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DETECTION OF *BACILLUS ANTHRACIS* IN THE AIR, SOIL AND ANIMAL TISSUE

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The objective of the present work was to establish effective and rapid diagnostic methods for the detection of Bacillus anthracis, a highly virulent zoonotic pathogen, in the air, soil and animal (or human) tissue samples. Liquid culture of B. anthracis was aerosolized and four air sampling procedures were employed. Detection of B. anthracis in the air samples was successful with RCS High Flow sampler (culture-based detection) and when sampling through the air filter (molecular detection using SmartHelix Complex Samples DNA Extraction Kit). Liquid B. anthracis culture was also employed for spiking the homogenised bovine lymphatic gland tissue and soil samples. DNA extraction was performed using three different commercial kits for each sample type. High Pure PCR Template Preparation Kit was the most effective for DNA extraction from animal tissue samples. Detection in the soil was successful when PowerSoil DNA Isolation Kit was used. Our results indicate that B. anthracis can be monitored in different matrices by rapid molecular methods when appropriate sampling and DNA extraction procedures are employed prior to PCR assay. The selected rapid protocols can be implemented in specialized veterinary or human diagnostic laboratories with moderate costs.

Key words: Bacillus anthracis, detection, DNA extraction, PCR

INTRODUCTION

Bacillus anthracis is the causative agent of anthrax, an acute zoonosis that occurs when *B. anthracis* spores enter the body by inhalation, ingestion or through skin lesions (Hanna, 1998). The respiratory form of the disease has a mortality rate approaching 100% (James *et al.*, 1998). Due to survival of anthrax spores for extremely long periods of time and their easy dispersal (Meselson *et al.*, 1994; Watson and Keir, 1994), *B. anthracis* has been regarded and used as a bioterroristic agent (Jernigan *et al.*, 2001). As early symptoms of anthrax in humans are nonspecific, a rapid detection of deliberate or non-deliberate release of *B. anthracis* is of great importance for the prevention of infection.

Detection of bacteria in aerosols and in the environmental samples is usually hampered because of demanding sampling and due to interference of organic or inorganic compounds with downstream enzymatic reactions in the detection protocol, respectively. Attempts to detect *B. anthracis* spores in the air (Makino *et al.*, 2001; Makino and Cheun, 2003; Campbell *et al.*, 2007) and in the environmental or tissue samples (Beyer *et al.*, 1995; Cheun *et al.*, 2001; Cheun *et al.*, 2003; Zdovc *et al.*, 2007) have been described. However, detection often requires labour-intensive procedures and no standardized protocols are available.

The aim of the present study was to implement the current diagnostic methods employed in biosafety level 3 (BSL-3) laboratories with more effective and/or rapid sampling or extraction procedures for the detection of *B. anthracis* in different matrices. Air and soil samples were investigated as representing potential sources of infection. In addition, the study was performed on animal tissue samples as resected animal or human tissues enable the assessment of infection in anthrax suspects.

MATERIALS AND METHODS

B. anthracis strain for sample preparation

An overnight culture of non-pathogenic *B. anthracis* vaccinal strain Sterne 34 F2 (NCTC 8234) (Sterne, 1939) was grown in tryptic soy broth (TSB; Oxoid, UK) at 37°C. Bacterial culture was diluted in 10-fold series and enumerated on blood agar plates (BBL Columbia Agar Base supplemented with 5% of sheep blood; Becton Dickinson, USA) applying the standard plate count technique (Madigan *et al.*, 2003). The selected dilution of *B. anthracis* culture was used for subsequent preparation of air, soil and tissue samples.

Detection in air samples

Aerosolization and sampling. In order to detect enough bacteria to provide statistically relevant data, five millilitres of bacterial suspension containing 9.2×10^4 cells were aerosolized in the aerosolization chamber of 40 m³ at a rate of 2.5 mL min⁻¹ with aerosol drops measuring 5-10 µm in diameter (airbrush DG-35; Fimotool, Slovenia) according to results obtained in a preliminary study. After aerosolization, the chamber was ventilated for 5 min to ensure even dispersion of the aerosolized suspension. Larger droplets were allowed to sediment for 10 min prior to continual sampling of aerosolized *B. anthracis* for 20 min.

Four different sampling procedures were employed: (i) onto solid medium (total count tryptic soy agar (TSA) strips; Biotest AG, Germany) by RCS High Flow microbial air sampler (Biotest AG, Germany), (ii-iii) into liquid medium (physiological saline solution (PS); 0.9% NaCl) by SAS PCR sampler (International PBI, Italy; into 45 mL of PS to obtain 3 × 15 mL of sampling liquid for comparison of three different detection methods) and by BioSampler (SKC, UK; into 15 mL of PS), and (iv) through the polyethersulfone air filter with pore size of 0.22 µm (TPP, Switzerland) by pump (KNF Neuberger, Germany). Samplings were

performed according to the instructions provided by the sampler/pump manufacturers. All samplings were performed simultaneously and samplers were placed at a maximum possible distance from each other (into the corners of the chamber). During the 20-min sampling, different volumes of aerosols were processed according to instrument capacity: 2000, 1000, 250 and 450 l by RCS High Flow, SAS PCR, BioSampler and air pump, respectively.

Detection. *B. anthracis* from the aerosols was detected indirectly by growth on TSA strips after 24-h incubation at 37°C and directly after DNA extraction from the filtered sampling liquid (0.45 µm membrane filters; Sartorius, Germany) or from the air filter.

Randomly selected suspect colonies grown on TSA strips were resuspended in sterile water and subjected to fast lysis with 15-min boiling followed by centrifugation for 2 min at 14000 g. Supernatants were used as a source of DNA for confirmation by PCR employing *B. anthracis*-specific primers PA8/PA5 targeting *pag* gene (Beyer *et al.*, 1995) as recommended by the World Organization for Animal Health (OIE, 2010). A 50-µL reaction mixture contained 5 µL of DNA, 1.25 U of Platinum *Taq* DNA Polymerase (Invitrogen, USA), 1.5 mM MgCl₂ and 1×PCR buffer supplied by the manufacturer, 50 pmol of each primer (Invitrogen, USA) and 0.2 mM of each dNTP (Applied Biosystems, USA). Amplification was performed in GeneAmp PCR System 2700 (Applied Biosystems, USA) following the previously described amplification protocol (15). PCR products were electrophorised in 2% agarose gels, stained with ethidium bromide (10 µg mL⁻¹) and visualized using GeneGenius bio-imaging system (Syngene, UK).

Extraction from the filter-captured *B. anthracis* was performed by the method developed in our laboratory, SmartHelix Complex Samples DNA Extraction Kit (Institute of Physical Biology, Slovenia; more information on the SmartHelix technology is available at <http://smart-helix.com/>); air filter or 15 mL of the sampling liquid were processed. For the SAS PCR sampling, extraction was also performed using Adiapure Water DNA Extraction and Purification Kit (Adiagene, France) according to the manufacturer's instructions. After DNA extraction using commercial kits, real-time PCR determination was performed by LightCycler 1.2 (Roche Diagnostics, Germany) using LightCycler *Bacillus anthracis* Detection Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions; as the employed *B. anthracis* strain lacked the pXO2 virulence plasmid, detection targeted the *pag* gene only. In addition, cultivation-dependent detection after SAS PCR sampling was performed (15 mL of the sampling liquid were filtered for the overnight cultivation at 37°C in 35 mL of TSB, which was followed by the spectrophotometric culture turbidity-measurement at 600 nm).

Detection in tissue and soil samples

Sample preparation. The overnight *B. anthracis* culture was enumerated and diluted in 10-fold series to obtain 10⁻² to 10⁻⁶ dilutions. After spiking with 1 mL of each culture dilution, samples (3 mL of homogenized tissue of bovine lymphatic

gland or 10 g of Green Plant Soil by Klasmann-Deilmann, Germany) were thoroughly mixed for 30 s using a stomacher (Compact Micro; IUL Instruments, Spain). Tissue samples were spiked with 26700, 2670, 267, 26.7 and 2.67 CFU per 50 mg of sample, and soil samples with 40000, 4000, 400, 40 and 4 CFU per 250 mg of sample (50 and 250 mg being the starting weights of tissue and soil samples for DNA extraction, respectively).

Detection. Three different commercial DNA extraction kits were selected for each sample type according to the manufacturers' instructions. Each extraction was performed in three parallels. The extracted DNA was subjected to classical (two replicates) and real-time (one replicate) PCR amplification as described above for the air samples.

For animal tissue samples, (i) SmartHelix Complex Samples DNA Extraction Kit (Institute of Physical Biology, Slovenia; abbreviated as SH), (ii) InviMag Forensic Kit/ KFmL (Invitek, Germany; abbreviated as IM) for automatic DNA extraction using the KingFisher mL workstation (Thermo Fisher Scientific, USA), and (iii) High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany; abbreviated as HP) were used. For soil samples, (i) SmartHelix Complex Samples DNA Extraction Kit, (ii) QIAamp DNA Stool Mini Kit (Qiagen, Germany; abbreviated as QS), and (iii) PowerSoil DNA Isolation Kit (MoBio Laboratories, USA; abbreviated as PS) were used.

RESULTS

Detection in air samples

Detection of *B. anthracis* in the air samples was successful with RCS High Flow sampler and when sampling through the air filter (Table 1). After RCS High Flow sampling, 123 bacterial colonies were counted, which were all morphologically consistent with *B. anthracis*.

Table 1. Cultivation-dependent and molecular detection of *Bacillus anthracis* in air samples

Air sampler	Cultivation-dependent detection		Molecular detection	
	Cultivation medium	Result (growth)	DNA extraction kit	Result (LC)***
RCS High Flow	TSA	pos*	-	-
SAS PCR	-	-	SH	neg
	-	-	AW	neg
	TSB	neg**	-	-
BioSampler	-	-	SH	neg
Pump	-	-	SH	pos

*123 CFU; **according to spectrophotometric culture turbidity-measurement at 600 nm; ***LC results shown in Figure 1 (abbreviations: SAS-SH, SAS PCR sampling followed by SH extraction; SAS-AW, SAS PCR sampling followed by AW extraction; Bio-SH, BioSampler sampling followed by SH extraction; filter-SH, sampling by pump through air filter followed by SH extraction)

TSA, tryptic soy agar; TSB, tryptic soy broth; LC, real-time PCR amplification by LightCycler; SH, SmartHelix Complex Samples DNA Extraction Kit; AW, Adiapure Water DNA Extraction and Purification Kit; *pos*, positive result (bacterial growth or LC amplification); *neg*, negative result (no bacterial growth or LC amplification).

The selected colonies were confirmed as such by PCR. Sampling into the liquid medium by the other two samplers generated negative results (Table 1). Although exhibiting a relatively high crossing-point value (Ct), results of PCR amplification by LightCycler indicated a successful detection of *B. anthracis* after filtration through the air filter (Figure 1).

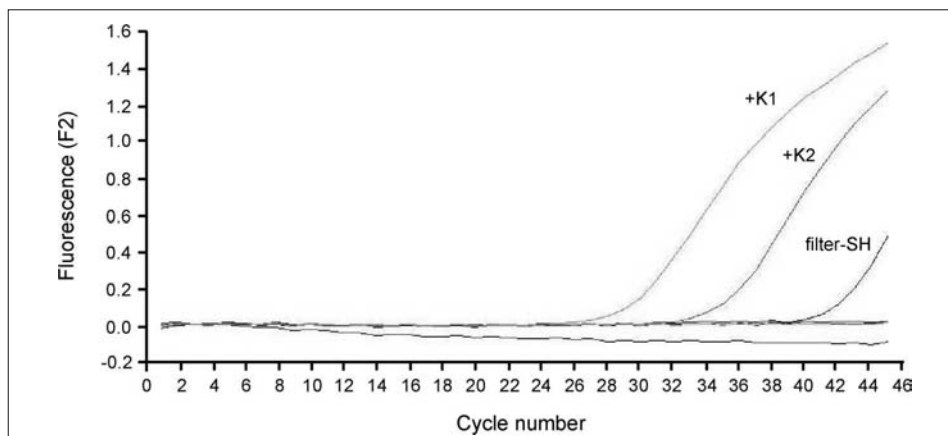


Figure 1. Results of *Bacillus anthracis*-specific real-time PCR amplification by LightCycler for air samples (for details on abbreviations see Table 1): negative for samples SAS-SH, SAS-AW, Bio-SH and negative amplification control (flat curves), positive for samples filter-SH, +K1 and +K2 with the reported Ct values 41.00, 29.56 and 35.16, respectively; +K1, in-house positive amplification control (DNA of *B. anthracis* strain from laboratory collection); +K2, positive amplification control from kit contents (*B. anthracis* control *pag* template)

Detection in tissue samples

Detection of *B. anthracis* in animal tissue samples was successful when HP (Figure 2/A) or IM kits were used, but not with SH (Table 2). For the former two, the limit of detection (LOD; with 100% detection probability) was $26.7 < \text{LOD} \leq 267$ CFU per 50 mg of tissue. However, HP was found to be the most effective commercial kit for DNA extraction from tissue samples in our study, since it enabled *B. anthracis* detection in more samples with lower bacterial loads than IM; only HP enabled detection in one sample exerting the lowest contamination level of 2.67 CFU per 50 mg (Table 2).

Detection in soil sample

Detection of *B. anthracis* in soil samples was successful when PS kit (Figure 2/B) was used with $40 < \text{LOD} \leq 400$ CFU per 250 mg of soil; PS also enabled detection of 4 CFU per 250 mg in 33% and 40 CFU in 78% of samples (Table 3). QS and SH kits did not prove to be appropriate for *B. anthracis* detection in soil samples (Table 3).

Table 2. Molecular detection of *Bacillus anthracis* in tissue samples

DNA extraction kit	BA load*	2.67			26.7			267			2670			26700		
		E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3
SH	Sample															
	PCR ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IM	PCR ₃ (LC)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HP	PCR ₃ (LC)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₃ (LC)**	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

*CFU per sample (50 mg); **LC results: the reported Ct values for IM and HP were close to their upper limit for samples with the lowest BA load detected (i.e. approx. 43.5) and decreasing with the 10-fold increasing BA load added to samples according to approx. 90% amplification efficiency (i.e. for 1.8-root of 10 per BA load)

BA, *B. anthracis*; SH, SmartHelix Complex Samples DNA Extraction Kit; IM, InviMag Forensic Kit/ KFmL; HP, High Pure PCR Template Preparation Kit; PCR, amplification replicates 1-3; LC, real-time PCR amplification by LightCycler; E, DNA extraction parallels 1-3; -, negative result (no PCR amplification); +, positive result (PCR amplification).

Table 3. Molecular detection of *Bacillus anthracis* in soil samples

DNA extraction kit	BA load*	4			40			400			4000			40000		
		E ₁	E ₂	E ₃	E ₁	E ₂	E ₃	E ₁	E ₂	E ₃	E ₁	E ₂	E ₃	E ₁	E ₂	E ₃
SH	Sample															
	PCR ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QS	Sample															
	PCR ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS	Sample															
	PCR ₁	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+
	PCR ₂	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	PCR ₃ (LC)**	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+

*CFU per sample (250 mg); **LC results: the reported Ct value for QS was 39.98 / for PS 41.56 for sample with the lowest BA load detected and reaching an average of 27.34 in sample with the highest BA load

SH, SmartHelix Complex Samples DNA Extraction Kit; QS, QIAamp DNA Stool Mini Kit; PS, PowerSoil DNA Isolation Kit; for other abbreviations, see notes in Table 2.

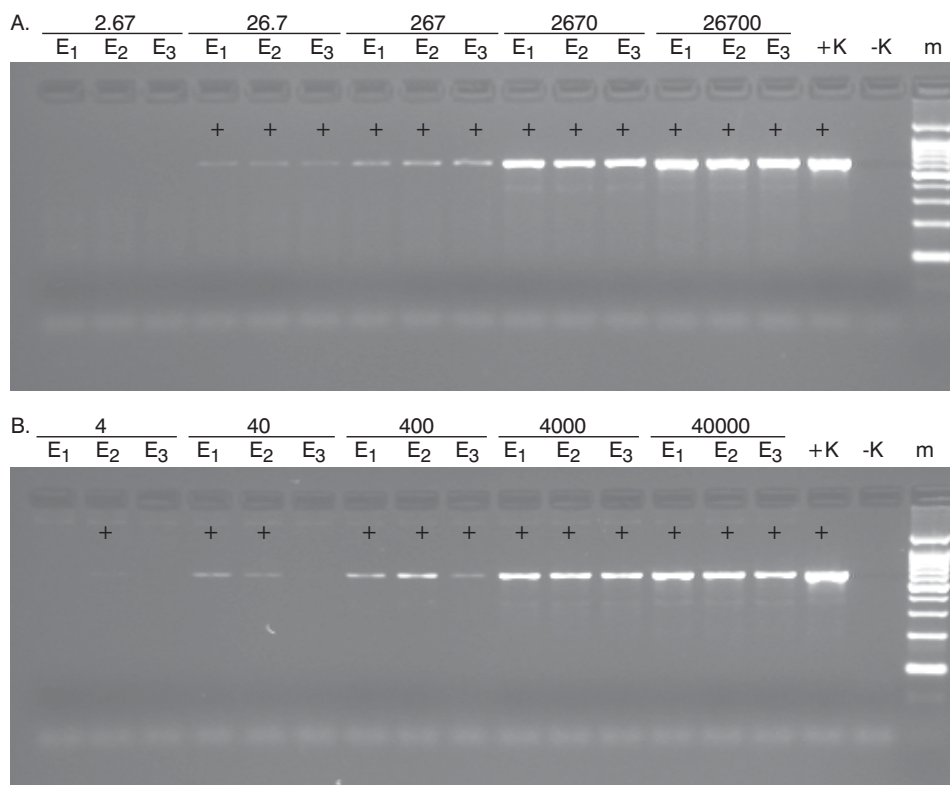


Figure 2. Results of *Bacillus anthracis*-specific PCR amplification using PA8/PA5 primers (for details on abbreviations see Tables 2 and 3): A. PCR₁ after HP extraction from tissue samples, B. PCR₁ after PS extraction from soil samples; +K, in-house positive amplification control (DNA of *B. anthracis* strain from laboratory collection); -K, negative amplification control (sterile water); m, molecular standard (GelPilot 100 bp Plus Ladder; Qiagen, Germany); +, results denoted as positive

DISCUSSION

Our results implicate that current diagnostic methods employed in BSL-3 laboratories for the detection of *B. anthracis* in different matrices can be successfully implemented with more rapid molecular methods. It was reported before that bacteriological culture examination alone may not be sufficient for the correct diagnosis (Zimmermann *et al.*, 2010). Molecular detection is based upon DNA extraction, but has to be adapted for the specific type of samples. In addition to classical phenol/chloroform extraction (Wilson, 1994), different commercial kits are available, which have been developed and improved if required regarding the characteristics of the sample material. Extraction of DNA for the detection of *B. anthracis* from the air samples could be accelerated by employing a rapid

thermal lysis procedure in place of commercial kits (Makino *et al.*, 2001). However, more complex samples like tissue and soil containing potential PCR inhibitors should be processed with more sophisticated extraction methods as it was reported that the limit of detection greatly depends on the sample pre-treatment and/or extraction process (Herzog *et al.*, 2009). Different commercially available kits were selected for our study according to their applicability for the sample types; QS kit that was designed for DNA extraction from faecal material was selected for soil samples, since soil can be contaminated with faeces from the infected animals.

Detection of *B. anthracis* in the air was successful after sampling onto solid medium and through the filter trap. It was confirmed that RCS High Flow sampler, handling high flow rates and large sample volumes, provides an efficient collection mechanism for the airborne microbes. The benefit of samplers employing a solid or adhesive medium is incubation, which enables a direct quantitative estimate of the number of CFU in the volume of sampled air (Lawley, 2009). As chains of *B. anthracis* cells impair the plate counting, not a true cell number could be reported for air samples or culture enumeration, but only the CFU value indicating an underestimated count.

Combined to good growth on ordinary nutrient media (Sleigh and Timbury, 1998), the successful cultivation-dependent detection of *B. anthracis* in the air was not surprising. However, when a faster response is needed in the case of a suspected bioterroristic incident or occupational exposure, more rapid molecular detection techniques should be employed as in the case of *B. anthracis* sampling through a filter by the air pump. According to a similar study, a single *B. anthracis* spore per 100 L of air was detected by the real-time PCR assay although employing a different sample processing (Makino *et al.*, 2001). The detection based on molecular methods employed in our study could probably be expected for lower contamination levels after adequate evaluation as vegetative cells and spores behave differently during aerosolization. In our study, a dilution of *B. anthracis* overnight broth culture was employed for sample preparation but spore content was not studied. However, as *B. anthracis* vegetative forms are dependent on sporulation for survival and the stringent response in stationary growth phase signals sporulation (van Schaik *et al.*, 2007), we expected to find both vegetative cells and spores of *B. anthracis* in culture suspensions prepared in aerobic conditions for aerosolization and sample inoculation. In addition, one-day old *B. anthracis* culture was tested for the presence of spores: after 15-min boiling, a dense bacterial growth was observed on the inoculated agar plates, and after 1.5-h boiling, viability was still not completely reduced. Therefore, *B. anthracis* spores were expected in the culture suspensions, albeit in lower amounts as in the studies concerning inoculation with spores alone.

The results of *B. anthracis* detection in the air were negative for both samplers using the collection fluid principle, although the portable SAS PCR sampler, which would be the sampler of choice for field analyses, processed a larger volume than BioSampler handling the smallest volume of air. Since the contamination level of air was presumably comparable for all samplers and the volume of sampled air was markedly larger only for the sampler employing solid

medium, negative results of the sampled liquid can not be properly explained by differences in the volume of sampled air. Further, physiological saline was selected according to the manufacturers' instructions, thus we can not question the suitability of the sampling liquid. Three different detection methods were employed after SAS PCR sampling, including molecular detection applying the extraction SH kit that proved to be appropriate for the detection of *B. anthracis* after sampling through the air filter. Long silicone tubes of SAS PCR sampler, enabling the patented collection mechanism, could represent a drawback due to possible electrostatic attachment of a certain portion of contaminating airborne microbes (Rooks, 1948). However, as detection failed in all three cases in addition to detection employing BioSampler, the lack of success in *B. anthracis* detection after sampling into the collection fluid might not depend on the selection of detection methods but on the collecting principle itself as the air sampler must be selected according to the concentration and type of bioaerosol. When air contamination levels are not expected to be high, methods using sampling liquids should be avoided (Consenza Sutton, 2004).

Although SH kit for DNA extraction from complex samples was shown to perform well for the extraction from filter-concentrated bacterial cells prior to PCR detection (from air samples - this study; from water samples - unpublished data), detection from animal tissue or soil samples was not successful. A more cell-disruptive procedure was needed for the extraction from tissue or soil, complementing thermal or mechanical disintegration with chemical lysis, in addition to sufficient removal of inhibitors of the subsequent PCR detection, being dependent on the purity of DNA extracted from sample material.

Detection of *B. anthracis* in animal tissue samples was successful with HP and IM kits. In comparison to IM kit, HP enabled detection in more sample extractions or amplification parallels with the lowest bacterial loads. However, when a large number of samples should be processed, IM kit can also be employed with satisfactory results as it is designed for the automated DNA extraction. Detection of *B. anthracis* in soil samples was successful with PS kit, which also enabled the detection of *B. anthracis* in soil samples with the lowest bacterial loads. The actual detection limits, or more objectively defined detection probabilities (Knutsson *et al.*, 2002), would be obtained with studies of additional contamination levels of tissue and soil in the selected range. Our results showed that HP and PS commercial kits enabled detection of *B. anthracis* in animal tissue and soil samples with comparable efficiency regarding the contamination level. The obtained limit of detection was comparable to the literature data for soil samples applying different extraction procedures in combination with PCR, nested PCR or real-time PCR (Kuske *et al.*, 1998; Cheun *et al.*, 2003; Ryu *et al.*, 2003). Pre-treatment of samples by one or two cultivation enrichments would improve the limit of detection (Cheun *et al.*, 2003), but the time-to-result demand would also increase.

We can conclude that *B. anthracis* can be monitored in the air, soil or animal tissue by rapid methods. However, an appropriate sampling and DNA extraction procedure must be selected prior to PCR assay. For the air samples, cultivation-dependent detection remains the gold standard, but rapid molecular techniques

can well be employed after filtration of the contaminated air through membrane filters. SmartHelix Complex Samples DNA Extraction Kit proved to perform well for DNA extraction from bacteria concentrated on the air filters. For the animal tissue samples, High Pure PCR Template Preparation Kit performed well, detecting 50 CFU g⁻¹ of tissue. On the other hand, InviMag Forensic Kit can be employed successfully when an automated extraction is preferred. For the soil samples, PowerSoil DNA Isolation Kit performed very well, detecting 15 CFU g⁻¹ of soil. All these protocols can be successfully introduced into BSL-3 diagnostic laboratories for the detection of *B. anthracis* at reasonable expenses.

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DETEKCIJA *BACILLUS ANTHRACIS* U VAZDUHU, ZEMLJIŠTU I ŽIVOTINJSKOM TKIVU

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SADRŽAJ

Cilj ovog istraživanja je bio da se uvedu efikasne i brze dijagnostičke metode za detekciju *Bacillus anthracis*, visokovirulentnog zoonotskog patogena, u vazduhu, zemljištu i uzorcima tkiva životinja i čoveka.

Tečna kultura *B. anthracis* je rasprskavana u obliku aerosola i zatim je vršeno uzorkovanje iz vazduha. Detekcija *B. anthracis* u uzrocima vazduha je bila uspešna kada je korišćen RCS High Flow sampler (detekcija bazirana na kultivisanju bakterija) i prilikom uzorkovanja kroz filter za vazduh (molekularna detekcija korišćenjem SmartHelix Complex Samples DNA Extraction Kit). Tečna kultura *B. anthracis* je takođe korišćena za kontaminaciju homogenizovanih limfnih žlezda goveda i uzoraka zemljišta. Ekstrakcija DNK je obavljena pomoću tri različita komercijalna kompleta za svaki tip uzoraka. Pri ekstrakciji DNK iz uzoraka tkiva, najefikasniji je bio High Pure PCR Template Preparation Kit. Detekcija u zemljištu je bila uspešna kada je korišćen PowerSoil DNA Isolation Kit. Naši rezultati ukazuju da *B. anthracis* može biti detektovan u različitim materijalima pomoću brzih molekularnih metoda kada su pre primene PCR testa uzorkovanje i ekstrakcija DNK izvedeni pravilno. Izabrani brzi protokoli mogu biti implementirani u specijalizovanim veterinarskim ili humanim dijagnostičkim laboratorijama uz razumne troškove.