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How safe is your DNA extract?

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Abstract

Three of four extraction methods yielding high quality DNA from blood failed to remove all live *Bacillus anthracis* from the extraction arm of a molecular assay that provided a partial molecular fingerprint of endemic *B. anthracis* in Israel and distinguished *B. anthracis* from closely related gram-positive bacteria.

Key words: *Bacillus anthracis; DNA extraction; biosafety, sequence analysis*

Introduction

Anthrax is an infectious disease caused by the non-motile, gram-positive, spore forming bacterium, *Bacillus anthracis.* Three forms of infection occur depending on the route of infection; cutaneous (skin), inhalation (lungs) and gastrointestinal. Edema toxin, lethal toxin, protective antigen, and capsular antigen are the virulence factors associated with *B. anthracis* pathogenesis. These factors are encoded on two plasmids, pX01 [1] and pX02 [2, 3]. The pX01 plasmid (185 kb) encodes the protective antigen, *pag* [4], the lethal factor, *lef* [5], and the edema factor, *cyc* [6], while the pX02 plasmid (95 kb) encodes three genes required for capsule formation, *Cap A*, *Cap B*, and *Cap C* [7]. Both plasmids must be present for *B. anthracis* to be pathogenic. Non-pathogenic, live *B. anthracis* agricultural vaccines have been produced from *B. anthracis* strains that lack either the plasmid pX01 (Pasteur vaccine strains) or pX02 (Sterne vaccine strains).

B. anthracis produces very stable spores when growth conditions become less than optimal. These spores remain viable in the soil for years and can infect domestic and wild animal. Humans can become infected with anthrax accidentally after coming in contact with the spores, by handling products from infected animals, by inhaling anthrax spores from contaminated animal products, and by eating undercooked meat from infected animals. Exposure can also be deliberate by acts of war or bioterrorism.

In any suspected anthrax outbreak (infection of one or more organism in an anthrax free region) it is important to know within a clinically relevant time whether pathogenic *B. anthracis* is actually present and in which organisms. A rapid molecular identification technique involves extracting DNA and characterizing it after PCR amplification using published *B. anthracis* specific primers validated for natural and weaponized anthrax and using commercially available extraction systems. The first requirement when establishing such an identification protocol is to determine whether the extracted DNA needs to be treated as a potential biological hazard (e.g., still contained infectious bacteria or spores) or just as a biochemical hazard (e.g., non-infectious DNA that might produce a false positive if reaction mixtures became contaminated).

Methods

Extraction of DNA from clinical samples spiked with *B. anthracis*

Bacteremia was mimicked by spiking fresh human blood from blood count Vacutainer® (Becton, Dickinson and Company, USA) tubes with bacteria from overnight liquid broth cultures of seven Israeli veterinary bovine isolates of *B. anthracis* isolated between 1980 and1990, obtained from the Clinical Bacteriology Laboratory, The Kimron Veterinary Institute, Israel, from seven non-*B. anthracis*, gram-positive clinical bacterial isolates obtained from the Bacteriology Laboratory, Sheba Medical Center, Tel Hashomer, Israel, and from Pasteur and Stern *B. anthracis* vaccine strains. PCR-quality DNA was prepared using four different procedures: DNA extraction using GeneReleaser (Bio Ventures, Incorporated, Murfreesboro, TN, USA), High Pure DNA Extraction Kits (Roche Diagnostics, Mannheim, Germany), and DNA Easy Tissue Kits (QIAGEN GMBH, Hilden, Germany), or by pre-heating aliquots of spiked blood at 95°C for 15 minutes before adding the PCR reaction mix.

Biosefety of DNA extracts

Aliquots of DNA from each procedure were shaken overnight in broth at 37C to determine whether they still contained any viable *B. anthracis.*

Molecular identification of *B. anthracis* genomic and plasmid DNA

The genomic and plasmid primers used in this study for PCR amplification, listed in Table 1, were chosen for the reasons outlined below.

Table 1. PCR primers used to amplify *Bacillis anthracis* genomic and plasmid DNAs.

Primer name	Primer sequence
Genomic: vrrA [Ref (8, 9)]	
GPR1	5'-CGT AGT TCA CGA ACT GCA TCT-3'
GPR2	5'-ATG ATG TAT CTA ATG CGG CGT-3'
EWA1	5'-TAT GGT TGG TAT TGC TG-3'
EWA2	5'-ATG GTT CCG CCT TAT CG-3'
GPR4	5'-ACA ACT ACC ACC GAT GGC-3'
GPR5	5'-TTA TTT ATC ATA TTA GTT GGA TTC g-3'
Genomic: BA813 [Ref (11, 15, 14)]	
Ba813 R1	5'-TTA ATT CAC TTG CAA CTG ATG GG-3'
Ba813 R2	5'-AAC GAT AGC TCC TAC ATT TGG AG-3'
Plasmid X01: *pag* [Ref (11, 15, 14)]	
pag67	5'-CAG AAT CAA GTT CCC AGG GG-3'
pag68	5-'TCG GAT AAG CTG CCA CAA GG-3'
Pag23	5'-CTA CAG GGG ATT TAT CTA TTC C-3'
Pag24	5'-ATT GTT ACA TGA TTA TCA GCG G-3'
Plasmid X02: *Cap A* [Ref (8)]	
CapA-F	5'-CAG AAG CAG TAG CAC CAG TAA-3'
CapA-R	5'-ATT TTC ACC AGC ACC CAC-3'
CapA-Fnes	5'-TGA CGA TGG TTG GTG ACA-3'
CapA-Rnes	5'-CCT TAT TGT ATC TTT AGT TCC C-3'

B. anthracis Genomic DNA

The 1110 nt vrrA template defined by primer pair GPR1 / GPR2 was chosen for the genomic template since it was reported to contain two to six copies of a variable number tandem repeat (VNTR) of 5'caatatcaacaa-3' and primers recognizing this template had been shown to distinguished *B. anthracis* from closely related gram positive bacteria such as *Bacillu*s *cereus, B. thuringiensis* and *B. mycoides* [8, 9]. A further advantage is that since the copy number is conserved in progeny [9], the VNTR vrrA copy number would provide a partial *B. anthracis* fingerprint. A full molecular fingerprint of any *B. anthracis* isolate would require a series of PCR reactions targeting this vrrA template and 5 additional genomic and 2 plasmid VNTR sites [10]. While these additional reactions might help distinguish endemic strains from introduced strains, they are not necessary for rapid primary identification of *B. anthracis* infections. Two internal primer pairs were chosen. Depending on VNTR copy number, the GPR4 / GPR5 primer pair amplifies a 378 to 426 nt sub-fragment of vrrA, while the EWA1 / EWA2 pair amplifies a 142 to 190 nt sub-fragment within the GPR4 / GPR5 template. The advantage of using the GPR4/ GPR5 primer pair stems from the fact that it had been validated for weaponized anthrax in an outbreak in the USSR [8], whereas it is easier to distinguish VNTR copy number by gel electrophoresis with the shorter EWA1 / EWA2 pair. Results were compared with the BA813R1 BA3R2 primer pair that amplified another genomic template BA813.

B. anthracis Plasmid DNA

One genomic template from each plasmid was chosen since pathogenicity required the presence of both plasmids. Specifically, *pag* and *Cap A* were chosen to represent the pX01 and pX02 plasmids, respectively, from among published PCR and nested PCR procedures for identifying *pag, lef, cyc*, and *Cap A* genes [11–14] since the primers for *pag* had been validated for many diverse strains including suspected weapon-modified organisms and a large database of sequence information existed for comparative molecular epidemiology of both [13, 7].

Preparation of positive control DNA for PCR

PCR amplification products from genomic DNA, *Cap A*, and *pag* from a field isolate of *B. anthracis* amplified using GPR-F / GPR-R , CAP-R / CAP F, and PAG67 / PAG 68 primer pairs, respectively, were cloned in pGEM-T-easy plasmids (Promega, Madison WI, USA) and transfected into JM109 competent bacteria (Promega, Madison WI, USA) according to manufacturers instructions. Plasmid DNA purified using Wizard Plus SV Minipreps DNA Purification System. (Promega, Madison, WI) and overnight cultures of transfected bacteria served as positive controls for all PCR reactions. The expected sizes were 377–425 nt, 397 nt, and 747 nt, respectively.

PCR Amplification

Two different PCR reactions were chosen, one based on a single tube Ready-to-go PCR bead assay (GE Healthcare Amersham Biosciences, Piscataway, NJ, USA) where all reagents except primers are stored at room temperature and the other using a commercial combination of Taq polymerases, in this study AmpliTaq Gold (Applied Biosystems by Life Technologies, Foster City, CA, USA), and optimized five-fold concentrated Taq reaction buffer chosen from among buffers A to H from a PCR Optimizer Kit (Invitrogen Ltd, Paisley, UK). The optimal buffers for PCR for genomic DNA were buffers E and to a lesser extent B for primer pair EWA1 / EWA2, buffer B for *pag* primers, and buffers A and B for *Cap A* primers (12 - 25 pmol of each primer per reaction mix). To simplify and unify procedures, all further amplifications with AmpliTaq Gold were with 5x B buffer (300 mM Tris-HCl, 75 mM ammonium sulfate, and 10 mM magnesium chloride at pH 8.5). The following amplification conditions were used for PCR: Activation at 93°C for 10 min; 60° for 2 min; 72° for 2 min; 35 cycles of 93°C for 45 seconds, 55°C for 45 seconds, and 72°C for 90 seconds; and a final elongation at 72°C for 10 minutes. PCR products were visualized by ethidium bromide staining after gel electrophoresis on 2% agarose gels.

DNA sequencing

The consensus sequences for pag and Cap A amplification products of 701 and 359 nt, respectively, were determined for

templates amplified with external primer pairs. PCR products were purified after gel electrophoresis using QIAgen MiniElute PCR product kits (QIAgen GMBH, Hilden, Germany), and sequenced on an automatic ABI sequencer (Applied Biosystems Inc., Foster City, CA) by the Biological Services Department of the Weizmann Institute of Science, Rehovot, Israel. The Cap A, *pag* and vrrA sequences from two isolates have been deposited in the GenBank (accession numbers HQ536626 to HQ53631.

Results

Biosafety of DNA preparations

Aliquots of DNA were incubated to determine whether the biohazardous mixture of blood and *B. anthracis* had been converted into a non-viable biochemical by each of four DNA extraction procedures. Aliquots of DNA were incubated overnight in broth. No viable bacteria were recovered from DNA solutions extracted with the QIAgen DNA Easy Tissue Kit when manufacturers' instructions were followed. In contrast, viable *B. anthracis* was recovered in overnight cultures of DNA prepared from *B. anthracis*-spiked blood cultures using GeneReleaser (Bio Ventures, Incorporated , Murfreesboro, TN, USA) and High Pure DNA Extraction Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturers' recommendations or after incubation at 95°C for 15 minutes. To further reduce the chance for viable bacteria remaining in extracted DNA and to increase DNA yield from gram-positive bacteria, we used the QAIgen DNA Easy Tissue Kit for all further preparations and added a manufacturer-suggested option of a 30-minute pre-digestion with 20 mg/ml lysozyme (Sigma) as a mandatory part of the DNA preparation protocol.

All four DNA preparation procedures yielded PCR quality DNA that was amplifiable by all of the primer sets described in Table 1 in both PCR assays. BA813 genomic primer pairs were able to detect as few as 15 to 40 colony-forming units, whereas vrrA, *pag* (PAG67/PAG68) and *Cap A* (EWA1/EWA2) primer pairs required ten-fold more bacteria in both PCR systems. In non-nested single reaction AmpliTaq Gold PCR, internal primer pairs were much better than external pairs when intensities of amplification products were compared. There was a lower threshold of detection when nested PCR was used for both assays, however in the Ready-to-go assay, a single PCR using internal primers gave bands only slightly less intense than those for nested PCR.

PCR of DNA from all blood samples spiked with Israeli field isolates of *B. anthracis* yielded bands of the expected sizes for vrrA, *pag* and *Cap A* for each pair of template specific primers. Those spiked with vaccine strains yielded vrrA and only the appropriate plasmid-encoded genes. Specifically *pag* template was absent for Pasteur vaccine and *Cap A* template was absent for Stern vaccine. The consensus sequences for *pag, Cap A,* and genomic DNA amplification products of 701nt, 348 nt, and 127 nt respectively, from seven Israeli veterinary *B. anthracis* strains isolated between 1980 and 1990 were determined for templates amplified with external primer pairs for *pag* and *Cap A,* and internal primers WA1 and WA2 for genomic DNA. All seven Israeli isolates had identical *pag* sequences, except for nucleotide 50 that was either a C or a T. All seven isolates had identical *Cap A* sequences. Finally, there were four perfect repeats of a 5'-CAATATCAACAA-3' VNTR in the vrrA genomic sequence as determined by electrophoresis of GPR4 / GPR5 PCR products on 2% agarose gels and by sequencing. The four perfect repeats were flanked by imperfect repeat elements 5'- CAATATCAACA**g**-3' and 5'-CAATA**c**C**cg**CAA-3' upstream and downstream of the 4 perfect repeats, i.e. the sequence was 5'- CAATATCAACA**g**__CAATATCAACAA CAATATCAACAA CAATATCAACAA CAATATCAACAA CAATA**c**C**cg**CAA-3'. Sequences for all three regions from isolates representing the two variants of *pag* are available from the GenBank nucleotide sequence database HQ536626 to HQ536630.

Discussion

We have described conditions for extraction of DNA for *Bacillus anthracis* diagnosis that can be performed in level 2 national clinical and veterinary laboratories using easily acquired commercial kits and components that can be easily transported to level 1 hospital or field hospitals in an emergency. All four DNA preparation procedures produced PCR quality DNA from spiked blood samples designed to mimic *B. anthracis* bacteremia. Both sets of *B. anthracis* genomic primers amplified the correct template in DNA from all Israeli *B. anthracis* isolates.

Diagnostic results should be provided in a clinical relevant time within the framework of practical biosafety procedures. Biosafety is always an issue when using a procedure to convert a biohazardous biological into a non-biohazardous biochemical. Preparation of PCR–quality *B. anthracis* DNA is no exception. Three of the four DNA preparation procedures evaluated left viable *B. anthracis* in the DNA solution. The addition of a pre-extraction lysozyme digestion step to further insure destruction of viable bacteria adds only 45 to 60 minutes to a PCR diagnostic procedure that can be completed within five and a half hours.

All of the four DNA extraction procedures may be used, provided that appropriate levels of personal protective equipment and environmental protective measures suitable for potential biohazards from viable *B. anthracis* are used at all times. Equipment must be decontaminated immediately after use and all biological and biochemical material must be disposed under strict isolation and decontamination procedures in less than 24 hours to prevent spore formation by any bacteria that remained viable. DNA solutions may be stored frozen, but unless specifically tested must be considered as biohazardous even when a given procedure has been repeatedly proven safe in the past. This is best illustrated by a recent PubMed notification (X-Promed-Id: 20090331.1226) from March 31, 2009 7: 22: 47 AM IDT, entitled ANTHRAX, LABORATORY EXPOSURE - FRANCE (02) that described exposure from an inadequately heat inactivated sample where "As before, a check loopfull was plated out on sheep agar for each supernatant, but because of the many hundreds of times this had been done before without anything growing [the culture had always been killed], the technician took the 6 vials of heated supernatant out of the Level 3+ lab and went to the Level 2 DNA laboratory before she had read the check plates the next day…" which in this instance were positive. Testing aliquots for viable *B. anthracis* delays results by a day and precludes moving the assay to

level 1 laboratory. When overnight broth cultures are positive, the amplified stock of *B. anthracis* must be safely disposed.

In conclusion, don't assume that your DNA extract is free from infectious pathogens; test it routinely to be sure.

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Conflicts of Interest: The authors affirm that there are no conflicts of interest.

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