PITFALLS IN THE DIAGNOSIS OF *B. ANTHRACIS* FROM ENVIRONMENTAL SAMPLES – LESSONS FROM INVESTIGATIONS OF SUSPICIOUS SPECIMENS

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INTRODUCTION

The recently reported cases of anthrax in the United States that were related to terrorist attacks, strongly support the demand for a validated procedure for the detection of *Bacillus anthracis* in environmental samples. Most procedures described in the literature are designed for use with clinical specimens and have not been validated with a broad range of environmental samples. This applies both to the procedures recommended by the CDC (Atlanta, GA, USA), as well as to most of the published monographs. In cases in which procedures for environmental samples are described, these do not imply modern diagnostic tools, or no in-test validation of the applied techniques is described. There are several reasons why protocols for clinical specimens are not applicable to environmental samples. The main causes of wrong results in cultural techniques are based on the physiological properties of bacterial spores and on effects related to microbiological ecology.

Bacterial spores, as opposed to vegetative bacteria, are under no pressure to germinate or propagate. In cases where growth conditions are suboptimal, the bacteria cannot be cultivated, even though spores are present and viable in the sample. Since most environmental samples contain substances inhibitory for germination and growth, these samples remain negative in culture. This effect is even more common in samples consisting of artificially prepared materials like meals and powders. Under laboratory cultivation conditions, *B. anthracis* is easily overgrown by the autochthonous bacterial flora of the soil and plants. Very often, detection of *B. anthracis* is hampered by overgrowth of hemolysing B. *cereus*. In the latter case *B. anthracis* will not be detected as colonies on agar media containing blood. Such conditions will inevitably lead to false negative results if a sample-specific positive control is not included. Until today there is no agar medium available which could select against the closest relatives of *B. anthracis* like *B. cereus, B. thuringiensis* and *B. mycoides*. But even more different *Bacillus spp*. like *B. megaterium* can be confused with *B. anthracis* and can therefore lead to false positive reporting.

The following procedures were developed and tested over a 10 year period and reflects our experience in detecting small amounts of spores of *B. anthracis* in samples from former tannery sites. The method has recently been adapted to the requirements of processing specimens derived from suspected terrorist attacks, which may vary heavily in composition and sporostatic properties, especially if they contain detergents. The procedure is recommended by the consulting anthrax laboratory of the veterinary diagnostic working group (AVID) in Germany and consists of a combination of cultural techniques with an in-test validated PCR.

SAMPLE PREPARATION AND CULTURAL TECHNIQUES

Samples processed in this test can vary greatly - from suspicious sheets of paper, to all kinds of powders on nearly any surface or even voluminous liquids such as drinking water. Swabs from surfaces were first placed in 3 ml of sterile water in a screw capped tube and shaken vigorously on

a Vortex® mixer. Fluid samples were filtered through membranes. If drinking water wasinvestigated, a volume of at least one liter was used. In general all sample materials were divided into two parts before processing, one defined as sample and one used as the spiked positive control. The basic procedure was as follows:

1–5 g of sample (1 ml liquid from the swab preparation or half a membrane filter, respectively) were suspended in a sufficient volume of sterile water (3 to 100 ml), depending on the composition of the materials (detergents and other growth inhibitors must be washed out) and sample size. The same was done in parallel for an artificially contaminated (spiked) control, into which 5 x 10^2 – 1 x 10^3 spores of an avirulent strain of *B. anthracis* harboring only the plasmid pXO1, not pXO2, are added (the Sterne strain 34F2 can be used for this purpose). Both samples were then heated at 65 °C – 70 °C in a water bath for 30 min to inactivate vegetative bacteria. This step can generally be omitted when powders, swabs, drinking water or similar materials are processed, but is absolutely necessary when soil or fecal samples are tested. If a larger volume is used in the first step, the sample must be centrifuged at 3000 x g for 15 min. The pellet was resuspended in 2-4 ml of deionized sterile water. If the sample contained detergents as indicated by the formation of foam, a second washing step was performed.

The first culture was done by streaking out a loopful (10 µl disposable loops) of each of the preparations (i) onto blood agar (BA) containing 5-10 % defibrinated sheep blood, (ii) onto blood trimethoprim agar (TMPA) to which 0.08 ml/l of an injectible pharmaceutical preparation had been added (consisting of 40 mg Trimethoprim, 200 mg Sulfamethoxazol, and 9 mg Benzylalkohol per ml , e.g. Veyx – Pharma GmbH, D- 34639 Schwarzenborn), and (iii) onto a chromogeneous "Cereus-Ident Agar" , CIA (Heipha GmbH, Germany). The polymyxin-lysozyme-EDTA-Ttalleous acetate agar (PLET) according to KNISELY (1966), which is recommended by the WHO (WHO, 1998) was found to add no further or new informations in comparison to the before mentioned media and therefore was not longer used in our laboratory. The remaining suspension from the original sample and the spiked sample were each transferred into 50 ml of trypton soybean broth (TSB) or heart infusion broth (HIB) and incubated over night. If membrane filters were used, these were also transferred into the nutrient medium. BA, TMPA and CIA plates were evaluated on the following day and after an additional 24 h incubation. PLET plates must be incubated for at least 48 h at 37 °C before they can be evaluated. Pure cultures were then established from suspicious colonies by subcultivation. These subcultures or re-suspended materials thereof were then processed by additional diagnostic procedures including PCR and phage analysis. The avirulent control strain must be visible on plates containing the spiked sample. To evaluate the flora grown in the 50 ml cultures about 0.1 ml of a dilution was evenly spread onto the surface of TMPA. Plates were evaluated after at least 12 h of incubation at 37 °C. Suspicious colonies from the sample were picked and pure cultures established on BA. Colonies of the avirulent control strain must be identifyable on the plates where the spiked sample was processed. If the spike could not be reisolated, the sample was considered not judgeable by cultural methods and was further investigated by a combination of PCR analysis and inoculation of mice.

Parallel to culturing described above, a diagnostic PCR was carried out with DNA prepared from the 50 ml original sample and the spiked sample after incubation. Details are given below.

PCR DIAGNOSIS OF *B. ANTHRACIS*

Polymerase chain reaction (PCR) offers an excellent accompanying diagnostic method following nonselective enrichment, as well as to confirm suspicious colonies. It is important to note that a negative PCR result is conclusive, if all necessary controls are carried out. A positive result,

however, must always be verified by isolation of the bacteria causing the PCR signal. This is inevitable because none of the known PCR targets can be relied on to be exclusively specific for *B. anthracis*.

The use of PCR as a diagnostic tool is necessary if (i) there is no growth of the control strain added to the sample material (spike) and plated directly onto appropriate agar plates, due to inhibition of germination or of growth of *B. anthracis*, and (ii) if plates, which should contain the spike, cannot be interpreted because *B. anthracis* is overgrown by hemolysing *Bacillus spp*. In both cases analysis of the direct cultures on plates would yield false negative results.

Of the primer systems described in the literature, only primers for the protective antigen (pag) and the lethal factor gene (lef) have not yet been shown to yield unspecific results. Many primers decribed for the cap B and cap C genes (Beyer et al., 1999) and all currently published primers targeting the chromosome of B. anthracis (Ramisse et al., 1999) were shown to produce false positive results with the natural soil flora.

More recent protocols for light-cycler (LC) PCR have not yet been sufficiently evaluated. The "SybrGreen" protocol for detection of the pag-gene, described by Makino et al. (2001) cannot be considered suitable for diagnostic purposes due to certain intrinsic problems with specificity of the SybrGreen protocol. Also, using the LC-protocol for detection of the rpoB-gene (Qi et al., 2001), we and others have found unspecific reactions with *B. cereus, B. thuringiensis*, and *B. mycoides* (Ellerbrok, 2002). This may be due to the fact, that any of the nucleotide exchanges used by the authors to differentiate between B. anthracis and related isolates are located in the third position of the appropriate codons. These nucleotides may be exchanged in many different Bacilli containing a related rpoB gene because their exchange have no influence on the corresponding amino acid sequence of the enzyme.

DNA preparation

When PCR is used only to confirm suspicious colonies, short boiling of resuspended vegetative bacteria in PCR buffer is sufficient to extract DNA. To prepare DNA from a non-selective enrichment culture or germinated spore suspensions, a DNA-preparation kit is recommended for use. Depending on the target sequence of the PCR, the DNA preparation should either enrich for plasmid DNA or genomic DNA. In the first case, the procedure should be able to isolate large, low copy number plasmids. For the isolation of PCR compatible DNA from environmental samples, the procedure must furthermore be able to sufficiently remove polymerase inhibitors. Our laboratory has successfully used the DNA preparation kits Nucleo Spin Plasmid, NucleoSpin Plant and Nucleobond (all from Macherey-Nagel, Dueren, Germany) or the DNeasy Plant (Quiagen) for environmental samples. It should be noted that preparation of genomic DNA from pure cultures of bacteria, using the NucleoSpin Plant kit, can be perfomed using the lysis buffer (CO) provided with the kit, followed by incubation at 60 °C for 30 min. DNA isolation from environmental samples, even when dealing with a liquid culture, however, requires the use of a special extraction buffer and heating in a microwave. If the culture has not been killed prior to DNA isolation heating of the suspension in the microwave may cause a severe biological hazard.

The DNeasy Plant kit was used as recommended by the provider with the following minor modifications:

- DNA preparation started with the pellet from 1 ml of the enrichment broth, resuspended in 400 µl of buffer AP1
- After the second wash with buffer AW, an additional washing step with pure ethanol was performed,
- DNA was eluted in 50 µl of prewarmed buffer AE.

Controls to be included in the diagnostic PCR

The following control reactions are necessary in order to verify diagnostic findings.

1. DNA from the sample material spiked with a positive control strain of *B. anthracis*. This control will provide information about possible inhibition of germination or growth during the culture of the sample materials.
2. Addition of 100 pg to 1 ng of purified genomic DNA of *B. anthracis* to the sample DNA. This reaction will detect possible inhibition of the PCR by potential polymerase inhibitors. It also serves as a control to determine the sensitivity of the PCR.
3. DNA (1 pg and 1 ng) of a pure culture of *B. anthacis*. This reaction serves as a positive control in the PCR. An amplicon should be visible for 1 ng of input DNA after the first PCR, whereas 1 pg DNA would be detectable after the nested PCR step only.
4. DNA derived from the negative 'in-process' control, e.g. *E. coli* cells. This reaction serves as a control for contaminations throughout the entire process of culture, DNA isolation, and PCR.
5. PCR pre-mix without DNA. This reaction serves as a control for contamination of the preparation of pre-mixes only.

Protocol for PCR in a block cycler instrument to detect the pag or the cap genes

A pre-mix consisting of a volume of 50 µl per reaction was prepared containing 200 µmol/l dNTP's, 1.5 mmol/l $MgCl_2$, 1 µmol/l of each primer, and 2.5 U polymerase. One microgram of T4 gene32 protein (Roche) was added to the standard pre-mix during the first PCR. This single-strand binding protein enhances the efficacy of PCR's by a factor of 100 if the reaction is influenced by polymerase inhibitors (Beyer et al., 1995). The use of a 'hot start' protocol is advisable. Running a second PCR (nested PCR) is necessary (i) if there is only weak sensitivity of the first of PCR, e.g. due to inhibition of growth in the spiked control culture or (ii) as a control reaction to verify the specificity of a positive result of a sample after the first PCR. The nested PCR step may be omitted if the first PCR yields a negative result for the sample and the inhibition control, and the corresponding positive control reacts positive. Listed below are the PCR conditions for for a thermal block cycler running under block control.

I. 94 °C – 4 min
II. 25 cycles (1st PCR); 30 cycles (nested PCR):
- 83 °C – 1 min
- 55 °C – 1 min 30 sec
- 73 °C – 1 min 30 sec
III. 72 °C – 9 min
IV. hold at 8 °C

Primers used in the diagnostic PCR are shown in table 1. Additional primer systems are provided in table 2. It has been seen, however, that primers (until now with the exception of primers targeting the pag and lef genes) may react unspecifically with various *Bacillus spp*.

Table 1. *Primers for nested PCR to detect the pag-gene*

Primer	Sequence (5`- 3`)	Reference	Binding site	Gene	Annealing
PA5	TCCTAACACTAACGAAGTCG	Beyer et al. (1995)	2452- 2471 [a]	pag	55° C
PA8	GAGGTAGAAGGATATACGGT	Beyer et al. (1995)	3048- 3029 [a]	pag	55° C
PA6	ACCAATATCAAAGAACGACG C	Beyer et al. (1995)	2631- 2651 [a]	pag	55° C
PA7	ATCACCAGAGGCAAGACAC CC	Beyer et al. (1995)	2841- 2821 [a]	pag	55° C

primer pairs are: PA5/PA8 and PA6/PA7

Table 2. *Additional published primer systems*

Primer	Sequence (5`- 3`)	Reference	Binding site	Gene	Annealing
CAP6	TACTGACGAGGAGCAACCGA	Beyer '95	506- 525 [a]	Cap (B)	55 °C
CAP103	GGCTCAGTGTAACTCCTAAT	Beyer '95	1541- 1522 [a]	Cap (B)	55° C
CAP9	ATGTATGGCAGTTCAACCCG	Beyer '95	617- 636 [a]	Cap (B)	55° C
CAP102	ACCCACTCCATATACAATCC	Beyer '95	1394- 1375 [a]	Cap (B)	55° C
R1	TTAATTCACTTGCAACTGATGGG	Patra `96	227- 249 [b]	Ba813	55° C
R2	AACGATACGTCCTACATTTGGAG	Patra `96	98- 120 [b]	Ba813	55° C
C1 [c]	GCCAGGTGCTATACCGTATCAGCAA	Patra `96	179- 203 [b]	Ba813	37 °C
3D [c]	GAGTAACTCGTTAATGCTTCAAATT	Patra `96	139- 163 [b]	Ba813	37 °C
BA17	GAAATAGTTATTGCGATTGG	Sjöstedt `95	1230- 1249 [a]	Cap (B,C)	54 °C [e]
BA20	GGTGCTACTGCTTCTGTACG	Sjöstedt `95	2102- 2083 [a]	Cap C,A)	62 °C [e]
BA57	ACTCGTTTTTAATCAGCCCG	Sjöstedt `97	1603- 1622 [a]	Cap (C)	52 °C
BA58	TGGTAACCCTTGTCTTTGAAT	Sjöstedt `97	1867- 1847 [a]	Cap (C)	58 °C [f]
17	GAAATAGTTATTGCGATTGG	Ramisse `96	1230- 1249 [a]	Cap (B,C)	58 °C
20	GGTGCTACTGCTTCTGTACG	Ramisse `96	2102- 2083 [a]	Cap C,A)	58 °C
57	ACTCGTTTTTAATCAGCCCG	Ramisse `96	1603- 1622 [a]	Cap (C)	58 °C
58	GGTAACCCTTGTCTTTGAAT	Ramisse `96	1866- 1847 [a]	Cap (C)	58 °C
MO1	GCTGATCTTGACTATGTGGGTG	Makino `93	2452- 2473 [a]	Cap (A)	65 °C
MO2	GGCTTCCTGTCTAGGACTCGG	Makino `93	2739- 2719 [a]	Cap (A)	65 °C
BACA1FI	ACAACTGGTACATCTGCGCG	Reif `94	470- 489 [a]	Cap (B)	58 °C [g]
BACA 6RI	GATGAGGGATCATTCGCTGC	Reif `94	1073- 1092 [a]	Cap (B)	58 °C [g]
67	CAGAATCAAGTTCCCAGGGG	Ramisse `96	1925- 1944 [a]	Pag	58 °C
68	TCGGATAAGCTGCCACAAGG	Ramisse `96	2652- 2671 [a]	Pag	58 °C
23	CTACAGGGGATTTATCTATTCC	Ramisse `96	2006- 2027 [a]	Pag	58 °C

24	ATTGTTACATGATTATCAGCGG	Ramisse `96	2135- 2156 [a]	Pag	58 °C
Cvi	CACTCGTTTTTAATCAGCCC	Beyer `99	1602- 1621 [a]	Cap (C)	55 °C
Cri	CCTGGAACAATAACTCCAATACC	Beyer `99	1808- 1830 [a]	Cap (C)	55 °C
PA-S	CGGATCAAGTATATGGGAATATAGCAA	Ellerbrok '02	3245-3271	pag	60 °C
PA-R	CCGGTTTAGTCGTTTCTAATGGAT	Ellerbrok '02	3448-3425	pag	60 °C
Cap-S	ACGTATGGTGTTTCAAGATTCATG	Ellerbrok '02	1673-1696	cap C	60 °C
Cap-R	ATTTTCGTCTCATTCTACCTCACC	Ellerbrok '02	1993-1940	cap C	60 °C

[a] Positions derived from Makino et al. (1989) and Welkos et al. (1988).

[b] GenBank accession No. U46157

[c] The oligonucleotides C1 and 3D are not used as primers, but as probes for the PCR-ELISA described in Beyer et al. (1999), they will hybridise with the PCR product at 37 °C; Patra et al. (1995) first published the sequence for primers C1 and D3. Probe 3D is a reverse complementary copy of the oligonucleotide D3 and will not hybridise with the same strand of DNA as C1.

[d] Positions derived from Bragg et al. (1989).

[e] These temperatures were published by Söstedt et al. (1997) as *T*m.

[f] Sjöstedt et al. (1997) described 52 °C as the annealing temperature.

[g] Reif et al. (1994) described 55 °C as the annealing temperature.

Protocol for PCR in a LC instrument to detect the pag gene (variant 1)
The premix (20 µl) consisted of:
- 4 mM $MgCl_2$
- 0,5 µmol of each primer:
PA8F2: 5'-CGGCTTCTGATCCGTACAGT-3'
PA8R2: 5'-ATTCTGTGTGGATTGATCCTCA-3'
- 0,2 µmol of probe FL: 5'-GGGTGTCTTGCCTCTGGTGATACAT- X
- 0,2 µmol of probeLC: (red 640) 5'-TTATCAATCCGTCCTGTAACCTTTTCG-P
- 1/10 vol. of FastStart mastermix
- 5 µl DNA

Protocol for PCR in a LC instrument to detect the pag gene (variant 2)
The premix (20 µl) consisted of:
- 4 mM $MgCl_2$
- 0,5 µmol of each primer: (Ellerbrok et al. 2002)
BAPA-S: 5'-CGGATCAAGTATATGGGAATATAGCAA-3'
BAPA-R: 5-CCGGTTTAGTCGTTTCTAATGGAT-3'
- 0,2 µmol of probe BAPA-FL 5'-TGCGGTAACACTTCACTCCAGTTCGA-X
- 0,2 µmol of probe BAPA-LCRed 640 5'-CCTGTATCCACCCTCACTCTTCCATTTTC-P
- 1/10 vol. of FastStart mastermix
- 5 µl DNA

Protocol for PCR in a LC instrument to detect the pag gene (variant 2)
The premix (20 μl) consisted of:
- 4 mM $MgCl_2$
- 0,5 μmol of each primer:
CapS: 5'-ACGTATGGTGTTTCAAGATTCATG-3' (Ellerbrok et al., 2002)
Cap A**: 5-GATTGCAAATGTTGCACCACTTA-3'
- 0,2 μmol of probe CapC-FL+ 5'-TATTGTTATCCTGTTATGCCATTTGAGATTTTT- X
- 0,2 μmol of probe CapC-LC Red640 5'-AATTCCGTGGTATTGGAGTTATTGTTCC- P
- 1/10 vol. of FastStart mastermix
- 5 μl DNA

The experimental protocol was set as follows:
- Pre-incubation step: 95 °C for 10 min, slope at 20 °C/sec
- Amplification (45 cycles): 95 °C for 10 sec; 55 °C for 20 sec, 72 °C for 30 sec, slope 20 °C/sec; one single signal aquisition at the end of annealing
- Denaturation: 95 °C for 0 sec, slope 20 °C/sec; 40 °C for 30 sec, slope 20 °C/sec; 80 °C for 0 sec, slope 0.1°C /sec with continuous acquisition of the signal
- Cooling to 40 °C for 30 sec, slope 20 °C/sec

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