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In silico and in vitro evaluation of PCR-based assays for the detection of *Bacillus anthracis* chromosomal signature sequences

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Abbreviations: qPCR, quantitative real time polymerase chain reaction; WHO, World Health Organization; OIE, World Organisation for Animal Health; *B.*, *Bacillus*; EU, European Union; SE, sensitivity; SP, specificity; CFU, colony forming unit; IAC, internal amplification control; Cq, quantification cycle (or threshold cycle); FRET, fluorescence resonance energy transfer; LOD, limit of detection; SNP, single nucleotide polymorphism; HRM, high resolution melting; RAPD, random amplification of polymorphic DNA; SD, standard deviation; DNA, deoxyribonucleic acid; BLAST, Basic Local Alignment Search Tool; NCBI, National Center for Biotechnology Information; FTP, file transfer protocol; SVA, National Veterinary Institute in Sweden; RIVM, National Institute for Public Health and the Environment in the Netherlands; CVI, Central Veterinary Institute of Wageningen; Anses, French agency for food, environmental and occupational health & safety; DTU, Technical University of Denmark

Bacillus anthracis, the causative agent of anthrax, is a zoonotic pathogen that is relatively common throughout the world and may cause life threatening diseases in animals and humans. There are many PCR-based assays in use for the detection of *B. anthracis*. While most of the developed assays rely on unique markers present on virulence plasmids pXO1 and pXO2, relatively few assays incorporate chromosomal DNA markers due to the close relatedness of *B. anthracis* to the *B. cereus* group strains. For the detection of chromosomal DNA, different genes have been used, such as BA813, *rpoB*, *gyrA*, *plcR*, S-layer, and prophage-lambda. Following a review of the literature, an in silico analysis of all signature sequences reported for identification of *B. anthracis* was conducted. Published primer and probe sequences were compared for specificity against 134 available *Bacillus* spp. genomes. Although many of the chromosomal targets evaluated are claimed to be specific to *B. anthracis*, cross-reactions with closely related *B. cereus* and *B. thuringiensis* strains were often observed. Of the 35 investigated PCR assays, only 4 were 100% specific for the *B. anthracis* chromosome. An interlaboratory ring trial among five European laboratories was then performed to evaluate six assays, including the WHO recommended procedures, using a collection of 90 *Bacillus* strains. Three assays performed adequately, yielding no false positive or negative results. All three assays target chromosomal markers located within the lambdaBa03 prophage region (PL3, BA5345, and BA5357). Detection limit was further assessed for one of these highly specific assays.

Introduction

B. anthracis, the etiological agent of anthrax, is a zoonotic pathogen that can cause life threatening diseases in animals and humans.[1] Virulent strains of *B. anthracis* harbor two plasmids, pXO1 and pXO2, carrying unique genes that confer toxin production and capsule synthesis, respectively.[2-4] Due to its possible use as an agent for bioterrorism, *B. anthracis* is one of the most feared microorganisms.

The major challenge of developing a reliable assay for the detection of *B. anthracis* stems from its high similarity to other strains in its genus. *B. anthracis* is a member of the *Bacillus cereus* group of bacteria (*B. cereus sensu lato*) which comprises 6 genetically related species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. weihanstephanensis*, and *B. pseudomycoides*. An extremely high degree of genomic homology exists between *B. cereus*, *B. anthracis*, and *B. thuringiensis*, which some authors consider genetically just one species.[5,6] The main difference between these

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species is the presence of unique virulence plasmids. However, data gathered in the last decade have shown that *B. cereus* strains that contain anthrax-specific pXO-like plasmids exist[7-12] which further obscure the much intermixed phylogenetic structure of the *B. cereus* group.

Some PCR-based assays in use for detection of *B. anthracis* rely on plasmid-encoded targets in conjunction with a chromosomal marker to correctly differentiate pathogenic from apathogenic *B. anthracis* strains and *B. anthracis* from non-anthracis *Bacillus* species, respectively (for a review see ref. 13). The importance of including a chromosomal assay to verify the presence of *B. anthracis* independently of plasmid occurrence was emphasized by the discovery of forms of *B. anthracis* isolates lacking plasmids, *B. cereus* isolates harboring anthrax-like virulence plasmids, and pXO2 gene homologs in environmental Bacillus isolates.[7-12] Several chromosomal targets have been investigated for identification purposes, but most of the markers reported to be unique for *B. anthracis* were in fact common to both *B. anthracis* and a subpopulation of closely related *B. cereus* and *B. thuringiensis* strains.[13-15] Few chromosomal sequences that provide sufficient polymorphism to unambiguously distinguish *B. anthracis* from its near neighbors have been identified.[14,16-22] Some of these assays rely upon single-nucleotide differences for discrimination and are therefore sensitive to assay conditions and PCR cycling parameters. Small alterations in these conditions can result in the loss of specificity, especially with hydrolysis probes, i.e., TaqMan chemistry.[18,23-25]

To evaluate the wide range of PCR methods used in laboratories for *B. anthracis* identification, a computer-based comparative analysis of more than 300 PCR-target sequences reported in the literature was conducted. All sequences were compared against all publicly available *Bacillus* genomes and sorted for specificity. The three assays with highest in silico specificity, together with three assays with lower specificity, were evaluated in an international ring trial using DNA of *Bacillus* strains exchanged in the framework of the EU AniBioThreat project. The best chromosomal signatures for reliable *B. anthracis* genome detection are discussed for the purpose of selecting an assay as international standard for *B. anthracis* detection.

Results

Literature survey of PCR-based detection methods

The literature survey showed that at least 20 different chromosomal markers have been described (**Table 1**).[13-15] The first DNA signatures that were developed for anthrax PCR detection methods independently of plasmids occurrence were DNA fragments used to genotype *B. anthracis*. They include the *vrrA* marker,[26-28] the AC-390 gene,[29] and the SG-850/749 fragment.[30] These genetic markers provide limited specificity and require additional time-consuming and labor-intensive post-PCR analysis steps. Other areas of the chromosome have also been investigated as potential DNA-targets for identification purposes, including the so-called BA813[31-38] and BA5510 sequences,[19] genes *bclB*,[39] *sap*,[40,41] *saspB*,[5,42] and *sspE*,[22,43] the B-type small acid-soluble spore protein gene (SASP),[44] a glycosyltransferase group 1 family protein,[45] a protein showing similarities with an abhydrolase,[18] and several DNA loci located on prophage regions,[17] i.e., BA5345,[21] BA5357,[46] and PL3.[47] Although most of these regions have been claimed to be anthrax-specific, *B. cereus* strains sometimes yield false-positive results.[13-15] Finally, a few single nucleotide polymorphisms (SNP) have also been considered for PCR markers. Target genes include *rpoB*,[24,48-51] *gyrA*,[25,52,53] *gyrB*,[54,55] *plc*,[20,23,53,56] *purA*,[57] and the 16S-23S rDNA internal spacer sequences.[58-60] But, so far, only the nonsense mutation in the global regulator PlcR, which controls the transcription of secreted virulence factors in *B. cereus* and *B. thuringiensis*, have proved to be truly unique to *B. anthracis* strains.[16,20,59] False-positive signals have sometimes been recorded with closely related strains of the *B. cereus* group using the other published SNPs.[24,49,52,59,61-63]

In silico analysis

About a hundred sequences corresponding to all primers and probes currently published were compiled and compared using the primer alignment function of the Gegenees software (www.gegenees.org).[64] Each sequence was tested against all available *Bacillus* spp. genomes and scored for specificity (**Table 1**). *Bacillus* is one of the largest genera represented in the bacterial genome database, with about 140 distinct members of the *B. cereus* group sequenced (www.ncbi.nlm.nih.gov).

Excluding SNP discrimination assays, it was found that out of the 35 PCR assays analyzed in silico, only four were specific for the *B. anthracis* chromosome, with a minimum unalignment value for background genomes higher than zero (**Table 1**). These assays target the markers BA5345,[21,65] PL3,[47] and BA5357,[46] respectively. Three of these assays are based on hydrolysis probe ("TaqMan assay"); the fourth uses SYBR Green chemistry. These primer/probe sequences showed a perfect match to all *B. anthracis* genomes, and very poor matches to *B. thuringiensis* and *B. cereus* strains, including strains that are known to be phylogenetically very closely linked to *B. anthracis*. All other assays were found to be prone to false positive identification, as perfect matches were found for several *B. cereus* and *B. thuringiensis* strains.

To illustrate the complexity of the *B. cereus* group and why PCR-markers cross-react with some *B. cereus* and *B. thuringiensis* strains, we compared the genomes of 22 strains that were later used for PCR assays assessment in the ring trial (see below). **Table 2** shows a similarity matrix that gives a phylogenomic overview of the 22 genomes. We considered an 80% average core genome similarity as threshold for a strain to be called a near neighbor as genomes passing this criterion produced most cross-reactions. Assessment of several in silico primer alignments showed that the vast majority of the cross reactions occurred within the near-neighbor group, at least for the better performing assays.

Regarding assays relying upon single-nucleotide differences for discrimination, the in silico investigation confirmed that the *plcR* and *purA* point mutations were unique to *B. anthracis* strains (data not shown). The SNP at position 1668 of *gyrA* was also found to be a relatively specific marker for *B. anthracis* identification as only one genome (*B. thuringiensis* serovar monterrey BGSC 4AJ1) contained the C variant specific for *B. anthracis*. Screening other published SNPs resulted in false-positive signals for several strains of the *B. cereus* group (data not shown).

Table 1. Specificity of primer/probe sequences published

Reference	Target (loci tag^{Ames})	Technique		Primer/probe DNA sequence (5′-3′)	Perfect match in target genomes	Min unalignment in background genomes	Number of hits in background at that level
Hurtle et al.[52]	*gyrA*	qPCR	p	GGGAACAAAT GATGATGATT TCGT	Yes	0	>10
	(BA_0006)	HP-MGB	p	ACTCTGGGAT TTCATATCCT TTCGT	Yes	0	>10
			s	CGCATGACCA TATTC	Yes	0	1
Antwerpen et al.[21]	BA5345	qPCR	p	CGTAAGGACA ATAAAAGCCG TTGT	Yes	2	2
	(BA_5345)*	HP	p	CGATACAGAC ATTTATTGGG AACTACAC	Yes	7	1
			s	TGCAATCGAT GAGCTAATGA ACAATGACCC T	Yes	3	1
Hadjinicolaou et al.[60]	16s rRNA	qPCR	s	TTACCTCACC AACTAGCTAA TGCGA	Yes	0	~50
		Beacon	p	TTCGGCTGTC ACTTATGGAT G	Yes	0	~50
			p	TCGGCTACGC ATCGTTGCCT TG	No	0	~50
Irenge et al.[57]	*purA*	qPCR	p	CAACACTTAA AATTTGTGTT GCTTACAA	Yes	0	>10
	(BA_5716)	HP-LNA	p	TCACATTTCG CTAAAATGTT TAAGTTTG	Yes	0	>10
			s	TCGATAACTT TCCCATCGCA	Yes	1	18
	ptsI	qPCR	p	GCTTGACGGA AYTCATCAAG AGT	ND	1	~40–50
	(BA_4267)	HP-LNA	p	TATGYCTTGA WGARCAAGAT GTGTTC	ND	3	~40–50
			s	GTACACAACT TCGTGCATT	Yes	0	~40
Vahedi et al.[38]	BA813	PCR	p	AATGATAGCT CCTACATTTG GAG	No	3	~20
	(BA-5031)		p	TTAATTCACT TGCAACTGAT GGG	Yes	0	1
Qi et al.[24]	*rpoB*	qPCR	p	CCACCAACAG TAGAAAATGC C	Yes	0	2
	(BA_0102)	FRET	p	AAATTTCACC AGTTTCTGGA TCT	Yes	0	2
			s	TCCAAAGCGC TATGATTTAG CAAATGT	Yes	0	4
			s	GGTCGCTACA AGATCAACAA GAAGTTACAC	Yes	0	~20
Oggioni et al.[48]	*rpoB*	qPCR	p	TTGCTTGAAA TTTATGAGCG TCTAC	Yes	0	~50
	(BA_0102)	FRET	p	ATTGTTCCTT CTGCCGCTAA AA	Yes	0	~50
			s	TGTAGGTCGC TACAAGATCA ACAAG	Yes	0	21
			s	AAGCGCTATG ATTTAGCAA	Yes	0	5
Easterday et al.[20]	*plcR*	qPCR	p	CCAATCAATG TCATACTATT AATTTGACAC	Yes	0	19
	(BA_5595)	HP-MGB	p	ATGCAAAAGC ATTATACTTG GACAAT	Yes	0	8
			s	CAAAGCGCTT ATTCGTATT	Yes	1	25
			s	AAAGCGCTTC TTCGTATT	No	0	~30
Lewerin et al.[65]	BA_5345	qPCR	p	GAAGGACGAT ACAGACATTT ATTGG	Yes	5	2
	(BA_5345)*	SybrGreen	p	ACCGCAAGTT GAATAGCAAG	Yes	0	2
Wielinga et al.[47]	PL3	qPCR	p	AAAGCTACAA ACTCTGAAAT TTGTAAATTG	Yes	5	1
	(BA_5358)*	HP	p	CAACGATGAT TGGAGATAGA GTATTCTTT	Yes	6	2
			s	AACAGTACGT TTCACTGGAG CAAAATCAA	Yes	4	1
Kim et al.[43]	*sspE*	qPCR	p	GAGAAAGATG AGTAAAAAAC AACAA	Yes	0	~50
	(BA_0523)	SybrGreen	p	CATTTGTGCT TTGAATGCTA G	Yes	0	11
Coker et al.[35]	BA813	qPCR	s	AATGCCAGGT TCTATACCGT ATCAGCAAGCTATTC	Yes	0	~20
	(BA-5031)	HP-MGB	p	GGAGGGAATA CAGCAAACAC AGA	Yes	0	~15
			p	TGCAACTGAT GGGATTTCTT TCT	Yes	0	~15

ND, BLAST could not handle Y, W and R; s, probe; p, primer; np, nested primer; HP, hydrolysis probes; MGB, minor-grove-binding; FRET, hybridization probes; RAPD, random amplification of polymorphic DNA; LNA, locked nucleic-acid; GT, glycosyltransferase. *DNA located on prophage region.

Table 1. Specificity of primer/probe sequences published (continued)

Reference	Target (loci tag[Ames])	Technique		Primer/probe DNA sequence (5′-3′)	Perfect match in target genomes	Min unalignment in background genomes	Number of hits in background at that level
Bode et al.[18]	B26	qPCR	p	TGGCGGAAAA GCTAATATAG TAAAGTA	Yes	0	7
	(BA_2686)	HP-MGB	p	CCACATATCG AATCTCCTGT CTAAAA	Yes	0	6
			s	ACTTCTAAAA AGCAGATAGA AAT	Yes	0	7
Ryu et al.[41]	*sap*	qPCR	p	CAATCGAAAT GGCTGACCAA A	Yes	0	6
	(BA_0885)	HP	p	ACCCTCTGGT GAAACAACTT CAGT	Yes	0	4
			s	TAGCTGATGA GCCAACAGCA TTACAATTCA CAGT	Yes	0	4
Ellerbrok et al.[49]	*rpoB*	qPCR	p	CCACCAACAG TAGAAAATGC C	Yes	0	2
	(BA_0102)	HP	p	AAATTTCACC AGTTTCTGGA TCT	Yes	0	2
			s	ACTTGTGTCT CGTTTCTTCG ATCCAAAGCG	Yes	0	~40
Luna et al.[36]	Ba813	qPCR	p	AATTTGAAGC ATTAACGAGT T	Yes	0	~20
	(BA-5031)	HP	p	TTCTTTCTGA CTTGGAATAG C	Yes	0	~20
			s	GCCAGGTTCTA TACCGTATCA GCAA	Yes	0	~20
Letant et al.[46]	BA5357	qPCR	p	TTTCGATGAT TTGCAATGCC	Yes	2	10
	(BA_5357)*	HP	p	TCCAAGTTAC AGTGTCGGCA TATT	Yes	5	3
			s	ACATCAAGTC ATGGCGTGAC TACCCAGACT T	Yes	6	1
WHO[44]	B-type SASP	qPCR	p	GCTAGTTATG GTACAGAGTT TGCGAC	Yes	0	15
	(BA_0524)	FRET	p	CCATAACTGA CATTTGTGCT TTGAAT	No	3	11
			s	CAAGCAAACG CACAATCAGA AGCTAAG	Yes	0	10
			s	GCGCAAGCTT CTGGTGCTAG C	Yes	4	~40
Jackson et al.[27]	*vrrA*	PCR	p	ACAACTACCA CCGATGGC	Yes	0	~40
	(BA_4509/11)		p	TTATTTATCA TATTAGTTGG ATTCG	Yes	0	32
			np	TATGGTTGGT ATTGCTG	Yes	0	16
			np	ATGGTTCCGC CTTATCG	Yes	0	32
Ramisse et al.[31]	BA813	PCR	p	TTAATTCACT TGCAACTGAT GGG	Yes	0	1
	(BA-5031)		p	AACGATAGCT CCTACATTTG GAG	Yes	0	19
WHO[40]	S-Layer, *sap*	PCR	p	CGCGTTTCTA TGGCATCTCT TCT	Yes	0	13
	(BA_0885)		p	TTCTGAAGCT GGCGTTACAA AT	No	2	3
Daffonchio et al.[30]	SG-850/749	RAPD (*Alu*I)	p	ACTGGCTAAT TATGTAATG	No	2	~50
	(BA_1584/85)		p	ATAATTATCC ATTGATTTCG	Yes	0	~30
Wang et al.[37]	BA813	microarray	p	CATTTAGCGA AGATCCAGT	Yes	0	~20
	(BA-5031)		p	CTTGCTGATA CGGTATAGAA C	Yes	0	~20
			s	TTTTTTTTTT CATTTAGCGA AGATCCAGT	Yes	0	~20
Brightwell et al.[33]	Ba81	PCR	p	TTAATTCAC TTGCAACTG ATGGG	Yes	0	1
	(BA-5031)		p	AACGATAGC TCCTACATT TGGAG	Yes	0	~20
Nubel et al.[58]	16–23S tRNA	microarray	s	GCAACGAGC GCAACCC	Yes	0	~140
			s	CTGAGCTAT AGSCCCATA	No	1	~80
			s	CCATACAAAT TTCAGGATTT A	Yes	0	2
			s	CCATACAAAT TTCAGGATTT	Yes	0	2
			s	CATACAAATT TCAGGATTT	Yes	0	2
Daffonchio et al.[59]	16–23S tRNA	PCR	p	GATATGATAT AAATAAATCG CG	No	2	2
			p	GTGGGTTTCC CCATTCGG	No	0	~100

ND, BLAST could not handle Y, W and R; s, probe; p, primer; np, nested primer; HP, hydrolysis probes; MGB, minor-grove-binding; FRET, hybridization probes; RAPD, random amplification of polymorphic DNA; LNA, locked nucleic-acid; GT, glycosyltransferase. *DNA located on prophage region.

Table 1. Specificity of primer/probe sequences published (continued)

Reference	Target (loci tag[Ames])	Technique		Primer/probe DNA sequence (5′-3′)	Perfect match in target genomes	Min unalignment in background genomes	Number of hits in background at that level
Ko et al.[50]	*rpoB*	PCR	p	TTCGTCCTGT TATTGCAG	Yes	1	~40
	(BA_0102)		p	GACGATCATY TWGGAAACCG	ND	ND	ND
			p	GGNGTYTCRA TYGGACACAT	ND	ND	ND
Cheun et al.[34]	BA813	nested PCR	p	ACTAACGAAT CTTTCATTTA GCG	Yes	0	~20
	(BA-5031)		p	ATTGCACTTG CATAATATCC TTG	Yes	0	~20
			np	AACGATAGCT CCTACATTTG GAG	Yes	0	~20
			np	TTAATTCACT TGCAACTGAT GGG	Yes	0	1
	S-Layer	nested PCR	p	CGCGTTTCTA TGGCATCTCTT CT	Yes	0	13
	(BA_0885)		p	TTCTGAAGCT GGCGTTACAA AT	No	2	2
			np	CGGRACAGAA GCAGCAAAA	No	1	5
			np	GCTGTTGGCT CATCAGCTA	Yes	0	3
Park et al.[55]	*gyrB*	PCR	p	GGTAGATTAG CAGATTGCTC TTCAAAAGA	No	1	12
	(BA_0005)		p	ACGAGCTTTCT CAATATCAAA ATCTCCGC	Yes	0	11
Kim et al.[45]	GT	PCR	p	TCTTCAGTGA CAAAACCACA	Yes	0	2
	(BA_5519)		p	CAAGAAATCT TTTTCGAAGG	Yes	0	3
Olsen et al.[19]	*tagH*	qPCR	p	CTGCATTGAT AGCAATTTCT TCA	Yes	0	2
	(BA_5510)	FRET	p	CAGGTTGATA CATAAACTTT CCA	Yes	0	2
			s	GTAATTCCCA TCATTAAACC TTTTAATTCG ATAT	Yes	0	2
			s	CAATCCCTGT TAATTGACCA TTAAGCC	Yes	0	2
Leski et al.[39]	*bclB*	PCR	p	AGGCCCAGAA AATATTGGAC	Yes	0	22
	(BA_2450)		p	GAGTTCCTCC CACACCTGG	Yes	0	8
Cherif et al.[29]	AC-390	PCR	p	GAAAATGGCC GGATGAGT	No	0	9
	(BA_5406)		p	GACGTTGAAA CATTTATGCA	No	0	11

ND, BLAST could not handle Y, W and R; s, probe; p, primer; np, nested primer; HP, hydrolysis probes; MGB, minor-grove-binding; FRET, hybridization probes; RAPD, random amplification of polymorphic DNA; LNA, locked nucleic-acid; GT, glycosyltransferase. *DNA located on prophage region.

Ring trial

The three hydrolysis probe assays with highest specificities in the in silico analysis (BA5345, PL3, and BA5357) were evaluated in vitro using a panel of 90 *Bacillus* strains in a laboratory ring-trial performed at 5 European laboratories (RIVM, DTU, SVA, ANSES, and CVI). Assays mentioned by the World Health Organization (WHO)[31,40,44] were also included in the ring trial, as well as a hydrolysis probe assay[35] that targets the often used BA813 marker[31-38] (**Table 3**). The latter marker has shown in silico cross-reactions toward the near-neighbor strains in use in this trial and was included for this reason. The two WHO procedures tested are, respectively, a formerly used conventional gel-based PCR assay targeting the S-layer gene *sap*[40] and a dual hybridization probes qPCR assay targeting a gene encoding the small acid-soluble spore protein SASP.[44]

Results of the ring trial confirmed the results obtained in the in silico analysis (**Table 4**). The three assays with highest in silico specificity (BA5345,[21] PL3,[47] and BA5357[46]) all performed well in the ring trial, with diagnostic sensitivity and specificity values close to 1 (**Table 5**). Furthermore, these assays were found to be robust and provided consistent results between laboratories (kappa values of 0.9–1.0). All 31 *B. anthracis* strains were correctly detected, except in one laboratory that failed to detect one sample with a lower DNA content using the BA5345 assay. None of the non-anthrax strains gave false-positive results for these assays for any of the participating laboratories.

The results obtained using the S-layer,[40] BA813,[35] and SASP[44] assays displayed a lower agreement among laboratories (k values of 0.5–0.8). In general, the three methods had relative low diagnostic sensitivity and specificity compared with the BA5345, PL3, and BA5357 assays, indicating that these methods have a lower performance both in detecting *B. anthracis* in truly contaminated samples and in declaring truly non-contaminated samples as free of *B. anthracis*. Although the BA813 assay was found to be quite effective in identifying true *B. anthracis* strains—except for laboratory 2, which failed to detect two strains—it yielded a number of false-positive results (ranging from 11 to 23 strains) in all laboratories. As for the former WHO recommended S-layer assay,[40] this conventional PCR method was apparently not as sensitive as several of the others (**Table 5**), producing false-negative results in

Table 2. Similarity matrix created by Gegenees over a set of 22 *Bacillus* strains used in this study.

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. *B. anthracis* Vollum	**100**	**100**	**100**	**95**	**95**	**93**	**94**	**94**	**93**	**91**	**91**	**84**	**83**	**81**	74	73	74	73	70	68	69	56
2. *B. anthracis* Sterne	**100**	**100**	**100**	**95**	**95**	**93**	**95**	**94**	**93**	**91**	**91**	**84**	**84**	**82**	74	74	74	74	71	68	69	57
3. *B. anthracis* CNEVA9066	**100**	**100**	**100**	**95**	**95**	**93**	**94**	**94**	**93**	**91**	**91**	**84**	**83**	**81**	74	74	74	73	71	68	69	57
4. *B. thuringiensis* BGSC 4AJ1	**94**	**95**	**94**	**100**	**95**	**93**	**93**	**93**	**92**	**91**	**90**	**83**	**83**	**81**	74	73	74	73	70	68	68	55
5. *B. thuringiensis* BGSC 4BA1	**95**	**95**	**95**	**95**	**100**	**93**	**93**	**94**	**92**	**91**	**91**	**83**	**83**	**81**	74	73	74	73	72	68	68	56
6. *B. thuringiensis* 97–27	**92**	**93**	**93**	**93**	**93**	**100**	**92**	**93**	**92**	**91**	**91**	**83**	**83**	**82**	74	73	74	74	71	68	69	57
7. *B. thuringiensis* BGSC 4CC1	**93**	**94**	**94**	**93**	**93**	**92**	**100**	**92**	**92**	**90**	**90**	**83**	**83**	**81**	74	73	74	73	70	68	68	56
8. *B. thuringiensis* BGSC 4AW1	**93**	**94**	**94**	**94**	**94**	**93**	**93**	**100**	**93**	**91**	**91**	**83**	**83**	**81**	74	73	74	73	70	68	68	56
9. *B. cereus* NVH0597–99	**92**	**92**	**92**	**92**	**92**	**92**	**92**	**93**	**100**	**91**	**91**	**83**	**83**	**81**	74	73	74	73	71	69	69	57
10. *B. cereus* SJ1	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**100**	**91**	**83**	**83**	**82**	74	73	74	73	70	68	68	56
11. *B. cereus* BGSC 6E1	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**91**	**100**	**83**	**83**	**81**	74	73	74	73	70	68	68	56
12. *B. cereus* 4342	**83**	**84**	**84**	**84**	**83**	**83**	**84**	**83**	**83**	**83**	**83**	**100**	**94**	**83**	75	74	75	74	71	68	69	56
13. *B. thuringiensis* BGSC 4Y1	**83**	**83**	**83**	**83**	**83**	**83**	**83**	**83**	**83**	**83**	**83**	**94**	**100**	**83**	75	74	74	74	71	68	68	56
14. *B. cereus* ATCC 10987	**81**	**81**	**81**	**81**	**81**	**82**	**81**	**81**	**81**	**81**	**81**	**83**	**83**	**100**	74	73	73	73	70	68	68	57
15. *B. cereus* ATCC 14579	74	74	74	74	74	74	74	74	74	74	74	75	75	74	**100**	**89**	**88**	**89**	**83**	69	69	57
16. *B. cereus* ATCC 10876	73	73	73	73	73	74	73	73	73	73	73	74	74	73	**89**	**100**	**88**	**86**	**82**	68	68	56
17. *B. thuringiensis* BGSC 4BD1	74	74	74	74	74	74	74	74	74	74	74	74	74	73	**88**	**88**	**100**	**85**	**81**	69	69	55
18. *B. thuringiensis* ATCC 10792	73	73	73	73	73	73	73	73	73	73	73	74	74	73	**88**	**86**	**85**	**100**	**83**	68	68	56
19. *B. thuringiensis* ATCC 35646	71	71	71	71	73	71	71	71	71	71	71	72	72	71	**84**	**83**	**83**	**85**	**100**	67	67	54
20. *B. mycoides* ATCC 6462	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	66	**100**	**91**	56
21. *B. weihenstephanensis* KBAB4	68	69	68	68	68	68	68	68	68	68	68	69	68	68	69	68	68	68	66	**91**	**100**	58
22. *B. pseudomycoides* DSM 12442	55	56	56	55	56	56	55	56	55	56	55	56	56	56	56	55	56	56	53	56	57	**100**

Anthrax and its close neighbors are indicated in bold. The phylogenomic overview is based on average genomic core genome similarity values.

Table 3. Selected PCR assays for the *B. anthracis* ring trial

Reference	Marker	Primer/probe name	Sequences (5′-3′)	End concentration (uM)	PCR size (bp)	Cycling program		Final vol (ul)
Antwerpen et al.[21]	BA5345*	dhp61_183–113F	CGTAAGGACA ATAAAAGCCG TTGT	0.9	96	15 s 95 °C 1 min 55 °C	45×	20
		dhp61_183–208R	CGATACAGAC ATTTATTGGG AACTACAC	0.3				
		dhp61_183–143T	TGCAATCGAT GAGCTAATGA ACAATGACCCT	0.25				
Wielinga et al.[47]	PL3*	PL3_f	AAAGCTACAA ACTCTGAAAT TTGTAAATTG	0.2	139	5 s 95 °C 35 s 60 °C	45×	20
		PL3_r	CAACGATGAT TGGAGATAGA GTATTCTTT	0.2				
		Tqpro_PL3	AACAGTACGT TTCACTGGAG CAAAATCAA	0.1				
Letant et al.[46]	BA5357*	Forward	TTTCGATGAT TTGCAATGCC	1	105	5 s 95 °C 20 s 60 °C	45×	20
		Reverse	TCCAAGTTAC AGTGTCGGCA TATT	1				
		Probe	ACATCAAGTC ATGGCGTGAC TACCCAGACT T	0.08				
WHO[40]	*sap* (S-layer)	Upper 391–413	CGCGTTTCTA TGGCATCTCT TCT	0.2	639	30 s 95 °C 30 s 55 °C 30 s 72 °C	30×	20
		Lower 1029–1008	TTCTGAAGCT GGCGTTACAA AT	0.2				
Coker[35]	BA813	BA813-FP	GGAGGGAATA CAGCAAACAC AGA	16	123	15 s 95 °C 1 min 60 °C	40×	20
		BA813-RP	TGCAACTGAT GGGATTTCTT TCT	16				
		BA813-PR	AATGCCAGGT TCTATACCGT ATCAGCAAGCT ATTC	0.1				
WHO[44]	B-type SASP	ANT-F	GCTAGTTATG GTACAGAGTT TGCGAC	0.5		10 s 95 °C 20 s 57 °C 30 s 72 °C	45×	20
		ANT-Amt	CCATAACTGA CATTTGTGCT TTGAAT	0.5				
		ANT-FL	CAAGCAAACG CACAATCAGA AGCTAAG-FL	0.2				
		ANT-LC:Red640	LC RED640-GCGCAAGCTT CTGGTGCTAG C-P	0.2				
IAC	Bfp	ABbfp_F	TCATGGCCGA CAAGCAGAA	0.2	170	Assay dependence		
		ABbfp_R	GCTCAGGGCG GACTG	0.2				
		ABbfp_Tq	CGACC ACTACCAGCA GAACACC	0.2				

IAC, internal amplification control; Bfp, blue fluorescence protein. *DNA located on prophage region BA03.

all laboratories. In contrast, higher specificity (specificity ranging from 0.88 to 0.95, depending on laboratory, **Table 5**) was obtained with the current WHO recommended SASP assay.[44] This assay correctly identified most of the closely related strains, even though improper but late amplifications were sporadically observed for a few strains (ranging from 3 to 5). All *B. anthracis* strains were tested PCR-positive by two of the three laboratories that had succeeded to implement the assay on their PCR platforms. The WHO protocol relies on fluorescence resonance energy transfer (FRET) probes chemistry, but not all real-time PCR instruments have detection systems including a channel designated for FRET experiments. The third laboratory equipped with FRET-capabilities failed to detect five samples with lower DNA concentration (**Table 4**).

Limit of detection of the PL3 assay

In order to propose a single reference method for *B. anthracis* chromosome detection to diagnostic laboratories throughout Europe, we further assessed the laboratory sensitivity of one of the best performing assays identified in this work, the PL3 assay.[47] Serial dilutions of genomic DNA from *B. anthracis* strain 17JB were tested to determine the lowest concentration of DNA that could be detected at 95% probability. The detection limit (LOD_{PCR} at 95% confidence interval) was found to be 2 genome equivalents. Performance in artificially contaminated organs (wild boar spleen) was also examined using 10-fold dilutions of calibrated suspensions of vegetative cells. Non-inoculated samples were confirmed to be negative. A reproducible detection (100%, $n = 9$) of samples containing 11 vegetative cells/PCR was observed, corresponding to 10^3 *B. anthracis* CFU per ml of spleen homogenates. Samples containing fewer targets (i.e., 10^2 CFU/ml) could be sporadically detected (data not shown).

Discussion

PCR-based identification assays are fast and sensitive methods, widely used in food, clinical or veterinary laboratories to detect the presence of pathogens or to confirm species identity. Reliable detection requires the selection of primers and probes that hybridize efficiently and specifically with DNA from the targeted bacterium, in order to prevent false negative or

Table 4. Strain identities and PCR results of the ring trial on *B. anthracis* genome detection by PCR. Five laboratories participated in the ring trial

Species	Strain name	DNA ng/μl	BA5345 Antwerpen					PL3 Wielinga					BA5357 Letant					BA813 Coker					*sap* (S-layer) WHO 1998					B-type SASP WHO 2008				
B. anthracis	17JB	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	u	+	u
B. anthracis	08-1298	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	u	+	u
B. anthracis	09-1122	0.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	07-1371	0.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	u	+	u
B. anthracis	07-1167	0.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	−	u	+	u
B. anthracis	92-9066	0.1	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	+	−	+	−	u	+	u
B. anthracis	CIP 53.169	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	CIP 74.12	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	u	+	u
B. anthracis	CIP 81.89	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	−	u	+	u
B. anthracis	CIP A204	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	u	+	u
B. anthracis	CIP A205	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	u	+	u
B. anthracis	CIP A206	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	u	+	u
B. anthracis	CIP A211	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	u	+	u
B. cereus	ATCC 14579	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	u	+	u
B. cereus	06.1248	0.2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	u	−	u
B. cereus	08.1458	0.5	−	−	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. cereus	97-BC14	0.2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	−	−	−	−	−	u	−	u
B. cereus	00.624.49	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	−	−	−	−	−	−	−	u	−	u
B. cereus	97-BC17	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	u	−	u
B. cereus	97-BC18	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	u	+	u
B. cereus	97-BC59	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. cereus	CIP A28	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. cereus	CIP 63.81	0.1	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	u	−	u
B. cereus	CIP 70.1	0.5	−	−	−	−	−	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. gibsonii	CIP 104.720	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. licheniformis	ATCC 14580	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	u	−	u
B. subtilis	ATCC 6051	0.3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	d	−	−	−	−	+	−	−	−	−	−	−	−	u	−	u
B. anthracis	23932	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	56430	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	131959-5	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	u	+	u
B. anthracis	127491	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	188678-1	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	13185	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u

d, doubtful; u, unsuccesfully analyzed; +, PCR positive; –, PCR negative; ser, serovar; var, variant

Table 4. Strain identities and PCR results of the ring trial on *B. anthracis* genome detection by PCR. Five laboratories participated in the ring trial (continued)

Species	Strain name	DNA ng/μl	BA5345 Antwerpen					PL3 Wielinga					BA5357 Letant					BA813 Coker					*sap* (S-layer) WHO 1998					B-type SASP WHO 2008				
B. anthracis	128268	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. cereus	1847	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	u	–	u
B. anthracis	132064-1	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. atrophaeus	ATCC 9372	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	ATCC 11778	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10530	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10536	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10583	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10619	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	NVH0597-99	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	–	–	+	–	+	–	–	u	–	u
B. cereus	ATCC 10702	0.5	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10286	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	u	–	u
B. cereus	WSBC 10483	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10566	0.5	d	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	u	–	u
B. cereus	WSBC 10572	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	–	+	+	+	+	–	–	u	–	u
B. cereus	WSBC 10705	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	+	–	–	–	–	u	–	u
B. cereus	WSBC 10763	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–	u	–	u
B. cereus	WSBC 10770	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	–	–	–	–	–	–	+	u	–	u
B. cereus	ATCC 10987	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	+	–	–	–	–	–	–	–	u	–	u
B. coagulans		0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. pumilus	ATCC 27142	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. megaterium	ATCC 8245	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. subtilis	ATCC 6633	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis var *galleriae*	ATCC 29730	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *thuringiensis*	NRRL HD-2	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *aizawai*	NRRL HD-11	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *kurstaki*	NRRL HD-73	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. anthracis	NCTC 109	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	NCTC 8234	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	u	+	u
B. anthracis	NCTC 7753	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u

d, doubtful; u, unsuccesfully analyzed; +, PCR positive; –, PCR negative; ser, serovar; var, variant

Table 4. Strain identities and PCR results of the ring trial on *B. anthracis* genome detection by PCR. Five laboratories participated in the ring trial (continued)

Species	Strain name	DNA ng/μl	BA5345 Antwerpen					PL3 Wielinga					BA5357 Letant					BA813 Coker					*sap* (S-layer) WHO 1998					B-type SASP WHO 2008				
B. anthracis	NCTC 7752	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	NCTC 5444	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	NCTC 2620	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	u	+	u
B. anthracis	NCTC 1328	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	NCTC 10340	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	u	+	u
B. cereus	BGSC 6E1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *pulsiensis*	BGSC 4CC1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	u	–	u
B. thuringiensis ser *andalousiensis*	BGSC 4AW1	0.5	–	–	–	–	d	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	u	+	u
B. thuringiensis ser *pondicheriensis*	BGSC 4BA1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	u	+	u
B. thuringiensis ser *monterrey*	BGSC 4AJ1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	–	–	u	–	u
B. thuringiensis ser *huazhongensis*	BGSC 4BD1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	d	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *tochigiensis*	BGSC 4Y1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	+	–	u	+	u
B. megaterium	DSM 319	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	+	–	–	–	–	–	–	–	u	–	u
B. pumilus	ATCC 7061	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *Berliner*	ATCC 10792	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	+	–	–	–	–	+	–	–	u	–	u
B. weihenstephanensis	KBAB4	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	+	–	–	u	–	u
B. pseudomycoides	DSM 12442	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	d	–	–	–	–	–	–	+	–	–	u	–	u
B. cereus	ATCC 10876	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. mycoides	ATCC 6462	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. subtilis	NCTC 3610	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	u	–	u
B. subtilis	NCTC 10400	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	u	–	u
B. thuringiensis ser *israelensis*	ATCC 35646	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	u	–	u
B. cereus	ATCC 4342	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	+	–	–	u	–	u
B. thuringiensis ser *konkukian*	97-27	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	+	–	–	u	+	u
B. cereus	SJ1	0.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	d	+	–	–	–	–	+	–	–	u	+	u
B. anthracis	SVA-2008	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u
B. anthracis	SVA-2011	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	u	+	u

d, doubtful; u, unsuccesfully analyzed; +, PCR positive; –, PCR negative; ser, serovar; var, variant

Table 5. Diagnostic sensitivity (SE) and specificity (SP) values for the different assays and laboratories

PCR assay	Values for indicated laboratory # (95% confidence limits)									
	1		2		3		4		5	
	SE	SP	SE	SP	SE	SP	SE	SP	SE	SP
BA5345	1.00	0.98	0.94	1.00	1.00	1.00	1.00	1.00	1.00	0.97
	(0.89–1)	(0.91–1)	(0.79–0.99)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.88–1)
PL3	1.00	0.97	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.97
	(0.89–1)	(0.88–1)	(0.89–1)	(0.91–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.88–1)
BA5357	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95
	(0.89–1)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.94–1)	(0.89–1)	(0.86–0.99)
sap (S-layer)	0.97	0.69	0.52	0.81	1.00	0.56	1.00	0.69	1.00	0.58
	(0.83–1)	(0.56–0.81)	(0.33–0.70)	(0.69–0.90)	(0.89–1)	(0.42–0.69)	(0.89–1)	(0.56–0.81)	(0.89–1)	(0.44–0.70)
BA813	0.71	0.93	0.52	0.92	0.94	0.86	0.97	0.92	0.97	0.75
	(0.52–0.86)	(0.84–0.98)	(0.33–0.70)	(0.81–0.97)	(0.79–0.99)	(0.75–0.94)	(0.83–1)	(0.81–0.97)	(0.83–1)	(0.62–0.85)
B-type SASP	1.00	0.93	0.84	0.95	nd	nd	1.00	0.88	nd	nd
	(0.89–1.0)	(0.84-.98)	(0.66-.95)	(0.86-.99)			(0.89-.00)	(0.77-.95)		

nd, not determined

false-positive results. For the almost clonal species of *B. anthracis*, the selection of robust DNA signature sequences for the development of PCR assays has proven to be a very difficult task since few of the investigated markers proved to be truly unique for the species. At present, only three chromosomal features appeared to be useful to differentiate *B. anthracis* from the rest of the *B. cereus* group at the genetic level: (1) being part of the clonal cluster made up of highly monomorphic *B. anthracis* strains, as analyzed by MLST, MLVA or similar methods; (2) carrying a nonsense mutation at nucleotide position 640 of the *plcR* gene, introducing a premature TAA stop codon; and (3) presence of a unique combination of four excision-proficient, lambdoid prophages (lambda01–04).[4,16,66]

An unexpectedly high amount of PCR assays (~88 %) were found to be unspecific for *B. anthracis*. This is mostly because not much was known about the genetically closely related strains until the recent rapid increase in available genome sequences. The increasing use of Next Generation Sequencing technologies in systematic characterization of bacterial genomes has offered a powerful approach for large-scale genome comparisons and identification of specific DNA signatures. This is illustrated by the current study in which a thorough in silico analysis of published PCR assays for the detection of *B. anthracis* was possible due to the availability of manifold genome sequences. Conclusions drawn from this in silico analysis of the full set of *Bacillus* spp. genomes published to date were the following:

1) There was no PCR assay with superior specificity for any common target carried by the pXO1 or pXO2 virulence plasmids (*lef, cya, pag*, and *cap*), since several *B. cereus* strains were found to contain pXO-like plasmids carrying highly similar genes (data not shown), as was previously reported by others.[7-12]

2) Only two single-nucleotide differences appeared to be reliable markers for the specific identification of *B. anthracis*: a variant at nucleotide position 640 in the *plcR* gene or at position 1050 in the *purA* gene.

3) The four highly specific assays identified in silico (i.e., Antwerpen, Lewerin, Létant, and Wielinga) target three different loci located within the lambdaBa03 prophage region (ranging from BA5339 to BA5363 loci in the Ames annotated genome). All other markers that had been thought to discriminate *B. anthracis* from other *B. cereus* group bacteria were found in at least some closely related strains and could therefore result in erroneous species attribution, as exemplified by the BA813-targeted assays or the S-layer assay.[40]

Except for the recent SASP assay,[44] most of the published assays gave poor results in the in silico analysis (**Table 1**), including those referred to in the Terrestrial Manual of OIE,[67] i.e., Jackson et al.[27] and Ramisse et al.[31] However, to our knowledge, this is the first study addressing the in vitro evaluation of the SASP genomic markers. Our results should be confirmed on a larger panel of *Bacillus* strains to enable clear conclusions. Nevertheless, when standardizing PCR based detection methods for *B. anthracis*, the latter assay might be problematic with regard to its ease of implementation. The WHO protocol is based on a hybridization probes format for DNA detection and quantification by real-time PCR, and only a part of the qPCR instruments on the market currently includes detection system with decoupled excitation and emission filter channels that allow the use of hybridization probes (FRET) chemistry.[68] Hydrolysis probes are more commonly applied and thus form an alternative that should be more universally applicable.

Although excision proficient prophage sequences are generally not considered useful targets for bacterial identification because of their instability, the persistent presence of the four prophage regions in all *B. anthracis* genomes can be advantageously utilized for the definitive discrimination of *B. anthracis* from other *B. cereus* group bacteria.[66] Given the high impact of the anthrax identification issue, one must be cautious and avoid relying solely on assays based on SNP discrimination. Such assays are more sensitive to assay conditions compared with assays relying on unique

signature sequences, and the occurrence of false positive signals from *B. cereus* strains caused by mispriming is more likely. Even though various techniques have been evaluated to enhance the specificity of SNP-based PCR assays (including TaqMan mismatch amplification mutation assay,[23] restriction site insertion-PCR,[56] tentacle or locked nucleic acids probes-based PCR[25] or high resolution melting (HRM)-PCR[53]), they are neither as robust nor as user friendly as assays based on unique signature sequences. The chromosomal markers BA5345 (Antwerpen), PL3 (Wielinga), or BA5357 (Letant), enable unambiguous identification of *B. anthracis* strains, including plasmid-cured isolates. Moreover, the PL3 assay was confirmed to be sensitive enough to be used in biological samples. High diagnostic sensitivity of the assay reduces the occurrence of false-negative results, which can be further reduced by the use of an internal control to prevent pipetting errors. It should be emphasized that one of these assays should be implemented in conjunction with plasmid-encoded targets in *B. anthracis*-specific PCR methods to discriminate non-virulent from virulent strains.

In conclusion, this study highlights the importance of analyzing the diagnostic sensitivity and specificity of PCR assays designed for detection of *B. anthracis*, as many of particularly the older protocols produce both false negative and false-positive results. This is important with regard to the aim of standardization of a PCR assay for *B. anthracis* detection. Even though only slight differences regarding the analytical sensitivity were observed between the three highly specific chromosomal assays during the ring-trial, we propose the robust and sensitive PL3 assay as possible European standard to harmonize and improve PCR methods for detection of anthrax in animal, feed, environmental, and food samples based on results of this study.

Materials and Methods

Strains

DNA from a total of 90 *Bacillus* strains were used in this study, including 31 *B. anthracis* isolates, 44 strains of *B. cereus* or *B. thuringiensis*, and 15 strains encompassing 10 other bacterial species (**Table 4**). Strains came from the collections of *Bacilli* of the different partners: Anses (n = 27), SVA (n = 22), CVI (n = 9) and RIVM (n = 32). Of the 90 *B. cereus* group strains used for in vitro studies, 22 had publicly available whole genome sequences (**Table 2**), including 11 *B. cereus* or *B. thuringiensis* strains closely related to *B. anthracis* (**Table 2**) and reported as near-neighbors based on multilocus sequence typing analysis.[16] All DNA samples were randomly coded and sent to each of the 5 participating laboratories.

DNA extraction procedures

At Anses, *B. anthracis* suspensions were incubated at 100 °C in boiling water for 20 min. After cooling and centrifugation, viability testing was performed to verify absence of live *B. anthracis*. DNA from artificially contaminated samples was further purified using the High Pure PCR template Preparation Kit from Roche according to the manufacturer's recommendations. DNA from non-pathogenic non-*B. anthracis* bacilli cultures was alternatively extracted using a 200 µl aliquot of InstaGeneTM Matrix as described by the supplier (Bio-Rad Laboratories).

At CVI, bacterial suspensions were inactivated at 100 °C for 10 min and tested for absence of viable *B. anthracis* by plating aliquots on nutrient agar petri dishes. DNA was purified using the QIAamp DNA Mini Kit (Qiagen Benelux).

At RIVM, bacteria suspensions were incubated at 100 °C for 30 min, centrifuged at maximum speed for 1 min and the resulting lysates were transferred to a 0.22 µm sterile Ultrafree-MC spin filter (Millipore). The spin filter was then centrifuged for 4 min at maximum speed to clean the DNA lysate from left over cell debris. DNA lysates from *B. anthracis* and non-pathogenic bacteria were further purified or isolated, respectively using the NucliSENS Magnetic Extraction reagents (bioMerieux) following the manufacturer instructions.

At SVA, bacterial cultures were centrifuged and DNA extracted from the pellet using the MasterPure Gram positive kit (Epicenter Biotechnologies). The DNA was taken out of the BSL-3 facility by first passing it through an Ultrafree-MC 0.22 µm sterile filter (Merck Millipore).

Internal amplification control

A fragment of the blue fluorescent protein gene (*bfp*) was used as an internal amplification control (IAC). The IAC primers and probe were designed such that they do not interact with any of the primers and probes from the tested assays. Oligonucleotides design was performed by using the software package Visual Oligonucleotide Modeling Platform version 6 (DNA Software Inc.). The primers and probe were the following: ABbfp_F (5′-TCATGGCCGA CAAGCAGAA-3′), ABbfp_R (5′-GCTCAGGGCG GACTG-3′), and ABbfp_Tq (5′-Cy5-CGACCACTAC CAGCAGAACA CC-BHQ2-3′). Amplicons from the *bfp* gene were produced by using conventional PCR and were purified by using the Qiagen PCR purification kit. The amount of amplicons that need to be added to samples to obtain suitable Cq values for use as internal control was determined empirically from 10-fold serial dilutions. The developed real-time qPCR assays were used to determine the amplicon dilution needed for a Cq value between 32 and 35.

Conventional and real-time qPCR conditions

Participating laboratories were asked to investigate the complete set of blinded samples using the PCR platforms available at their institute. Real-time qPCR and conventional thermocyclers used were the following: Mx3005p (Stratagene); ABI 7500 Fast, StepOnePlus or AB9700 (Applied BioSystems); LightCycler 2.0 or LightCycler 480 (Roche Applied Science); C1000, iCycler or MyCycler (BioRad). Primers and probes were synthesized by each laboratory's usual suppliers (Eurogentec, Metabion, Sigma or Eurofins MWG operon). Total PCR reaction volume (20 µl) and template volume (2 µl of *Bacillus* DNA and 2 µl of the IAC DNA) were kept constant. Each laboratory also used the same qPCR kits and DNA polymerases as in their routine diagnostic activities. Five different commercially available or custom-made PCR kits (i.e., Taqman Universal PCR Master mix [Life Technologies], PerfeCta multiplex supermix [Quanta BioSciences], iQ Multiplex Powermix [Bio-Rad], VeriQuest qPCR fast master mix [affymetrix], and LightCycler FastStart DNA Master HybProbe [Roche Applied Science]) and 5 DNA polymerases (i.e., Fermentas true start, Quanta PerfeCta

Multiplex Super-mix, Tth DNA polymerase [Roche] in a custom-made mix [based on ref. 68], Go Taq DNA polymerase [Promega]) were used following manufacturer's instructions. The cycling program and primers/probe concentrations for each assay were those described in their original publication (as indicated in **Table 3**).

In silico analysis

Gegenees (http://www.gegenees.org) is open software that uses a fragmented alignment approach for the comparative analysis of hundreds of microbial genomes.[64] The genomes are fragmented and compared, all against all, by a multithreaded BLAST control engine. Each data point connecting two genomes is represented by a score. Although this genome alignment and data mining is the main application of Gegenees, it is also equipped with a primer alignment function that facilitates the alignment of several primers against a large amount of genomes for specificity testing.

The FTP-function of Gegenees was used to download all the available *Bacillus* spp. genomes from NCBI Genomes which, at the time of the study, amounted to 134 genomes. All primer/probe sequences from the literature survey were aligned to the 134 genomes with a short-sequence-setting (i.e., word length of 7) for the BLAST+ algorithm and the alignments were then sorted according to their "unalignment index". The unalignment index is the sum of non-aligned nucleotides and reported mismatches. A minimal unalignment index value of 0 for a primer corresponds to perfect sequence match with the genome the primer aligned to. Results have been acquired for all published sequences, regardless of the kind of assay reported (e.g., real-time qPCR, conventional PCR, LAMP, microarray, etc.) or targets used (pXO1-, pXO2-plasmid, or chromosomal DNA). Only data from chromosomal markers (n = 35) are reported in the present study (**Table 1**).

To illustrate the relatedness of the *B. cereus* group strains used in this study to *B. anthracis*, a whole genome comparison of the 22 available sequenced whole genome genomes was also performed (**Table 2**). Gegenees was set to perform an all-all fragmented alignment using 500 bp fragments. The average genomic core genome similarity values were also calculated (**Table 2**).

Ring trial

A ring trial was performed among 5 European laboratories in the framework of the EU AniBioThreat project (http://www.anibiothreat.com). Six published PCR-assays targeting different *B. anthracis* chromosomal markers were evaluated in vitro. The most specific methods according to in silico analysis[21,46,47] were compared with the assays recommended by the WHO[40,44] and a single assay targeting BA813.[35] Ninety blinded DNA samples were exchanged between partners and an IAC was distributed. A detailed standard operative protocol describing how to conduct and perform the ring trial was set up after consultation of all participating laboratories. Samples were re-tested in case of IAC inhibition. A reporting form file was distributed among participants to record the results.

Diagnostic sensitivity and specificity for all assays and laboratories were calculated together with the kappa values in SAS 9.1.3 (SAS Institute Inc.) using the FREQ procedure. The sensitivity was defined as the fraction of positive DNA samples which were known to contain *B. anthracis* (as determined by standard methods used by the different culture collections) that gave a positive PCR results by the different methods. Specificity was defined as the fraction of negative DNA samples which were known not to contain *B. anthracis* DNA that gave a negative PCR results by the different PCR methods. Kappa values measure the level of agreement between results obtained by the different participating laboratories and PCR methods combinations. The calculation is based on the difference between how much agreement is actually present ("observed" agreement) compared with how much agreement would be expected to be present by chance alone ("expected" agreement). A kappa value of 1 indicates perfect agreement, whereas a kappa of 0.5 indicates moderate agreement and a value of 0 indicates that the apparent agreement is only due to chance.[69]

Detection limit of the PL3 assay

The limit of detection of the PL3 assay[47] was determined by using serial dilutions of genomic DNA from *B. anthracis* strain 17JB. Six dilutions around the expected limit of detection (corresponding to 5, 2, 1, 0.5, 0.2, and 0.1 genome equivalents) were used to calculate a precise LOD_{PCR} value (3 runs, 24 replicates for each dilution).[70] Genomic DNA was quantified by fluorimetry using the Qubit® 2.0 Fluorometer (Invitrogen). The number of genomic copies was calculated as follows: $m = n \times (1.013 \times 10^{-21}$ g/bp), where m is the mass and n is the number of base pairs.

Wild boar spleen homogenates were used to assess the sensitivity of the assay in biological samples. Portions of 1 ml were artificially inoculated in triplicate at five contamination levels with calibrate suspensions of vegetative cells (ranging from 5.5×10^1 to 5.5×10^5 CFU/ml) from strain 17JB as previously described.[53] Samples were then incubated at 56 °C for 1 h in the presence of proteinase K and inactivated for 20 min at 100 °C in boiling water. After cooling and centrifugation, viability testing was performed to verify depletion of live *B. anthracis*. DNA was then extracted from 200 µl aliquots using the High Pure PCR Template Preparation Kit (Roche). Two microliter aliquots of the eluted DNA were used as template. The exact numbers of cells introduced into spleen homogenates were determined a posteriori by plating.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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