Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 3, 460–463
www.aaem.pl

ORIGINAL ARTICLE

Screening for anthrax occurrence in soil of flooded rural areas in Poland after rainfalls in spring 2010

Aleksandra A. Zasada[1], Kamila Formińska[1], Anna Ogrodnik[1], Rafał Gierczyński[1], Marek Jagielski[1]

[1] *National Institute of Public Health – National Institute of Hygiene, Department of Bacteriology, Warsaw, Poland*

Zasada AA, Formińska K, Ogrodnik A, Gierczyński R, Jagielski M. Screening for anthrax occurrence in soil of flooded rural areas in Poland after rainfalls in spring 2010. Ann Agric Environ Med. 2014; 21(3): 460–463. doi: 10.5604/12321966.1120584

Abstract

Introduction and objective. Anthrax spores remain viable and infectious in soil for decades. Flood water can percolate towards the surface the spores buried in soil. Moreover, the flood water might transport spores to areas previously unaffected. After the water recedes the spores located on the surface of the ground can be consumed by grazing animals and cause outbreaks of anthrax.

Materials and method. Soil samples were collected in areas of Poland most affected by floods in 2010 (Lubelskie, Świętokrzyskie, Podkarpackie and Mazowieckie provinces). After heating with the aim to kill vegetative forms of bacteria, the samples were cultured on PLET agar and the resulted colonies were investigated in terms of motility and presence of anthrax specific chromosomal (SG-749, plcR) and plasmid markers (capB, pagA).

Results. In total, 424 spore-forming, aerobically growing isolates were collected from the tested soil samples. Eighty-nine of them were non-motile. All the isolates were negative in PCR for anthrax specific chromosomal and plasmid markers.

Conclusions. Spores of *B. anthracis* that could be related to risk of anthrax outbreaks were not detected in soil samples tested in this study. The negative results presented may not be proof that Poland is country free of anthrax. The results, however, may suggest a relatively low risk of anthrax outbreaks being triggered in the sampled areas.

Key words

anthrax, spores, soil, flooding

INTRODUCTION

Anthrax is a zoonotic disease of mammalians, including humans, caused by *Bacillus anthracis*. The bacterium produces spores highly resistant to biological extremes of heat, cold, pH, desiccation, chemicals (and thus to disinfection), irradiation and other adverse environmental conditions. The spores may remain viable and infectious in the environment for decades [1, 2]. Contamination of the environment in natural ecosystems by anthrax spores usually begins with the release of the bacteria from the bodies of dead animals, and scavengers have been shown to play a supportive role in the local dissemination of the spores.

After death, body fluids – containing a large amount of *B. anthracis* cells – ooze from the rectum, nostrils or mouth of the animal carcass. The low CO_2 level in open air, when compared with the level in tissue, induces sporulation that enables transformation of weak *B. anthracis* vegetative cells into the highly resistant spores. The carcass blood and other tissues contain a high concentration of the bacteria and might contaminate the soil if not buried properly [3].

Anthrax spores were found to have a high floating capacity. It was observed that water can percolate the spores buried in soil toward the surface. Therefore, flooding is considered an important risk factor for natural anthrax outbreaks. The flood water might also transport spores to areas previously unaffected and the spores can be consumed by grazing animals after the waters recede [4, 5, 6].

Address for correspondence: Aleksandra A. Zasada, National Institute of Public Health – National Institute of Hygiene, Department of Bacteriology, Chocimska 24, 00-791 Warsaw, Poland
e-mail: azasada@pzh.gov.pl

Received: 05 July 2013; accepted: 06 May 2013

In the presented study, an effort was made to answer the question whether areas in Poland affected by floods in 2010 may pose an increased risk of anthrax outbreaks.

MATERIALS AND METHOD

Environmental sampling. Soil samples were collected in provinces most affected by floods in 2010: Lubelskie, Świętokrzyskie, Podkarpackie and Mazowieckie (Fig. 1). The samples were collected from the surface layer of the ground, from the low laying parts of the sampling areas. The sampling locations were specified according to the Global Positioning System (GPS), which enabled precise identification of the site in the case of detection of anthrax spores in the samples.

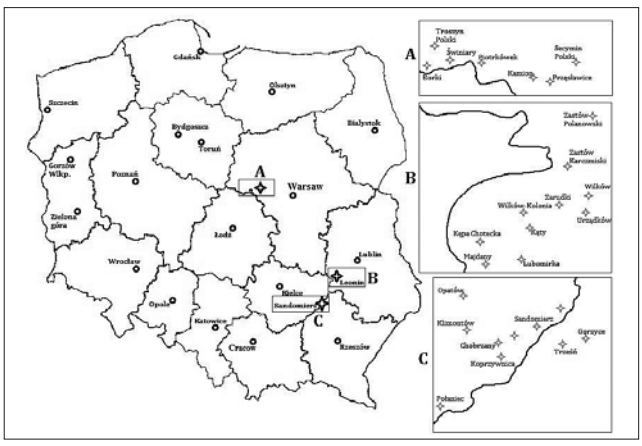

Figure 1. The main sampling points

Culture. 15 g of each soil sample were shaken vigorously with 30 ml of washing buffer (composition developed by Antonio Fasanella from the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Anthrax Reference Institute in Foggia, Italy; personal communication) for 30 min at room temperature. The samples were then centrifuged for 5 minutes at 2,000 rpm and the supernatants transferred to sterile falcon tubes. The supernatants were heated for 20 minutes at 64°C in a water bath. After cooling, 500 µl of each sample was spread onto selective polymyxin, lysozyme, EDTA, and thallous acetate (PLET) agar medium [7]. The PLET cultures were incubated at 35°C for up to 48 h. The resulting colonies were examined for motility.

Motility testing. The colonies grown on PLET agar were examined for motility using semisolid brain heart infusion (BHI) agar with tetrazolium salt. Addition of the salt allows tracking of bacterial movement through the medium, as bacterial metabolism reduces the colourless soluble tetrazolium salt to its insoluble, dark red form.

DNA extraction. Total DNA was extracted from non-motile isolates using GeneMATRIX Tissue & Bacterial DNA Purification Kit (EURX, Poland) according to manufacturer's instruction.

Molecular tests. Isolated strains were tested for *B. anthracis*-specific chromosomal markers SG-749 and *plcR* and for plasmid markers *pagA* and *capB*, as described earlier [8, 9, 10]. Briefly, in the PCR-RFLP for SG-749 method, PCR was conducted using DSF-Taq DNA Polymerase (Bioron) in conditions described by Daffonchio et al. [9]. Then the amplicons were digested by AluI restriction enzyme (Fermentas) and analyzed in 2% agarose gels (Sigma-Aldrich). RSI-PCR test for *plcR* marker was conducted, as previously described by Gierczyński et al. [8] using 4% agarose gels (Sigma-Aldrich) for separation of SspI restriction fragments. Plasmid markers *pagA* and *capB* were screened by PCR, as previously described by Zasada et al. [10]. The nucleotide sequences of primers used in the presented study are presented in Table 1, together with the expected size of the PCR amplicons.

RESULTS

Eighty-eight soil samples were collected. The applied procedure for soil samples investigation including killing of bacterial vegetative forms by heat and use of *B. anthracis* selective medium which enabled reduction in the total number of growing bacteria. The number of colonies recovered on PLET agar plates ranged from 0 – >300 (CFUs) per tested soil sample. Up to 13 morphologically diverse colonies could be distinguished per plate; however, in the majority of the tested samples, 5–8 different colonies were selected for further studies. In total, 424 spore-forming, aerobically growing isolates were collected from all the tested soil samples. The next step of the research was motility testing, as typical *B. anthracis* strains are non-motile. Eighty-nine of 424 isolates had been found to be non-motile and were tested by the afore-mentioned molecular approaches.

PCR amplicons of SG-749 marker were obtained for 61 isolates. All 61 isolates were considered potential *Bacillus cereus* group members. Further AluI digestion of the SG-749 marker amplicons revealed that none of the 61 isolates yielded *B. anthracis* specific restriction profile. A weak amplification of the *plcR* gene were observed for 71 isolates among the 89 non-motile spore-forming isolates in the first stage of the RSI-PCR test. No *B. anthracis* specific SspI restriction-profile of the *plcR* marker was detected, indicating that *B. anthracis*-specific nonsense mutation in *plcR* gene was absent in all of the 71 isolates positive in the first stage of the RSI-PCR test. PCR products with primers specific for *pagA* were obtained for 15 isolates. Two of these isolates yielded PCR products also for *capB*. The *pagA* and *capB* amplicons were weaker and in different size than the amplicons obtained for *B. anthracis* reference strains. Results of the molecular test are summarized in Table 2.

Table 2. Positive results obtained for investigated samples

SG-749	SG-749/ AluI	*plcR*	*plcR*/ SspI	*pagA*	*capB*	Total No. of investigated samples
61 (68.5%)	0 (0%)	71 (79.8%)	0 (0%)	15* (16.9%)	2* (2.3%)	89 (100%)

* weak positive

DISCUSSION

Today, in contrast to the beginning of the last century, anthrax cases in animals and humans are extremely rare in Poland. The majority of anthrax cases in livestock in Poland were recorded in the period 1922–1939. Human anthrax cases were most numerously reported in 1928 when 81 cases were recorded. A significant decrease in the incidence of anthrax in animals was observed from 1950 when a large number of animals had been covered by vaccination. Human anthrax in this period occurred in the form of sporadic incidences or small outbreaks of several cases per year. The last anthrax

Table I. Nucleotide sequences of primers used in the studies and expected size of amplicons.

Molecular marker	Primer designation	Nucleotide sequence 5'→3'	Amplicon size	References
SG-749	SG749f	ACTGGCTAATTATGTAATG	850 bp	[9]
	SG749r	ATAATTATCCATTGATTTCG		
plcR	AplF	GCTCAATCAACAATTGGCAGG	278 bp	[8]
	AplR	ATGTCATACTATTAATTTGACACGATAGTTCAATAGCTTTATTTGCATGACAAAGCGAAT		
pagA	PagAF	CCAGACCGTGACAATGATG	512 bp	[10]
	PagAR	CAAGTTCTTTCCCCTGCTA		
capB	CapBF	CTGACCAATCTAAGCCTGC	387 bp	[10]
	CapBR	TCGTTTCTCCAATCGCAAT		

outbreak in livestock in Poland was recorded in 2004 and the last human case of the disease was recorded in 2005 [11, 12, 13, 14]. However, due to the long persistence of *B. anthracis* spores in the environment, anthrax outbreaks may still occur in Poland.

In May and June 2010, floods affected Central Europe. In Poland, the biggest flood for the past 160 years occurred this time. The most affected parts of the country were the Świętokrzyskie, Lubelskie, Podkarpackie and Mazowieckie provinces. Intense flooding can unearth anthrax spores, and this could cause outbreaks of the disease, even far from areas affected by anthrax in former years [5, 15]. Exposure to anthrax spores in the soil and outbreaks of the disease associated with flooding were found, among others, in Canada [16], Australia [5] and Africa [17]. It is noteworthy that the outbreaks occurred even in areas where anthrax had not been reported for decades. Also in Europe, anthrax outbreaks were reported to have occurred as a result of floods. Lawerin et al. [6] described the anthrax outbreak in Sweden that occurred in 2008 after 27 years with no detected cases. Surprisingly, the outbreak occurred in winter when the animals were kept in a barn. Roughage contaminated with anthrax spores was the most likely source of the infection. The suspected feed, that was mixed with soil and dust, originated from fields where flooding had occurred the previous year. In July 2010, an animal anthrax outbreak was reported in Slovakia, in an area close to the Polish border. According to information from the Polish Press Agency (PAP), flooding prior to the outbreak in Slovakia was also considered the most likely cause.

In the presented study, soil samples taken from a surface layer of the ground in the areas affected by floods in Poland were investigated. In total, 89 samples were collected, mostly from the bottom of hollow places, where the trapped flood-water could concentrate spores by evaporation and slow soil penetration. A routine procedure was used for *B. anthracis* isolation from the soil samples that had been found to be effective in anthrax investigations conducted at the Anthrax Reference Institute In Foggia, Italy. Nevertheless, the attempts to detect viable anthrax spores for the presented study proved fruitless. Bielawska-Drózd et al. [18] have recommended a two-step culturing process in the non-selective enrichment broth before PCR for soil samples. However, this method is useful for direct PCR from soil samples, but not for the isolation of *B. anthracis* from the samples by culture. Using non-selective medium for *B. anthracis* isolation from soil samples by culture might be ineffective as other soil microorganisms could overgrow the anthrax bacilli. It was therefore decided to use the culture method for *B. anthracis* detection because culture of the bacteria gives opportunities for further studies, such as genotyping and virulotyping, and it is still regarded as the gold standard in microbiology.

Although PCR-amplicons with the primers for *B. anthracis* virulence-plasmid marker genes *pagA* or *capB* were detected, all the amplicons were weak and their size was distinct from the size expected for *B. anthracis*. It was proved that *B. anthracis* plasmids or parts of the plasmids might be transferred to other bacteria belonging to *B. cereus* group sensu lato [19]. The group consists of 7 genetically closely related species: *B. cereus* sensu stricto, *B. anthracis, B. thuringiensis, B. weihenstephanensis, B. mycoides, B. pseudomycoides* and *B. cytotoxicus* [1, 20]. Notably, all the species occur in the environment and could be isolated from soil. Hu et al. [21] have shown that *B. cereus* group isolates containing pXO1- and pXO2-like plasmids are widely distributed across a variety of environmental niches and geographical locations. Approximately 15% of a collection of 2,000 environmental isolates tested by the researchers contained either a pXO1- or pXO2-like plasmid. The highest overall prevalence of isolates carrying pXO1- and pXO2-like plasmids was found in soil samples (26%), but none of these isolates were found to carry the *B. anthracis* toxin or capsule virulence genes. In the presented study, however, there were weak positive results of PCR for *pagA* and *capB* markers are, but insufficient to prove the presence of virulent *B. anthracis*. This thesis is supported by the negative results for the 2 molecular test targeting chromosomal markers suitable for *B. anthracis* identification.

PCR is the only reliable method for anthrax detection when culture of the samples is impossible. But the investigators should bear in mind that weak positive results can occur, and such results should be verified using methods for the detection of *B. anthracis* chromosomal markers or other alternative methods for *B. anthracis* identification.

CONCLUSION

In the presented study, spores of *B. anthracis* that could be related to the risk of anthrax were not detected in the tested soil samples collected from areas of Poland that had been affected by floods in 2010. Despite the fact that the sampling was conducted in the most likely places of spores concentration, such as hollows and other low lying parts of the ground, the negative results presented herein may not argue that Poland is an anthrax free country. However, these results may suggest relatively low risk of flooding triggered anthrax outbreaks in the sampled areas. These findings have been additionally supported by the fact that no anthrax cases have been reported in this area from 2010 until today.

Acknowledgements
This study was supported by the Ministry of Science and Higher Education Research Grant No. N N404 082339 in years 2010–2013.

REFERENCES

1. Koehler TM. Anthrax. Springer-Verlsg Berlin Heidelberg New York, 2002.
2. De Vos V. The ecology of anthrax in the Kruger National Park, South Africa. Salisbury Med Bull. 1990; 68S: 19–23.
3. Turnbull PCB. Guidelines for the surveillance and control of anthrax in humans and animals. 3rd ed. WHO/EMC/ZDI./98.6
4. Fasanella A, Galante D, Garofolo G, Jones MH. Anthrax undervalued zoonosis. Vet Micorbiol. 2010; 140: 318–331.
5. Durrheim DN, Freeman P, Roth I, Hornitzky M. Epidemiologic questions from anthrax outbreaks, Hunter Valley, Australia. Emerg Infect Dis. 2009; 15: 840–842.
6. Lewerin SS, Elvander M, Westermark T, Hartzell LN, Norstrom AK, Ehrs S, et al. Anthrax outbreak in a Swedish beef cattle herd – 1st case in 27 years: case report. Acta Vet Scand. 2010; 52: 7.
7. Knisely RF. Selective medium for *Bacillus anthracis*. J Bacteriol. 1966; 92: 784–786.
8. Gierczyński R, Zasada AA, Raddadi N, Merabishvili M, Daffonchio D, Rastawicki W, et al. Specific *Bacillus anthracis* identification by a *plcR*-targeted restriction site insertion-PCR (RSI-PCR) assay. FEMS Microbiol Lett. 2007; 272: 55–59.
9. Daffonchio D, Borin S, Frova G, Gallo R, Mori E, Fani R, et al. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. Appl Environ Microbiol. 1999; 65: 1298–1303.

10. Zasada AA, Gierczyński R, Kałużewski S, Jagielski M. Virulotypes of *Bacillus anthracis* strains isolated in Poland. Med Dośw Mikrobiol. 2005; 57: 269–275.
11. Naruszewicz-Lesiuk D. Wąglik. In: Kostrzewski J. Choroby zakaźne w Polsce i ich zwalczanie w latach 1919–1962. Warszawa, PZWL, 1964. p. 380–389 (in Polish).
12. Anusz Z. Wąglik. In: Kostrzewski J. Choroby zakaźne w Polsce i ich zwalczanie w latach 1961–1970. Warszawa, PZWL, 1973. p. 292–297 (in Polish).
13. Anusz Z. Wąglik. In: Kostrzewski J. Choroby zakaźne w Polsce i ich zwalczanie w latach 1970–1979. Wrocław, Ossolineum, 1984. p. 292–294 (in Polish).
14. PZH. Meldunki o zachorowaniach na choroby zakaźne, zakażeniach i zatruciach w Polsce. http://www.pzh.gov.pl/epimeld/index_p.html (access: 2013.02.05) (in Polish).
15. Hamplson K, Lembo T, Bessell P, Auty H, Packer C, Holliday J, et al. Predictability of anthrax infection in the Serengeti, Tanzania. J Appl Ecol. 2011; 48: 1333–1344.
16. Epp T, Walander C, Argue CK. Case-control study investigating an anthrax outbreak in Saskatchewan, Canada – Summer 2006. Can Vet J. 2010; 51: 973–978.
17. Munang'andu HM, Banda F, Siamudaala VM, Munyeme M, Kasanga CJ, Hamududu B. The effect of seasonal variation on anthrax epidemiology in the upper Zambezi floodplain of western Zambia. J Vet Sci. 2012; 13: 293–298.
18. Bielawska-Drózd A, Niemcewicz M, Bartoszcze M. The evaluation of methods for detection of *Bacillus anthracis* spores in artificially contaminated soil samples. Pol J Environ Stud. 2008; 17: 5–10.
19. Van der Auwera GA, Timmery S, Hoton F, Mahillon J. Plasmid exchanges among members of the *Bacillus cereus* group in food-stuff. Int J Food Microbiol. 2007; 113: 164–172.
20. Scott II E., Dyer DW. Divergence of the SigB regulon and pathogenesis of the *Bacillus cereus sensu lato* group. BMC Genomics. 2012; 13: 564.
21. Hu X, Van der Auwera G, Timmery S, Zhu L, Mahillon J. Distribution, diversity, and potential mobility of extrachromosomal elements related to the *Bacillus anthracis* pXO1 and pXO2 virulence plasmids. Appl Environ Microbiol. 2009; 75: 3016–3028.