer of two plasmids, pXO1 (gene Pag), pXO2 (genes Cap) and S-layer locus (gene Sap) were used (Tables 1, 2).

Mapping method

The results of a qualitative detection method of *Bacillus anthracis* spores in the pastureland inserted as per their GPS coordinates and a map was created using Maps app of Microsoft cooperation 2020. Finally, Adobe Illustrator graphics editor and design program of Adobe Inc. was used to prepare a map for community used.

Results

Among the 50 soil samples, 7 samples of having GPS Coordinates (24°21'24.1"N 89°39'12.5"E, 24°21'26.1"N 89°39'21.9"E,24°21'35.3"N 89°40'08.5"E,24°21'25.8"N 89°39'

Table 1. Primers used for the detection of anthrax spores in soil sample

Primer	Sequence (5′-3′)	Target gene	Product size	Reference
Sap-1	CGCGTTTCTATGCCATCTCTTCT	Sap in genomic DNA	639 bp	Nazir *et al*. (2015)
Sap-2	TTCTGAAGCTGGCGTTACAAAT			
Pag-5	TCCTAACACTAACGAAGTCG	Protective antigen (PA) of pX01	596 bp	
Pag-8	GAGGTAGAAGGATATACGGT			
Cap-1	CTGAGCCATTAATCGATATG	capsule (pX02)	846 bp	
Cap-2	TCCCACTTACGTAATCTGAG			

Table 2. Thermal profile for the amplification of Sap, Pag and Cap in genomic DNA of *Bacillus anthracis* by PCR

PCR steps	Temperature (°C)			Time			Cycle		
	Sap	Pag	Cap	Sap	Pag	Cap	Sap	Pag	Cap
Initial denaturation	94	94	94	2 min	2 min	2 min	34	35	35
Denaturation	94	94	94	30 sec	30 sec	30 sec			
Annealing	52	55	55	30 sec	30 sec	30 sec			
Elongation	72	72	72	1 min	1 min	1 min			
Final extension	72	72	72	5 min	5 min	5 min			
Holding	4	4	4						

20.8"E, 24°21'34.2"N 89°40'06.5"E, 24°21'32.6"N 89°40'06.6 "E24°21'22.5"N 89°39'14.0"E) were showed a clear band (Fig. 1) through using genus specific Sap-1 and Sap-2 primers; Pag-5 and Pag-8 primers for Pag gene (representative of pX01 plasmid); Cap-1 and Cap-2 primers for Cap gene (representative of pX02 plasmid) of *B. anthracis* with amplification of 639 bp; 596 bp and 846 bp respectively after agarose gel electrophoresis. Details are given in Table 3. It undoubtedly proved that the soil of those area having pathogenic *Bacillus anthracis* spores and treated as positive or risky zone for grazing. On the other hand, which sample failed to show any band through using genus specific Sap-1 and Sap-2 primers; Pag-5 and Pag-8 primers for Pag gene (representative of pX01 plasmid); Cap-1 and Cap-2 primers for Cap gene (representative of pX02 plasmid) of *B. anthracis* with amplification of 639 bp; 596 bp and 846 bp respectively after agarose gel electrophoresis considered negative or free zone and their numbers were 43. In the next stage, GPS Coordinates values of all areas were inserted in the map and color segregation was used to define the risky and free grazing zone, and finally, the map (Fig. 2) was displayed for community use.

Table 3. Soil samples GPS Coordinates and Gel electrophoresis test result

Sample #	Name of the Place	Latitude	Longitude	Sap	Pag	Cap
1	Jamtoil	24°21'44.9"N	89°39'49.5"E	-	-	-
2	Pakuria	24°22'07.7"N	89°39'50.9"E	-	-	-
3	Jamtoil	24°21'26.1"N	89°39'21.9"E	+	+	+
4	Jamtoil	24°21'24.9"N	89°39'15.3"E			
5	Dhopakandi	24°21'35.3"N	89°40'08.5"E	+	+	+
6	Jamtoil	24°21'25.8"N	89°39'20.8"E	+	+	+
7	Dhopakandi	24°21'34.2"N	89°40'06.5"E	+	+	+
8	Kamarkhanda	24°21'58.3"N	89°39'09.9"E	-	-	-
9	Jamtoil	24°21'29.1"N	89°38'55.4"E	-	-	-
10	Dhopakandi	24°21'32.6"N	89°40'06.6"E	+	+	+
11	Jamtoil	24°21'22.5"N	89°39'14.0"E	+	+	+
12	Jamtoil	24°21'23.2"N	89°39'27.6"E	-	-	-
13	Jamtoil	24°22'02.2"N	89°39'31.1"E	-	-	-
14	Jamtoil	24°21'24.1"N	89°39'12.5"E	+	+	+
15	Jamtoil	24°21'37.5"N	89°39'57.1"E	-	-	-
16	Dhopakandi	24°21'37.7"N	89°40'06.1"E	-	-	-
17	Dhaleshwar	24°21'08.5"N	89°40'42.9"E	-	-	-
18	Dhopakandi	24°21'17.8"N	89°40'10.9"E	-	-	-
19	Dhopakandi	24°21'24.8"N	89°39'58.5"E	-	-	-
20	Dhopakandi	24°21'27.6"N	89°39'51.1"E	-	-	-
21	Jamtoil	24°21'33.8"N	89°39'41.8"E	-	-	-
22	Jamtoil	24°21'44.4"N	89°39'01.1"E	-	-	-
23	Jamtoil	24°21'46.7"N	89°39'01.1"E	-	-	-
24	Jamtoil	24°21'39.6"N	89°38'51.4"E	-	-	-
25	Jamtoil	24°21'40.0"N	89°38'32.3"E	-	-	-
26	Jamtoil	24°21'34.2"N	89°38'45.8"E	-	-	-
27	Jamtoil	24°21'26.4"N	89°39'06.8"E	-	-	-
28	Tengrail	24°21'24.7"N	89°40'46.3"E	-	-	-
29	Tengrail	24°21'21.4"N	89°40'41.5"E	-	-	-
30	Tengrail	24°21'20.3"N	89°40'44.5"E	-	-	-
31	Dhaleshwar	24°21'05.8"N	89°40'47.1"E	-	-	-
32	Dhaleshwar	24°20'58.4"N	89°40'44.6"E	-	-	-
33	Roy Daulatpur	24°20'45.4"N	89°40'52.9"E	-	-	-
34	Dhaleshwar	24°21'12.2"N	89°40'36.1"E	-	-	-
35	Dhaleshwar	24°21'15.2"N	89°40'21.2"E	-	-	-
36	Jamtoil	24°21'35.7"N	89°38'43.7"E	-	-	-
37	Jamtoil	24°21'46.9"N	89°39'15.3"E	-	-	-
38	Jamtoil	24°21'59.9"N	89°39'26.9"E	-	-	-
39	Jamtoil	24°21'55.3"N	89°39'32.1"E	-	-	-
40	Jamtoil	24°21'46.6"N	89°39'39.4"E	-	-	-
41	upazilla land office	24°21'46.5"N	89°39'36.0"E	-	-	-
42	Jamtoil	24°21'35.3"N	89°39'37.9"E	-	-	-
43	Dhaleshwar	24°21'03.7"N	89°40'39.9"E	-	-	-
44	Dhaleshwar	24°21'00.9"N	89°40'45.4"E	-	-	-
45	Dhopakandi	24°21'18.1"N	89°40'12.8"E	-	-	-
46	Dhaleshwar	24°21'10.1"N	89°40'22.7"E	-	-	-
47	Dhaleshwar	24°21'12.4"N	89°40'34.3"E	-	-	-
48	Jamtoil	24°21'59.9"N	89°39'17.0"E	-	-	-
49	Tengrail	24°21'25.2"N	89°40'19.9"E	-	-	-
50	Tengrail chowrasta	24°21'29.3"N	89°40'09.8"E	-	-	-

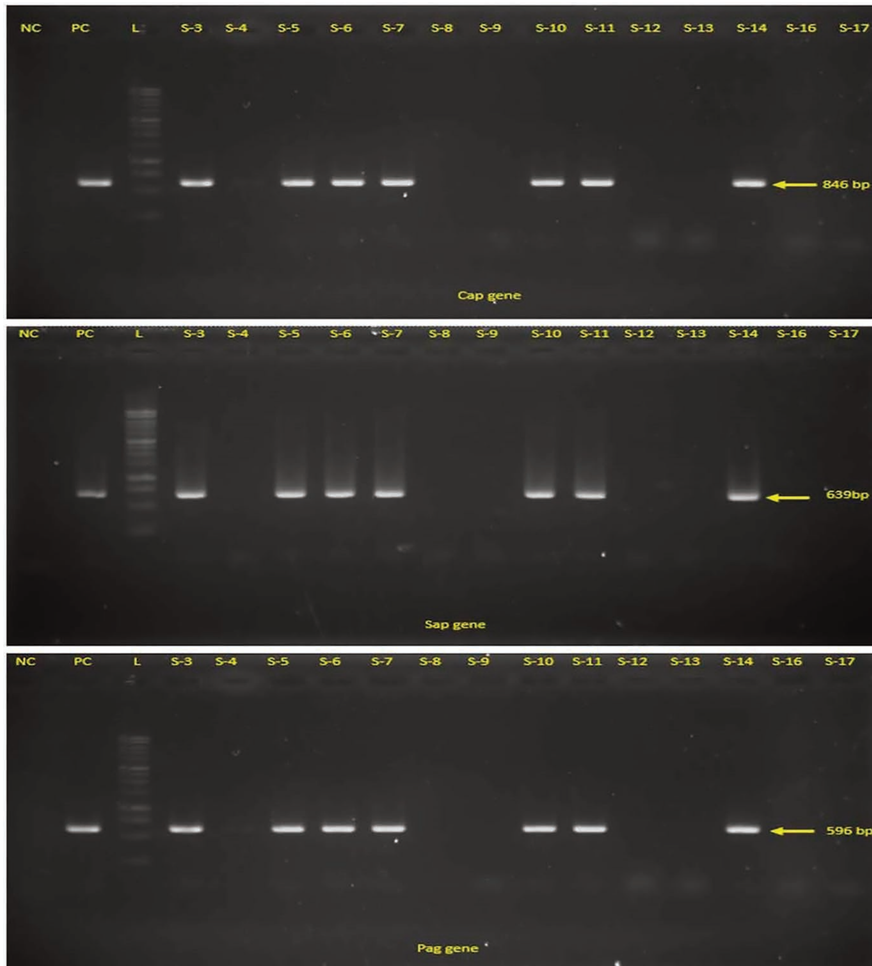

Fig. 1. Clear band (Figure 1) through using genus specific Sap-1 and Sap-2 primers; Pag-5 and Pag-8 primers for Pag gene (representative of pX01 plasmid); Cap-1 and Cap-2 primers for Cap gene (representative of pX02 plasmid) of *B. anthracis* with amplification of 639 bp; 596 bp and 846.

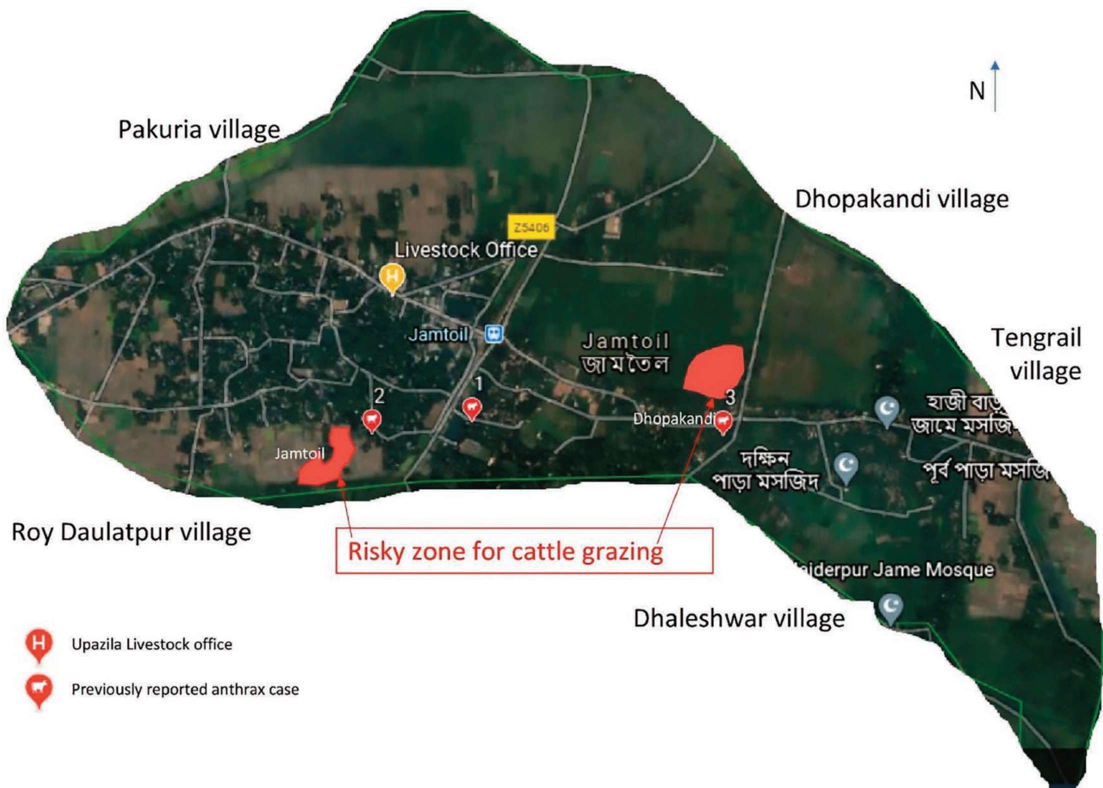

Fig. 2. Rich Map for community where the risky pasture zone for cattle has well marked.

Discussion

In 2009-2010 during repeated outbreak of anthrax disease in Bangladesh, isolation and detection of *Bacillus anthracis* spores from soil samples was first introduced by Ahsan *et al*. (2013) to evaluate the environmental parameters (Ahsan *et al*., 2013; Nazir *et al*., 2015).

In 2009-2010, when the prevalence of anthrax was high in Bangladesh, a team of researchers began research on epidemiological, etiological, ecological and also preventive measures of anthrax disease in both Human and livestock (Ahmed *et al*., 2010; Islam *et al*., 2013; Mondal and Yamage, 2014). At that time, it was seen that the disease was being transmitted repeatedly in some areas of Sirajganj district (Islam *et al*., 2015; Rume *et al*., 2016; Rume *et al*., 2020). They then speculated that since the disease was occurring in the same area, there might be an ecology issue (Rume, 2018). From such an idea, they researched and found that the soil and soil structure of those area were suitable for holding spores of *Bacillus anthracis*. In 2013, the research team was able to prove the presence of anthrax spores by testing soil samples in the area (Ahsan *et al*., 2013). In 2015, the research team discovered the PCR base spore detection protocol. They showed how accurately detecting pathogenic spores of *Bacillus anthracis* from the soil samples within very short time using sap, cap and pag gene formations (Nazir *et al*., 2015). In this study, we used the same protocol. For rapid and reliable qualitative detection system for Pathogenic *B. anthracis* spores isolated from soil samples, specific primer of two plasmids, pXO1 (gene Pag), pXO2 (genes Cap) and S-layer locus (gene Sap) were used. The use of this gene pattern was recognized not only in Bangladesh but also in other countries of the world. The names of some researchers like Mignot *et al*. (2003); Ryu *et al*. (2003); Almeida *et al*. (2006); Ogawa *et al*. (2015) and Ortatatli *et al*. (2019) are mentioned as references.

Although there was no previously published report on the presence of anthrax spores in the soil of the investigated areas (according to the available literatures), the presence of anthrax spores in the soil of nearby Upazila in the same district, is consistent with the obtained report (Ahsan *et al*., 2013; Fasanella *et al*., 2013; Ahsan *et al*., 2015). In map, all of the spots in the area that have seen anthrax outbreaks in the past are near the identified high-risk pastures. It also indirectly supports the outcome of the overall work from the current study. On the other hand, using scientific knowledge and GPS coordinates to create a map that makes it easily understandable to people in the area. Resource based union maps by identifying risk areas can be seen in many parts of Bangladesh. Union-based maps of disaster risk reduction in particular can be seen on each union council premises. Sociologists have long used the process of making common sense understandable by displaying maps, but such initiatives in the field of disease prevention are undoubtedly commendable. We hope that this will help in the formulation of area-based action plans and implementation of other programs including vaccine distribution, awareness raising.

Conclusion

An integrated approach at the country and community level can prevent anthrax disease in both animal and man. Promoting maps at the community level, which will play an important role in alerting cattle owners, especially in areas where there is an opportunity for open grazing. On the other hand, the map is a means of understanding for all people whether educated or uneducated. For that among the various effective anthrax prevention measures, rich mapping according to the presence of spores can be helpful at the community level that will protect people from anthrax disease in the future.

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