Title

Sensitive and specific recombinase polymerase amplification set of assays for fast screening, detection and identification of *Bacillus anthracis* in a field setting

Running title

Specific and fast RPA-identification of *B. anthracis*

Authors

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Abstract

Four isothermal recombinase polymerase amplification assays (RPA) were developed for fast in-field identification of *Bacillus (B.) anthracis*. RPAs targeted three specific sequences (i.e. BA_5345 chromosomal marker, lethal factor *lef*-pXO1, and capsule biosynthesis *capA*-pXO2), and a conserved sequence of the *B. cereus* group in the adenylate cyclase gene (*adk*). *B. anthracis*-specific RPA assays were first tested on purified genomic DNAs (n=60) containing 11 representatives of *B. anthracis*, then on soil (n=8) and white powder (n=8) samples spiked with inactivated *B. anthracis* spores and/or other biological agents. The RPA assays were also tested in another laboratory facility which blindly provided DNA and lysate samples (n=30, including 20 *B. anthracis* strains). RPA tests displayed 100% specificity and sensitivity. The hands-off turnaround time at 42°C ranged from 5 to 6 min for 10^2 genomic copies. The analytical sensitivity of each RPA was ~10 molecules per reaction. Besides, BA_5345 and *adk* RPA assays were assessed in field conditions on a series of surface swabs (n=13 with 11 swabs contaminated with *B. thuringiensis* spores) and blindly dispatched to the field laboratory by a CBRN sampling team. None of the 13 samples, expect the control, tested positive for *B. anthracis* and all samples harvested on spores-contaminated surfaces tested positive with *adk*-RPA assay. All three *B. anthracis*-specific RPA assays proved suitable for rapid and reliable identification of *B. anthracis* and could therefore easily be used by first responders in field conditions to quickly discriminate between a deliberate release of *B. anthracis* spores and a hoax attack involving “white-powders”.

Importance

In recent decades, particularly following 9/11 and the Amerithrax attack, the world experienced several attempts to sow panic and chaos in the society through thousands of white-powder copycats using household powders to mimic a real bioterrorism attack. In such circumstances, field deployable detection methods are particularly needed to screen samples

collected from the scene. The aim is to test them directly using a fast and reliable assay for detecting the presence of *B. anthracis*. While this does not prevent further confirmatory tests to be performed in reference laboratories, it would bring useful, timely relevant information to the local crisis managers, and help them to take appropriate decisions without having to wait for qPCR results (turnaround time: few hours) or phenotypic identification and sequencing (turnaround time: few days). In the current investigation, we developed a set of isothermal RPA assays for a rapid screening and identification of *B. anthracis* in powders and soil samples with the purpose to discriminate a deliberate release of *B. anthracis* spores from a hoax attack involving "white-powders". This would also apply to dispersion by spraying aerosolized forms of *B. anthracis*. Further work is now ongoing to confirm the first observations and validate the on-site use of these assays by first responders.

Introduction

B. anthracis, the etiological agent of anthrax, is a Gram positive endospore forming bacteria that can cause a life threatening disease in livestock and occasionally in humans (1). This zoonotic pathogen exerts its virulence activity through pXO1 and pXO2 plasmids carrying unique genes responsible for toxin production and capsule synthesis, respectively (2-4). Intentional dispersion of *B. anthracis* spores for bioterroristic purposes or in the context of military operations constitutes a major threat for both civilians and ground troops. In recent decades, particularly following 9/11 and the Amerithrax attack, the world experienced several attempts to sow panic and chaos in the society by a deliberate dispersion thousands of white-powder copycats using household powders to mimic a real bioterrorism attack (5-7). Under such circumstances, rapid and reliable detection and identification methods are particularly needed to fast screen samples collected from the scene, hence to test directly for the presence of *B. anthracis*. Conventional cultivation methods are clearly inappropriate in these conditions because they depend on highly skilled experts, require specific biosafety level 3 (BSL 3)

71 facilities and have a turnaround time of several hours or days (8). The high specificity,
72 sensitivity and speed of nucleic acid molecular methods, among which the quantitative real-
73 time polymerase chain reaction (qPCR), explain why these methods have become a
74 predominant diagnostic tool (9, 10). Besides, novel DNA-based technologies based on
75 isothermal amplification are now steadily gaining interest among first responders because of
76 their simplicity, speed and appropriateness for in-field use. Isothermal amplification enables
77 healthcare workers in remote locations to quickly analyze samples for the presence of nucleic
78 acid from a range of infectious agents, using field-deployable assays (11-15). Among these
79 methods, loop-mediated isothermal amplification (LAMP) (16) and recombinase polymerase
80 amplification (RPA) (17) assays have become increasingly popular and are now proposed in
81 the setting of bioterrorism for rapid biothreats detection including *B. anthracis* (18-20).

82 The major challenge for developing a *B. anthracis*-specific detection assay stems from the
83 genetic close relationship among a cluster of strains referred to as the *Bacillus cereus sensu*
84 *lato* group. This cluster comprises *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B.*
85 *pseudomycoides*, *B. weihenstephanensis* and *B. cytotoxicus* (21-23). The degree of genetic
86 similarity is substantially higher between *B. anthracis*, *B. cereus* and *B. thuringiensis*
87 compared to the other species. Some authors even proposed to consider them as a single
88 species (24). In that respect, the phylogenetic and taxonomic relationships of the bacteria of
89 the *B. cereus* group are still under intense discussion (25). To identify *B. anthracis*, PCR
90 assays commonly amplify target sequences from pXO1 and pXO2 virulence plasmids (10,
91 22). However, non-pathogenic *B. anthracis* isolates lacking these virulence plasmids as well
92 as few *B. cereus* strains harbouring anthrax-like plasmids have been characterized (6, 26).
93 These findings underscored the need for assays targeting unique chromosomal signatures
94 combined with plasmid genes to distinguish *B. anthracis* from closely related *B. cereus* group
95 isolates, and fully pathogenic from attenuated or non-virulent *B. anthracis* strains (27).

96 However, detecting *B. anthracis* chromosomal signatures sequences has proved to be very
97 challenging. Among all reported targets, only few were found to be unique to *B. anthracis*,
98 e.g. *purA* and *pclR* SNPs as well as BA_5345, *PL3* and BA5357 genes (9, 25).

99 We report here the design and validation of a set of three isothermal RPA assays that allows
100 for a fast and specific screening and identification of *B. anthracis* in powders and soil
101 samples. The assays amplify the BA_5345 chromosomal marker as well as *lef* (pXO1) and
102 *capA* (pXO2) virulence genes. The first validation was carried out on a well characterized
103 DNA collection of *B. anthracis* and *B. cereus* group members, and DNA strains outside the *B.*
104 *cereus* group. This was completed by a blind testing in an external laboratory facility, which
105 provided well-characterized DNA samples from its own collection of *B. anthracis* and *B.*
106 *cereus* group members. Optimized assays were subsequently used for detecting *B. anthracis*
107 in powder and soil samples spiked with inactivated *B. anthracis* spores and/or other biological
108 agents. A fourth RPA assay targeted a conserved sequence of *adk* gene which is shared
109 among all representatives of the *B. cereus* group. Finally, BA_5345 and *adk* RPA assays were
110 evaluated in field conditions by processing samples with and without *B. thuringiensis* strain, a
111 widely used simulant of *B. anthracis*. Data were compared to those obtained with our
112 previously validated duplex qPCR (28).

113 **Results**

114 **RPA assays optimization.** In the first optimization phase using the TwistAmp® Basic
115 amplification kit, three pairs of RPA primers targeting various sequence regions of BA_5345,
116 *lef*, *capA* and *adk* cloned target genes were selected based on the highest amplification
117 efficiency and a lowest background amplification on the gel electrophoresis (Figure 2). Unlike
118 set-1 and set-3 pairs of primers, the *lef* sequence region targeted by the set-2 pair of RPA

119 primers matched these criteria (Fig 2A). A similar approach was used to select the optimal
120 target region within the BA_5345, *capA* and *adk* sequences.

121 After the selection of the optimal target regions, optimization was carried out using the real-
122 time fluorescence DNA amplification TwistAmp® exo. RPA primers and probe were selected
123 according to the highest and fastest fluorescence amplification and negative NTC signals
124 (Table 1). The optimal temperature of the RPA reaction was set at 42°C. Following an initial
125 pre-incubation for 3 min, the tubes were taken out from the thermoblock, mixed vigorously,
126 spun and incubated further for the remaining period of time. Using $5x10^2$ genomic copies of
127 each BA_5345, *lef*, *capA* and *adk* cloned target in optimal reaction conditions, the detection
128 threshold time was 5.3, 6.3, 4.7 and 5.3 min, respectively. RPA results with pXO1 (*lef*) and
129 pXO2 (*capA*) targets were concordant with those obtained with our duplex qPCR designed for
130 detecting the same plasmid sequences (28).

131 **RPA assays sensitivity and speed**. Analytical sensitivity was assessed under optimal
132 conditions on tenfold serial dilution of recombinant plasmid standards carrying the BA_5345,
133 *lef* or *capA* targets. Recombinant a*dk* plasmid was not included in this assessment. For each
134 RPA assay, the concentration target of the DNA measured by fluorescence covered five
135 orders of magnitude (Fig 3A, 3B and 3C). The limit of detection (LOD) of each RPA assay at
136 a 95% detection probability, as estimated by a probit regression analysis on quintuplicate
137 tests, was 13.31, 11.61 and 7.43 molecules per reaction for BA-5345, *lef* and *capA*,
138 respectively (Fig 4A).The RPA assay showed excellent linearity over a dynamic range of 10^2
139 to 10^6 copies for each BA-5345, *lef* and *capA* target with a R-squared correlation of 0.97, 0.98
140 and 0.99, respectively (Fig 4B).

141 BA_5345, *lef* and *capA* RPA assays correctly identified *B. anthracis* reference DNAs (n=11),
142 powders containing a fixed concentration of *B. anthracis* Sterne spores (n=4) and soil samples

(n=3) spiked with different concentration of *B. anthracis* (Ames strain) spores with 100% sensitivity (n=18). The mean detection time for *B. anthracis* reference DNAs was 4.37, 6.80 and 4.23 min, respectively. Data from three replicates were reproducible. It is of note that powder samples spiked with *B. anthracis* spores were BA_5345- and *lef*-positive and *capA*-negative, in accordance with the *B. anthracis* Sterne (pXO1 + pXO2 -) strain.

RPA assays specificity and cross reactivity. Specificity was evaluated on a panel of 50 reference DNAs from strains phylogenetically distant or very close to *B. anthracis*, 4 negative powder, 5 soils spiked with single or multiple biological agents and 13 swab samples (with (n=11) or without (n=2) *B. thuringiensis* spores) tested in field conditions. Pooled results from all samples tested by RPA (n=72) indicated an overall specificity of 100 %. No non-specific signal was generated with 50 non–*B. anthracis* strains among which closely related *B. cereus* group, *Bacillus spp* and bacteria frequently encountered as contaminants during sampling. No background signal was detected with human DNA. No cross-detection was observed for the remaining samples containing either *B. thuringiensis* or other bacterial and viral biological agents.

Field testing

The BA_5345 and *adk* RPA assays were finally assessed on 13 swab samples collected and processed in field conditions under a laboratory tent. Data were compared to those obtained with a duplex qPCR for detecting *B. anthracis purA* SNPs and *B. cereus* group *ptsI* common marker (28) (Table 4). All swab samples, except the positive control, were negative with the BA_5345-RPA and the *purA* qPCR assays (Table 4). Results obtained with *adk*-RPA were identical to those generated by the *ptsI*-qPCR assay, except for sample 2 which was RPA-positive but qPCR-negative (Table 4). This latter turned out positive when tested on diluted extracted DNA.

Blind testing in the Institute of Veterinary Bacteriology, Bern, Switzerland

The three *B. anthracis* RPA-specific assays carried out on a panel of DNA and lysate samples (n=30 among which 20 *B. anthracis* and 10 *B. cereus* group members) were provided by the Institute of Veterinary Bacteriology and assessed in this facility. RPA results were 100% concordant with previous strain characterizations (Table 5). *B. anthracis*-specific RPA tests remained negative for all *B. cereus* DNA samples, which were efficiently amplified at equivalent concentration by a PCR of *panC* gene carried out in parallel to RPA testing.

Discussion

In the event of a natural, accidental or deliberate dispersal of *B. anthracis* spores, there is a crucial need for fast and reliable screening and detection of contaminated patients, animals, environment, or surfaces to immediately initiate appropriate countermeasures (29, 30). Overwhelming data published so far in this specific research area point out the lack of methods readily usable by first responders for on-site detection of *B. anthracis* spores, and the need for assays based on amplification of sequences from pXO1 and pXO2 virulence plasmids combined with specific chromosomal markers (9, 10). In the current study, isothermal amplification and detection were carried out on a set of DNA targets including pXO1 (*lef)* and pXO2 (*capA)* plasmid virulence targets combined with BA_5345, which is one of the most stable and specific chromosomal markers identified (31, 32). It is noteworthy that a panel of ten RPA assays was previously tested for detecting biothreat agents including *B. anthracis* (19). However, the focus was rather set on assay performance while *B. anthracis* detection was restricted to both virulence plasmids. The aim of the current study was to design a set of field-portable RPA assays usable in rapid diagnostic test (RDT), point of care testing (POCT) or point-of-need (PAN). This multi-target approach allowed for fast and

specific screening of samples with the detection of *B. anthracis* and its discrimination from closely related species belonging to the *Bacillus cereus sensu lato* group.

The current RPA assays were as sensitive as conventional qPCR with a LOD for each target gene being as low as 10 genome-equivalent copies. In terms of sensitivity and specificity, the current assays correctly identified all *B. anthracis* (11/11) among a panel of 60 reference DNAs including *B. anthracis* closely related species with no cross detection. Regarding the latter, it is of note that this panel included two *B. anthracis* phylogenetically-very close neighbors that we recently isolated from a soil sample in Namibia and that was further characterized by whole genome sequencing (manuscript in process). Moreover, the current assays correctly identified soil and powder samples containing *B. anthracis* spores. Finally, swab samples contaminated with *B. thuringiensis* used as *B. anthracis* simulant were rapidly and correctly identified in field conditions using a previous version of these RPA assays based only on BA_5345 and *adk* DNA targets.

Interestingly enough, as requested for a first screening and detection test, this RPA reaction was completed in less than 4 to 7 min depending on the target. This is significantly faster than conventional qPCR method with fluorescent probes which has a hands-off turnaround time of ~1.5 h. It is also faster than other isothermal amplification methods, e.g. LAMP which requires <45 min for signal build up (15, 19, 33). Moreover, RPA assays can be used on a small (74 x 178 x 188 mm), light (weight: 1 kg) and user-friendly portable device. Moreover, RPA kits are now commercially available in lyophilized format. Altogether, these assay characteristics make it particularly suitable and appropriate to in-field screening of suspicious materials like “white powders” and soil samples in suspected areas and for the detection of *B. anthracis* contaminated samples or surfaces. For these purposes, current assays may well be carried out in a van equipped with a glove box for sample inactivation and DNA extraction, as well as a dedicated space for performing analytical work and data processing. We believe that

such a lean organization based on a ultra-light deployable laboratory with simple, fast and robust screening tests would enable first responders to rapidly complete a first assessment, hence to save precious time and resources in case of "white powder" hoaxes. Recent reports highlighted the feasibility of this RPA-based RDT or POCT concept for detecting either *leishmania donovani* using a suitcase laboratory (13), Ebola in Guinea (14), Dengue (15), Chikungunya (11) or enteric viruses (12). Further work is now required to validate the current set of RPA assays on a broader panel of soil and powder samples contaminated both with *B. anthracis* spores and with closely related species, and to validate these assays during on-site operations in the framework of international exercises.

Initial *adk*-RPA tests carried out with earlier RPA-exo kit batches appeared to be very specific including when performed on-site during the exercise in Pionki, as reported in this work. Results showed indeed unambiguously that *pst1*-qPCR and *adk*-RPA assay carried out in field conditions provided similar results as qPCR, except for sample 2, which positivity was only detected by RPA. The latter result underscored the occurrence of a PCR inhibition leading to a false-negative result of the *pst1*-qPCR for this particular sample. RPA result obtained with the latter sample indicates that this isothermal amplification assay was more robust and less prone to matrix induced inhibition effects than qPCR (34). The potential of *adk* target gene to discriminate *B. cereus* group strains from bacteria of other genera including *Bacillus spp.* strains was also confirmed with a SYBR Green qPCR using the same primers as for the *adk*-RPA assay (Table 2). However, despite these very promising initial RPA results, artefactual amplification in NTC samples hampered further experiments.

In conclusion, three *B. anthracis*-specific RPA assays were developed for rapid and reliable screening of environmental samples by first responders in field conditions on samples commonly suspected to be contaminated by *B. anthracis* spores, i.e. suspicious white powders and soils.

Materials and methods

Bacterial strains and environmental samples. A first panel of 60 reference bacterial strains was used in this study comprising purified genomic DNA from 11 *B. anthracis*, 30 *B. cereus sensu lato* group, 9 *Bacillus* spp., as well as 10 Gram-positive and Gram-negative strains of other genera and human DNA. These purified genomic DNAs were obtained from the Centre d'Etudes du Bouchet (Vert-le-petit, France), the Bundeswher (Germany) and the collection of the Microbiology Unit (Université Catholique de Louvain, Belgium). The latter institution also kindly provided us with 22 additional strains of the *Bacillus cereus sensu lato* group and 8 *Bacillus* spp. Genomic DNAs of this panel of strains were characterized for the presence/absence of the virulence plasmids pXO1 and pXO2 using a previously described duplex qPCR (28).

A second sample collection tested in this study consisted of 8 soil and 8 powder environmental reconstituted samples. Soil samples (2g) were spiked either with *B. anthracis* Ames strain, Vaccinia virus, *B. thuringiensis*, *Francisella tularensis* Schu4 strain, *Burkholderia pseudomallei* or a mix of *Francisella* and *Burkholderia* at a concentration varying between 2 x 10^4 and 2 x 10^7 CFU/g of soil. Powder samples included DiPel® biological insecticide, yeast, cream and baking soda were either spiked or not with *B. anthracis* Sterne strain. Prior to spiking carried out at the US Army Dugway Proving Ground facility, all biological agents were inactivated with cobalt gamma-irradiation. These environmental samples were obtained in the frame of round robin SIBA (Sample Identification of Biological Agents) exercises organized on February 2006 (soils samples) and of January 2009 (powder samples) by the BioTesting & Production, Army Test & Evaluation Center, Developmental Test Command, West Desert Test Center, Life Science Test Facility, Dugway Proving Ground, UT 84022, USA for testing within the NATO Army

264 Armaments Group Joint Capability Group on CBRN Defence Group on *Sampling and*
265 *Identification of Chemical, Biological, and Radiological Agent* (SIBCRA – SG).

266 Another set of samples consisted of 13 swabs collected and analyzed on-site during the
267 PIONEX exercise organized in the framework of EC-FP7-PRACTICE project [European
268 Commission 7th framework programme, Preparedness and Resilience Against CBRN
269 Terrorism using Integrated Concepts and Equipment], from 22-25 April 2014, Pionki
270 (Warsaw), Poland (https://www.practice-fp7-security.eu/cms,article,82,final-full-scale-civil-
271 protection-cbrn-field-exercise-%7C-23-2....html) (35). One of the objectives of the exercise
272 was to test and validate rapid diagnostic tests usable in field conditions. In that respect, an
273 inflatable tent laboratory was deployed close to the scene to carry out DNA-based microbial
274 identification using qPCR and RPA (Figure 1, Pictures A-F). Two scenarios were executed by
275 Polish State Firemen CBRN sampling team: (a) swabbing barrels found with suspicious
276 substances in a nearby forest, and (b) swabbing surfaces contaminated by white powders after
277 intentional release inside a city building. After collection on site, samples were quickly
278 delivered to the laboratory. In the first scenario, swab samples (Table 4: samples n°1 to 3)
279 were processed for testing the presence of five different pathogens including the *B. anthracis*
280 simulant. In the second scenario, swab samples (Table 4, samples 4-13) were processed for
281 testing the presence of *B. anthracis*. In both scenarios, spores of *B. thuringiensis* ssp. *aizawai*,
282 strain ABTS-1857 (Xentari biological insecticide, Valent Biosciences Corporation, CA, USA)
283 were used as a *B. anthracis* simulant. Simulant and guidelines for contamination of both
284 scenes were provided by the CTMA/UCL laboratory staff and samples anonymized by the
285 PIONEX exercise controller.

286 The last validation of the RPA assays was carried out in the Department of Infectious
287 Diseases and Pathobiology, Institute of Veterinary Bacteriology, Bern, Switzerland where all
288 RPA reagents were shipped before hosting two scientists from CTMA (MB and JLG) for

carrying out the RPA assays. DNA samples from the Institute of Veterinary Bacteriology collection were prepared for blind testing by a local staff member (PP). DNA samples comprised *B. anthracis* (n= 20) and *B. cereus* (n= 10) group members (36-38). Whereas *B. anthracis* were previously characterized for the *B. anthracis* markers Sap, cap/pXO2 and pag/pXO1 (39). *B. cereus* DNA were assessed in parallel by the local scientist (PP) using the end-point PCR targeting the *panC* gene (40).

DNA extraction. All strains were cultivated in the CTMA laboratory except for *B. anthracis;* cultivated strains were extracted using the fully automated system EZ1 (Qiagen, The Netherlands). DNA from powder samples and swabs were extracted using the NucliSens miniMag semi-automated apparatus (Biomérieux Inc., Boxtel, The Netherlands). DNA from spiked environmental soil samples was extracted using the PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA). Extraction was carried out according to the manufacturer's instructions and the resulting DNA was eluted in Tris-HCl buffer (pH 8) and stored at -20°C. The DNA extractions performed at the Institute of Veterinary Bacteriology in Bern were performed with the DNeasy blood & tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Lysates were carried out as previously described (38).

RPA assays design. Three RPA assays were designed to detect and identify *B. anthracis* with a high specificity so as to distinguish it from closely related *B. cereus* pathogenic and non-pathogenic strains. These assays targeted the specific *B. anthracis* genomic marker BA_5345 (GenBank CP009981), together with the *lef* (GenBank CP009980) and *capA* (GenBank CP009979) genes located respectively on pXO1 and pXO2 virulence plasmids. A fourth assay targeted a highly conserved region of *adk* gene (GenBank CP009981), which is common to *B. cereus* group strains as deduced from a multiple sequence alignment performed on Genbank available *adk* sequences (41, 42). The sequence and localization of primers and probes used

for each RPA assay and the expected size of RPA amplicons are depicted in Table 1. Positions were numbered according to the reference genomic sequence of *B. anthracis* strain Ames_BA1004 (Table 1). The length of RPA primers and probes was set to 30-35 and ~45 nucleotides, respectively. Each probe was designed by introducing a THF abasic nucleotide analogue (tetrahydrofuran) at position 30 flanked by dT-FAM fluorophore (5-Carboxyfluorescein) and dT-BHQ1 quencher (black hole quencher) at position 28 and 32, respectively (Table 1). RPA primers and probes were designed based on rules and guidelines provided by the manufacturer, and purchased from Eurogentec (Liège, Belgium). The specificity of the primers and probes against their specific targets was studied in silico using Blast search tool as well as the Biostrings R package. The latter was used to identify a perfect match with whole- genome sequence of *B. anthracis* and closely related species (*B. cereus*, *B thuringensis*, *B. mycoides*, *B. cytotoxicus* and *B. weihenstephanensis*) which are available on genbank (n total = 171).

RPA assays conditions optimization. Initial optimization was carried out using the TwistAmp® Basic kit (TwistDx Limited, Cambridge, United Kingdom). A gel electrophoresis analysis was carried out after amplification with tested primers in order to improve the yield of amplification through selection of optimal target sequences. The final set of primers was selected based on the highest amplification efficiency and negative or lowest NTC value. After the selection of optimal target regions, a second phase of assay optimization was carried out using the real-time fluorescence DNA amplification TwistAmp® exo kit from the same manufacturer. The RPA reaction was performed in a final volume of 50 µL containing 420 nM each RPA primer, 120 nM RPA probe except for *lef*-probe that was 60 nM, 14 mM Mg-acetate and 1x rehydration buffer. All reagents except tested DNA sample and Mg-acetate were prepared in master mix, which was distributed into 0.2 ml reaction tube containing a dried enzyme pellet. A volume of 5 µL of sample DNA (5 pg) or control

standard was added to the tube. Next, Mg-acetate was pipetted into the tube lids. The lids were closed, the Mg-acetate was centrifuged into the tubes using a minispin centrifuge, and the tubes were immediately placed into a Twista portable real-time fluorometer (TwistDx, Cambridge, UK). Fluorescence measurements in the FAM channel were carried out at 42°C every 20 s for 20 min. To distinguish positive from negative results, a cutoff value was calculated for every individual sample according to the guidelines on threshold validation in the Twista Studio software manual. A sample was deemed positive if all replicates were above three standard deviations (3 SD) of the background during a defined time range (i.e., after 19 to 20 min of amplification). In addition, the slope validation was also carried out according to the guidelines.

Real-time PCR. Two duplex qPCRs described previously were used to identify *B. anthracis* and to distinguish *B. cereus* group strains from other species (28). The first qPCR was based on *purA* and *ptsI* targets while the second allowed detection of the *B. anthracis* virulence plasmids pXO1 and pXO2. A SYBR®-Green qPCR was developed to detect the *adk* target gene. This qPCR assay was carried out in 25 µL reaction volume containing 12.5 µL of 2X power SYBR Green master mix (Life technologies, Carlsbad, California, USA), 165 nM each primer and 2,5 µL of tested genomic DNA. The reaction was initiated at 50°C for 2 min, and 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 63°C for 1 min and extension for 90 seconds at 72°C. Each sample was tested in triplicate and data were recorded as Cycle threshold (Ct) on a Biorad CFX96 Detection System (Bio-Rad Laboratories N.V., Temse, Belgium) using the analytical software from the same manufacture.

Plasmid DNA and calibration curves. The *lef*, *capA*, BA_5345 and *adk* genes were amplified by PCR using specific pair of primers (Table 1) and genomic DNA from *B. anthracis* 9774 strain. Primers were designed using Primer 3 algorithm (43). Each reaction

PCR mixture (50 µl) contained 1 ng of genomic DNA, 250 nM each primer, 250 µM each desoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.7 U/reaction ampliTaq polymerase and 1X ampliTaq buffer II (Roche Molecular Systems, New Jersey, USA). The thermal amplification profile consisted of a denaturation step at 94°C for 3 min followed by 35 cycles thermal cycling at 94°C for 30 sec, 55 °C for 30 sec, and 72°C for 45 sec. After cycling, reaction tubes were further maintained at 72°C for 7 min. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into pCR®4-TOPO® and transformed in One Shot® TOP10 Electrocompetent *E. coli* bacterial cells using TOPO TA Cloning® Kit for Sequencing (Life technologies, Carlsbad, CA, USA). Resulting recombinant plasmids were purified using Invisorb® Spin Plasmid Mini Two (Invitek, Berlin, Germany) and verified by sequencing with a BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA), using an automated sequencer (3130 Genetic Analyser, Applied Biosystems). The BA_5345, *lef* and *capA* recombinant plasmids were quantified by UV with a NanoDrop® ND-1000 v3.5.2 spectrophotometer (Isogen Life Science, The Netherlands). Calibration curves were created from 10-fold diluted recombinant plasmids with a range of 10^6 down to 10^0 copies per reaction as calculated from plasmid concentration (OD 260 nm). The BA_5345, *lef* and *capA* gene plasmid standards were tested in five replicates, the threshold time in minutes was plotted against the number of molecules detected.

Statistical analysis of sensitivity. The LOD was determined using a probit-analysis. For each assay, a probit regression model was fitted with the "glm" function of the R statistical software (http://www.r-project.org/) using the target concentration as the independent variable and the detection event as the response variable. Each probit regression model was then used to determine the lowest concentration enabling target detection with a 95% probability (i.e. the LOD).

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401 **Author contributions**

402 Conceived and designed the experiments: MB. Performed the experiments and analyzed the
403 data: CD, MB, PP and JLG. Carried out the statistical analysis and manuscript revision: JA.
404 Wrote the paper: MB and JLG.

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538 **Figure legend**

539 Figure 1. Main steps carried out in a laboratory tent in field for samples processing from
540 receipt to the final analyses. Laboratory tent as deployed in the field (A). Samples were
541 brought to the laboratory by the Polish State Firemen CBRN sampling team (B). Samples
542 were processed in a glovebox to inactivate potential bio-agents present in the sample (C and
543 D) and nucleic acids were then extracted outside the glovebox (E). qPCR and RPA assays
544 were carried out and data analyzed (F).

545 Figure 2. The RPA assay optimization was achieved using TwistAmp basic amplification kit
546 as illustrated for the *lef* target gene. The upper part (A) shows the relative position of RPA
547 pair of primers (set-1 to 3) used in screening as well as the part of the target where the *lef*
548 probe was designed. The lower part (B) shows the results of gel electrophoresis analysis
549 following target amplification with the indicated set of primers. Samples 1 and 2 correspond
550 to NTC prepared in pre- and post-PCR rooms while sample 3 contained the target DNA.

551 Figure 3. RPA Amplification plots. Amplification carried out on 10 fold serial dilution
552 standards including NTC samples of BA_5345 (A), *lef* (B) and *capA* (C) target sequences.

553 Figure 4. Analytical sensitivity and quantitative range of BA_5345, *lef* and *capA* RPA assays.

554 Limit of detection (LOD) as determined by probit regression analysis (A) and assays linearity

555 range (B).

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556 Tables

557 Table 1: primers and probes used in this study

Type	Target	Name	Sequence (5'-3')	Start	End	Size (base)	amplicon size
PCR-primers	*lef*	lef-For	CGCTTCATTTGTTCTCCCATAC	139956	139935	22	860
		lef-Rev	CAACCCTAGGTGCGGATTTAG	139097	139117	21	
	capA	capA-For	GGTACAACGTACAGAAGCAGT	18512	18532	21	971
		capA-Rev	GAGCACCCTTGGATGTATCTTT	19482	19461	22	
	BA_5345	BA-For	CGATTTTGTGGATTGCGTATG	4873856	4873876	21	493
		BA-Rev	ACCGCAAGTTGAATAGCAAG	4874348	4874329	20	
	adk	adk-For	CCGAACAGATTGTTGCCAAG	4360272	4360253	20	600
		adk-Rev	ACGCTAAGCCTCCGATGAGA	4359673	4359692	20	
RPA-primers	*lef*	lef-58-For	TTAGAATTTGTAACTAAATCAGATTGGTTCT	139889	139859	31	146
		lef-60-Rev	CGTTCTATATTACTCCATGGACCTTCAAA	139744	139772	29	
	capA	capA-45-For	CGGATTATGGTGCTAAGGGAACTAAAGATAC	18838	18868	31	145
		CapA-63-Rev	CCAAGAGTAGCAACCCTAACACCATTTAC	18982	18954	29	
	BA_5345	BA-31-For	GTCTGGCACATGGTACTACTCAAACAAGAT	4874134	4874163	30	105
		BA-36-Rev	GAACAATGACCCTAGTGCATGTGTAGTTCC	4874238	4874209	30	
	adk	adk-27-For	GTGTGCGATAAATGTGGTGGCGAATTATATCAAC	4359877	4359844	34	137
		adk-28-Rev	CTTTGTAGGTAACCAAGCTCCTCGTAGAAATCAAG	4359741	4359775	35	
Probes	*lef*	lef-exo-55	AATTTGTAACTAAATCAGATTGGTTCTTATCTAATAGATATCCAG	139885	139841	45	
	capA	capA-exo-56	AAGGCCTTTAAAGAAGCTGATCTTGACTATGTGGGTGCTGGTGAA	18873	18917	45	
	BA_5345	BA-exo-54	CTCAAACAAGATTCAGAGACTCGTACATACATAGAAGGACGATAC	4874152	4874196	45	
	adk	adk-exo-53	TGATGACAATGAAGAAACTGTAGCAAATCGCTTAGATGTAAATATTA	4359839	4359793	47	

558

559 Table 2: Bacterial strains used for testing RPA assays specificity and sensitivity

[a]Name	Original identification	Source	RPA results (detection time in min)			qPCR results	
			BA_5345	*lef* (pXO1)	*capA* (pXO2)	*adk*	pXO1/pXO2
B. anthracis	9508	CEB	4,57 ± 0,23	6,80 ± 0,17	4,30 ± 0,00	+	+/+
B. anthracis	9531	CEB	4,20 ± 0,17	-	-	+	-/-
B. anthracis	9774	CEB	4,53 ± 0,40	6,23 ± 0,40	4,77 ± 0,40	+	+/+
B. anthracis	9506	CEB	4,10 ± 0,17	6,10 ± 0,17	4,67 ± 0,35	+	+/+
B. anthracis	9534	CEB	4,10 ± 0,17	-	4,67 ± 0,35	+	-/+
B. anthracis	9439	CEB	4,20 ± 0,17	6,53 ± 0,40	-	+	+/-
B. anthracis	9602	CEB	4,43 ± 0,51	6,43 ± 0,23	4,43 ± 0,23	+	+/+
B. anthracis	9440	CEB	4,30 ± 0,00	8,20 ± 0,17	4,57 ± 0,23	+	+/+
B. anthracis	VAR06/7570.4CAP	UCL	4,57 ± 0,23	7,00 ± 0,30	3,57 ± 0,23	+	+/+
B. anthracis	VAR06/1106.3#2	UCL	4,57 ± 0,23	6,90 ± 0,17	3,43 ± 0,23	+	+/+
B. anthracis	VAR06/5348.3#1	UCL	4,70 ± 0,00	7,00 ± 0,00	3,67 ± 0,35	+	+/+
B. cereus	ATCC 14579	UCL	-	-	-	+	-/-
B. cereus	ATCC 10987	UCL	-	-	-	+	-/-
B. cereus	DSM 2302	UCL	-	-	-	+	-/-
B. cereus	DSM345	BW	-	-	-	+	-/-
B. cereus	S3A	CTMA	-	-	-	+	-/-
B. cereus	ATCC 13061 (HT1-A1)	UCL	-	-	-	+	-/-
B. cereus	ATCC 10876 (HT1-A2)	UCL	-	-	-	+	-/-
B. cereus	ATCC 21282 (HT1-C1)	UCL	-	-	-	+	-/-
B. mycoides	MYC005	UCL	-	-	-	+	-/-
B. mycoides	ATCC 6463	UCL	-	-	-	+	-/-
B. mycoides	MYC003	UCL	-	-	-	+	-/-
B. mycoides	WSBC 10211	BW	-	-	-	+	-/-
B. mycoides	HBS 1-16	UCL	-	-	-	+	-/-
B. pseudomycoides	NRRL B-617	UCL	-	-	-	+	-/-
B. pseudomycoides	NRRL BD5	UCL	-	-	-	+	-/-
B. pseudomycoides	NRRL NRS 321N	UCL	-	-	-	+	-/-
B. pseudomycoides	6.A.3	CTMA	-	-	-	+	-/-
B. weihenstephanensis	WSBC 28005	BW	-	-	-	+	-/-
B. weihenstephanensis	WSBC 10204	UCL	-	-	-	+	-/-
B. weihenstephanensis	WSBC 10278	BW	-	-	-	+	-/-
B. weihenstephanensis	WSBC10207	UCL	-	-	-	+	-/-
B. weihenstephanensis	WS2481	UCL	-	-	-	+	-/-
B. weihenstephanensis	WSBC10201	UCL	-	-	-	+	-/-
B. thuringiensis	4Q2-72	UCL	-	-	-	+	-/-
B. thuringiensis	T03A016 = HD1	UCL	-	-	-	+	-/-
B. thuringiensis	WSBC 10206	BW	-	-	-	+	-/-
B. thuringiensis	ABTS-1857	CTMA	-	-	-	+	-/-
B. thuringiensis	HD73	UCL	-	-	-	+	-/-
B. thuringiensis	Bt5	UCL	-	-	-	+	-/-

B. thuringiensis	ATCC 39646	UCL	-	-	-	+	-/-
B. subtilis	ATCC 6633	UCL	-	-	-	-	-/-
B. subtilis	ATCC 12711	UCL	-	-	-	-	-/-
B. subtilis	ATCC 6051	UCL	-	-	-	-	-/-
B. subtilis	168 (Suxia)	UCL	-	-	-	-	-/-
B. subtilis	DSMZ 5934	CTMA	-	-	-	-	-/-
B. amyloliquefaciens	ATCC 23350	UCL	-	-	-	-	-/-
B. badius	ATCC 14574	UCL	-	-	-	-	-/-
B. sphaericus	ATCC 10208	UCL	-	-	-	-	-/-
B. atrophaeus	ATCC 9372	CTMA	-	-	-	-	-/-
Streptococcus pyogenes	ATCC 12344	CTMA	-	-	-	-	-/-
Enterococcus faecalis	ATCC 29212	CTMA	-	-	-	-	-/-
Stenotrophomonas maltophilia	ATCC 13637	CTMA	-	-	-	-	-/-
Escherichia coli	DSMZ 8579	CTMA	-	-	-	-	-/-
Enterobacter aerogenes	DSMZ 30053	CTMA	-	-	-	-	-/-
Serratia liquefasciens	ATCC 27592	CTMA	-	-	-	-	-/-
Staphylococcus aureus	NCTC 10442	CTMA	-	-	-	-	-/-
Streptococcus pneumoniae	DSMZ 20566	CTMA	-	-	-	-	-/-
Klebsiella pneumoniae	ATCC 13883	CTMA	-	-	-	-	-/-
Clostridium difficile	DSMZ 1296	CTMA	-	-	-	-	-/-

560 *BW* Bundeswehr (Germany), *CEB* Centre d'Etude du Bouchet (France), *UCL* Université Catholique de Louvain (Belgium), *CTMA* Centre de

561 Technologies Moléculaires Appliquées (Belgium). – not detected, + detected. [a]After extraction and measurement of DNA concentration,5 pg

562 were used in each RPA reaction and samples with negative result were amplified in parallel using a 16S rRNA PCR.

563

564 Table 3 : Environmental powder and soil samples (NATO SIBCRA exercise)

[a]Sample	matrix	spiking status	Concentration	RPA results		
				BA_5345	*lef* (pXO1)	*capA* (pXO2
[b]01	Dipel powder	*B. thuringiensis*	ND	-	-	-
02	Dipel powder	*B. thuringiensis* + *B. anthracis* Sterne	ND	+	+	-
[b]03	Backing soda powder	Nihil	ND	-	-	-
04	Backing soda powder	*B. anthracis* Sterne	ND	+	+	-
[b]05	Yeast powder	Nihil	ND	-	-	-
06	Yeast powder	*B. anthracis* Sterne	ND	+	+	-
[b]07	Cream powder	Nihil	ND	-	-	-
08	Cream powder	*B. anthracis* Sterne	ND	+	+	-
09	Soil	*B. anthracis*	($2X10^4$ cfu/g)	+	+	+
10	Soil	*B. anthracis*	($2X10^5$ cfu/g)	+	+	+
11	Soil	*B. anthracis*	($2X10^7$ cfu/g)	+	+	+
12	Soil	*B. thuringiensis*	10 mg/g	-	-	-
13	Soil	*Vaccinia virus*	$2X10^7$ cfu/g	-	-	-
14	Soil	*F. tularensis*	$2X10^7$ cfu/g	-	-	-
15	Soil	*B. pseudomallei*	$2X10^7$ cfu/g	-	-	-
16	Soil	*Mixed F. tularensis* + *B. pseudomallei*	$2X10^6$ cfu/g each	-	-	-

565 [a]Nucleic acid extract from these samples were investigated in the frame of SIBCRA exercises. Laboratory results were positive for all agents

566 other than *B. anthracis* (i.e. vaccinia virus, *F. tularensis*, *B. pseudomallei*, and mixed *F. tularensis* and *B. pseudomallei*) , [b]To confirm that

567 DNA in the extract is amplifiable, samples were assessed using the 16S rRNA PCR. – not detected, + detected

568

569 Table 4: Swab samples tested in the field (PIONEX exercise, Pionki, Poland, project FP7

570 PRACTICE)

Sample	Type	Scenario	RPA results		qPCR results	
			BA_5345	*adk*	*purA*	*pts*I
control 01	NTC	Nihil	-	-	-	-
control 02	Extraction	Nihil	-	-	-	-
control 03	BA 9602	Nihil	+	+	+	+
01	Swab	1	-	+	-	+
02	Swab	1	-	+	-	-
[a]03	Swab	1	-	-	-	-
[a]04	Swab	2	-	-	-	-
05	Swab	2	-	+	-	+
06	Swab	2	-	+	-	+
07	Swab	2	-	+	-	+
08	Swab	2	-	+	-	+
09	Swab	2	-	+	-	+
10	Swab	2	-	+	-	+
11	Swab	2	-	+	-	+
12	Swab	2	-	+	-	+
13	Swab	2	-	+	-	+

571 [a]Swab extracted samples were positive when assessed using the 16S rRNA PCR. – not detected, + detected

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582 Table 5: Blind testing at the Institute of Veterinary Bacteriology, Bern, Switzerland

Strain	Sample type	[a]Species	RPA			
			BA_5345	*lef*(pXO1)	*capA*(pXO2)	Identification
JF3788	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3786	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3852	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3787	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3785	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3784	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3853	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3854	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3783	DNA extract	*B. anthracis*	+	+	+	*B. anthracis*
JF3960	lysate	*B. anthracis*	+	+	+	*B. anthracis*
JF3965	lysate	*B. anthracis*	+	+	+	*B. anthracis*
JF3966	lysate	*B. anthracis*	+	+	+	*B. anthracis*
JF3963	lysate	*B. anthracis*	+	+	+	*B. anthracis*
JF3961	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A1	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A3	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A5	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A7	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A10	lysate	*B. anthracis*	+	+	+	*B. anthracis*
Chad A12	lysate	*B. anthracis*	+	+	+	*B. anthracis*
[b] JF4875	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF5512	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF4075	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] M2841	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF5881	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF3778	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF1887	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] M2089	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF4090	DNA extract	*B. cereus*	-	-	-	*B. cereus*
[b] JF4059	DNA extract	*B. cereus*	-	-	-	*B. cereus*

583 [a]Genomic DNA characterized by realtime PCR Taqman assays targeting *sap* chromosomal, *cap*-pXO2 and *pag*-pXO1genes, [b]End-point PCR

584 of the *panC* gene confirmed the presence of amplifiable DNA in all negative samples (*B. cereus* group). – not detected, + detected

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