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METHODS AND PROTOCOLS

Development and validation of a real-time quantitative PCR assay for rapid identification of *Bacillus anthracis* in environmental samples

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Abstract A real-time polymerase chain reaction (PCR) assay was developed for rapid identification of *Bacillus anthracis* in environmental samples. These samples often harbor *Bacillus cereus* bacteria closely related *to B. anthracis*, which may hinder its specific identification by resulting in false positive signals. The assay consists of two duplex real-time PCR: the first PCR allows amplification of a sequence specific of the *B. cereus* group (*B. anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus pseudomycoides*, and *Bacillus mycoides*) within the phosphoenolpyruvate/sugar *phosphotransferase system I* gene and a *B. anthracis* specific single nucleotide polymorphism within the adenylosuccinate synthetase gene. The second real-time PCR assay targets the lethal factor gene from virulence plasmid pXO1 and the capsule synthesis gene from virulence plasmid pXO2. Specificity of the assay is

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enhanced by the use of minor groove binding probes and/or locked nucleic acids probes. The assay was validated on 304 bacterial strains including 37 *B. anthracis*, 67 *B. cereus* group, 54 strains of non-*cereus* group *Bacillus*, and 146 Gram-positive and Gram-negative bacteria strains. The assay was performed on various environmental samples spiked with *B. anthracis* or *B. cereus* spores. The assay allowed an accurate identification of *B. anthracis* in environmental samples. This study provides a rapid and reliable method for improving rapid identification of *B. anthracis* in field operational conditions.

Keywords *Bacillus anthracis* · LNA probe · PCR · Environmental samples

Introduction

Bacillus anthracis is a Gram-positive, rod-shaped spore-forming bacterium, which causes anthrax, a serious and often fatal disease of livestock and humans (Dixon et al. 1999). The potential use of aerosolized anthrax causative agent as a biological weapon agent is well documented (Zilinskas 1997). Deliberate release of anthrax spores in the aftermath of September 11, 2001 in USA (Harling et al. 2001; Jernigan et al. 2002) and multiplication of hoaxes worldwide (Leask et al. 2003) have underscored the critical importance of rapid, and non-hazardous methods for identification of anthrax causative agent in whatever samples, including environmental samples. Conventional culture represents the "gold standard" for identification of *B. anthracis*, a process that may take up to 48 h as it requires primary culture of the sample and subsequent testing on isolated colonies. Moreover, the assay needs to be conducted in a biosafety level 3 laboratory by specialist trained staff experienced in working with *B. anthracis*. The method is time consuming, and sometimes uncertain because other *Bacillus* species closely related to *B. anthracis* that are abundant in the environment can display one or more of these features (Beyer et al. 1995; Hoffmaster et al. 2004; Klee et al. 2006; Marston et al. 2006; Hu et al. 2009). *B. anthracis* is indeed a member of a closely related phylogenetic cluster referred to as the *Bacillus cereus* group. This cluster includes *B. anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus mycoides, Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* (Helgason et al. 2000; Valjevac et al. 2005). Given their potential for diagnosis, molecular diagnostic tools have been developed and are now widely used by many laboratories for rapid identification of *B. anthracis* (Ramisse et al. 1996; Ryu et al. 2003; Kim et al. 2005; Park et al. 2007). Most of these methods are based on detection of *B. anthracis* pathogenicity-related plasmids pXO1 and pXO2. However, data gathered in the last decade have consistently shown that the pXO1 and pXO2 genes are not specific of *B. anthracis* (Pannucci et al. 2002; Hoffmaster et al. 2004, 2006; Klee et al. 2006). The use of chromosomal markers in the assay, in addition to virulence plasmids, would allow to get insight into the genetic background of the strain involved, and thus improve the discrimination between *B. anthracis* strains (virulent or non-virulent) and other *B. cereus* strains. Accordingly, several chromosomal markers have been described and used for specific identification of *B. anthracis* (Patra et al. 1996; Qi et al. 2001; Easterday et al. 2005; Antwerpen et al. 2008). However, experience showed that most of these chromosomal markers are common to both *B. anthracis* and *B. cereus* or at least a subpopulation of the *B. cereus* group and discrimination between them often relies upon single nucleotide polymorphisms (SNPs) within these genes. But so far, most of SNPs have failed to totally discriminate between *B. anthracis* and some *B. cereus* group members. We report here the design and validation of a new real-time polymerase chain reaction (PCR) assay for specific detection of *B. anthracis*. The assay is based on amplification of an original chromosomal marker harboring SNPs which allow the discrimination of *B. anthracis* strains from non-*B. anthracis*. The assay was validated on a collection of *B. anthracis* and *B. cereus* group members from various laboratories and subsequently used for detection of *B. anthracis* in 50 environmental masked samples (soils, burned leaves, powders, and air filter membranes) randomly spiked with inactivated *B. anthracis* spores and/or with *B. cereus* group spores. Data obtained show that this novel assay is a reliable surrogate for specific identification of *B. anthracis* in environmental samples.

Materials and methods

Strains used in this study

A panel of 304 bacterial strains was used in this study. These included 37 *B. anthracis*, 67 *B. cereus* group, 54 *Bacillus* spp. outside the *B. cereus* group (Table 1), as well as 146 strains of other Gram-positive and Gram-negative bacteria (Online Resource Table 1).

Spiking of inactivated *B. anthracis* spores in soil samples

Spiking of environmental samples was carried out at the US Army Dugway Proving Ground facility. Strains of *B. anthracis* spores (Ames A363001, Ames A3631750, Vollum 1B, Sterne and Delta-Sterne) were inactivated with cobalt (γ) irradiation: 4.1×10E+06 rad for 125 min (total dose). To check for viability, TSA plates were inoculated with 100 μL of inactivated product and

Table 1 *Bacillus* strains used in this study

Name	Original identification	Source	Plasmid pX01/ pXO2 content	RT-qPCR results			G→A Substitution *pur*A gene
				pXO1 target	pXO2 target	Chromosomal *pts*I allele	
Bacillus anthracis	A22	BW	+/+	+	+	+	+
Bacillus anthracis	A37	BW	+/+	+	+	+	+
Bacillus. anthracis	A40	BW	+/+	+	+	+	+
Bacillus. anthracis	A42	BW	+/+	+	+	+	+
Bacillus anthracis	A58	BW	−/−	−	−	+	+
Bacillus. anthracis	64/12B	BW	+/+	+	+	+	+
Bacillus. anthracis	JF3783	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3784	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3785	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3786	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3787	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3788	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3851	Ub	+/+	+	+	+	+
Bacillus. anthracis	JF3852	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3853	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3854	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3887	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3888	Ub	+/+	+	+	+	+
Bacillus anthracis	JF3889	Ub	+/+	+	+	+	+
Bacillus anthracis	A15	Ub	+/−	+	−	+	+
Bacillus anthracis	A73202	Ub	−/+	−	+	+	+
Bacillus anthracis	Sterne	Ub	+/−	+	−	+	+
Bacillus anthracis	Fildes	Ub	−/−	−	−	+	+
Bacillus anthracis	St-Mary	Ub	−/+	−	+	+	+
Bacillus anthracis	CEB 9434	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9439	BW	+/−	+	−	+	+
Bacillus anthracis	CEB 9440	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9501	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9506	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9508	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9602	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9606	BW	+/+	+	+	+	+
Bacillus anthracis	CEB 9774	BW	+/+	+	+	+	+
Bacillus anthracis	ATCC 6602	UCL	−/+	−	+	+	+
Bacillus anthracis	CEB 9606	UCL	+/−	+	−	+	+
Bacillus anthracis	CEB 8534	UCL	−/+	−	+	+	+
Bacillus anthracis	CEB 227	CEB	+/+	+	+	+	+
Bacillus cereus	DSM 345	BW	−/−	−	−	+	−
Bacillus cereus	DSM 3101	BW	−/−	−	−	+	−
Bacillus cereus	DSM 4384	BW	−/−	−	−	+	−
Bacillus cereus	DSM 4312	BW	−/−	−	−	+	−
Bacillus cereus	DSM 4313	BW	−/−	−	−	+	−
Bacillus cereus	DSM 4282	BW	−/−	−	−	+	−
Bacillus cereus	DSM 2301	BW	−/−	−	−	+	−
Bacillus cereus	DSM 2302	BW	−/−	−	−	+	−
Bacillus cereus	DSM 2046	BW	−/−	−	−	+	−

Table 1 (continued)

Name	Original identification	Source	Plasmid pX01/ pXO2 content	RT-qPCR results			G→A Substitution *pur*A gene
				pXO1 target	pXO2 target	Chromosomal *pts*I allele	
Bacillus cereus	DSM 1	BW	−/−	−	−	+	−
Bacillus cereus	DSM 2109	BW	−/−	−	−	+	−
Bacillus mycoides	DSM 43227 T	BW	−/−	−	−	+	−
Bacillus mycoides	NCTC 2603	BW	−/−	−	−	+	−
Bacillus mycoides	NCTC 926	BW	−/−	−	−	+	−
Bacillus mycoides	NCTC 7586	BW	−/−	−	−	+	−
Bacillus mycoides	NCTC 7973	BW	−/−	−	−	+	−
Bacillus mycoides	WSBC 10211	BW	−/−	−	−	+	−
Bacillus mycoides	WSBC 10294	BW	−/−	−	−	+	−
Bacillus mycoides	WSBC 10295	BW	−/−	−	−	+	−
Bacillus mycoides	WSBC 10296	BW	−/−	−	−	+	−
Bacillus mycoides	WSBC 10276	BW	−/−	−	−	+	−
Bacillus pseudomycoides	B520	BW	−/−	−	−	+	−
Bacillus pseudomycoides	B521	BW	−/−	−	−	+	−
Bacillus pseudomycoides	B522	BW	−/−	−	−	+	−
Bacillus thuringiensis	B508	BW	−/−	−	−	+	−
Bacillus thuringiensis	B509	BW	−/−	−	−	+	−
Bacillus thuringiensis	B510	BW	−/−	−	−	+	−
Bacillus thuringiensis	B511	BW	−/−	−	−	+	−
Bacillus thuringiensis	B512	BW	−/−	−	−	+	−
Bacillus thuringiensis	B513	BW	−/−	−	−	+	−
Bacillus thuringiensis	B526	BW	−/−	−	−	+	−
Bacillus thuringiensis	B538	BW	−/−	−	−	+	−
Bacillus thuringiensis	WSBC 10206	BW	−/−	−	−	+	−
Bacillus thuringiensis	WSBC 10208	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 10278	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 10279	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 10291	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 10292	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 10293	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 28001	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 28003	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 28005	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 28009	BW	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC 28012	BW	−/−	−	−	+	−
Bacillus thuringiensis	CEB 9727	CEB	−/−	−	−	+	−
Bacillus cereus	ATCC 10987	UCL	−/−	−	−	+	−
Bacillus cereus	ATCC 14579	UCL	−/−	−	−	+	−
Bacillus thuringiensis	4Q2-72	UCL	−/−	−	−	+	−
Bacillus mycoides	MYC003	UCL	−/−	−	−	+	−
Bacillus mycoides	NRS306	UCL	−/−	−	−	+	−
Bacillus thuringiensis	T03A016	UCL	−/−	−	−	+	−
Bacillus weihenstephanensis	WSBC10240	UCL	−/−	−	−	+	−
Bacillus mycoides	KNC 1-2	UCL	−/−	−	−	+	−
Bacillus mycoides	KNC 2-13	UCL	−/−	−	−	+	−
Bacillus mycoides	KNC 1-10	UCL	−/−	−	−	+	−

Table 1 (continued)

Name	Original identification	Source	Plasmid pX01/ pXO2 content	RT-qPCR results			G→A Substitution *pur*A gene
				pXO1 target	pXO2 target	Chromosomal *pts*I allele	
Bacillus mycoides	MYC 004	UCL	−/−	−	−	+	−
Bacillus mycoides	BD 23	UCL	−/−	−	−	+	−
Bacillus mycoides	KBS 1-4	UCL	−/−	−	−	+	−
Bacillus mycoides	KBS 2-12	UCL	−/−	−	−	+	−
Bacillus mycoides	KBS 317	UCL	−/−	−	−	+	−
Bacillus mycoides	KNC 2-16	UCL	−/−	−	−	+	−
Bacillus mycoides	KNC 2-18	UCL	−/−	−	−	+	−
Bacillus mycoides	MYC 005	UCL	−/−	−	−	+	−
Bacillus mycoides	B 3436	UCL	−/−	−	−	+	−
Bacillus mycoides	NRS 1216	UCL	−/−	−	−	+	−
Bacillus mycoides	NRS 321	UCL	−/−	−	−	+	−
Bacillus pseudomycoides	BD 5	UCL	−/−	−	−	+	−
Bacillus alvei	DSM 50905	BW	−/−	−	−	−	−
Bacillus alvei	WSBC 10365	BW	−/−	−	−	−	−
Bacillus alvei	NCTC 3349	BW	−/−	−	−	−	−
Bacillus brevis	WSBC 10379	BW	−/−	−	−	−	−
Bacillus brevis	NCTC 7577	BW	−/−	−	−	−	−
Bacillus circulans	DSM 8579	BW	−/−	−	−	−	−
Bacillus circulans	DSM 2046	BW	−/−	−	−	−	−
Bacillus circulans	WSBC 10088	BW	−/−	−	−	−	−
Bacillus circulans	NCTC 5846	BW	−/−	−	−	−	−
Bacillus circulans	ATCC 9966	BW	−/−	−	−	−	−
Bacillus circulans	NCTC 7577	BW	−/−	−	−	−	−
Bacillus coagulans	DSM 1103	BW	−/−	−	−	−	−
Bacillus coagulans	DSM 43214	BW	−/−	−	−	−	−
Bacillus coagulans	NCTC 3992	BW	−/−	−	−	−	−
Bacillus coagulans	ATCC 10545	BW	−/−	−	−	−	−
Bacillus firmus	DSM 2899	BW	−/−	−	−	−	−
Bacillus firmus	WSBC 10311	BW	−/−	−	−	−	−
Bacillus laterosporum	ATCC 9141	BW	−/−	−	−	−	−
Bacillus laterosporus	WSBC 10364	BW	−/−	−	−	−	−
Bacillus lentus	B371	BW	−/−	−	−	−	−
Bacillus lentus	B387	BW	−/−	−	−	−	−
Bacillus licheniformis	DSM 2048	BW	−/−	−	−	−	−
Bacillus licheniformis	B745	BW	−/−	−	−	−	−
Bacillus licheniformis	B746	BW	−/−	−	−	−	−
Bacillus megaterium	B327	BW	−/−	−	−	−	−
Bacillus megaterium	DSM 13	BW	−/−	−	−	−	−
Bacillus megaterium	B372	BW	−/−	−	−	−	−
Bacillus megaterium	WSBC 10380	BW	−/−	−	−	−	−
Bacillus megaterium	WSBC 10389	BW	−/−	−	−	−	−
Bacillus megaterium	B753	BW	−/−	−	−	−	−
Bacillus megaterium	DSM 333	BW	−/−	−	−	−	−
Bacillus polymyxa	DSM 20296 T	BW	−/−	−	−	−	−
Bacillus polymyxa	DSM 2048	BW	−/−	−	−	−	−
Bacillus polymyxa	WSBC 10029	BW	−/−	−	−	−	−

Table 1 (continued)

Name	Original identification	Source	Plasmid pX01/ pXO2 content	RT-qPCR results			G→A Substitution *pur*A gene
				pXO1 target	pXO2 target	Chromosomal *pts*I allele	
Bacillus polymyxa	WSBC 10256	BW	−/−	−	−	−	−
Bacillus polymyxa	WSBC 10275	BW	−/−	−	−	−	−
Bacillus polymyxa	WSBC 10359	BW	−/−	−	−	−	−
Bacillus polymyxa	WSBC 10360	BW	−/−	−	−	−	−
Bacillus pumilis	WSBC 10031	BW	−/−	−	−	−	−
Bacillus pumilis	WSBC 10045	BW	−/−	−	−	−	−
Bacillus pumilis	WSBC 10067	BW	−/−	−	−	−	−
Bacillus pumilis	WSBC 10396	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 2608	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 5896	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 7582	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 7585	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 9602	BW	−/−	−	−	−	−
Bacillus sphaericus	NCTC 10338	BW	−/−	−	−	−	−
Bacillus subtilis	DSM 4631 T	BW	−/−	−	−	−	−
Bacillus subtilis	DSM 5934	BW	−/−	−	−	−	−
Bacillus subtilis	ATCC10401	BW	−/−	−	−	−	−
Bacillus subtilis	WSBC 10202	BW	−/−	−	−	−	−
Bacillus subtilis	WSBC 10203	BW	−/−	−	−	−	−
Bacillus subtilis	DSM 1092	BW	−/−	−	−	−	−

BW Bundeswehr (Deutschland), *Ub* University of Berne (Switzerland), *UCL* Université Catholique de Louvain (Belgium), *CEB* Centre d'Etude du Bouchet (France)

incubated for 48 h at 37 °C. Plates were examined daily for growth detection viability.

The real-time PCR assay was used for *B. anthracis* identification on a panel of masked environmental samples 14 soils (2 g each), eight filter membranes (10 cm diameter), eight liquids filtrated from these membranes (2 mL each), ten liquids containing burned leaves (10 mL each), and ten powders (talcum, yeast, creamer, baking soda, and Dipel®; 2 g each) that were spiked with inactivated *B. anthracis* spores (2×10^7, 2×10^5, 2×10^4, and 2×10^3). These samples were obtained in the frame of round robin SIBA (Sample Identification of Biological Agents) exercises of identification of biological agents, which are annually performed by NATO member countries.

DNA extraction

DNA was extracted from cells of one single *B. cereus* American Type Culture Collection (ATCC) 14579 colony grown at 37 °C on blood sheep agar for 16 h, using the Nucleospin® kit (Macherey Nagel, Germany) according to manufacturer's instructions. The resulting DNA was eluted in Tris-HCl buffer (pH 8) and stored at −20 °C until use as template in PCR.

DNA from *B. anthracis* and *B. cereus* strains were obtained from the collection of the Microbiology Unit (Université catholique de Louvain, Louvain-la-Neuve, Belgium), Institut für Veterinär Bakteriologie (Berne Universität, Switzerland), Bundeswehr Institute of Microbiology (Munich, Deutschland), and Centre d'Etudes du Bouchet (Vert-le-petit, France). DNA from other bacteria was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen and from the ATCC.

DNA extraction from environmental samples

The PowerMax™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA) was used to extract DNA from the 14 soil samples according to the manufacturer's instructions. DNA from other environmental samples (36 samples) was extracted using the NucliSens® miniMag semi-automated apparatus (Biomérieux Inc., Boxtel, The Netherlands), according to the manufacturer's instructions. Filter membranes samples were first grinded using FastPrep® before DNA was extracted using the NucliSens® miniMag, as described

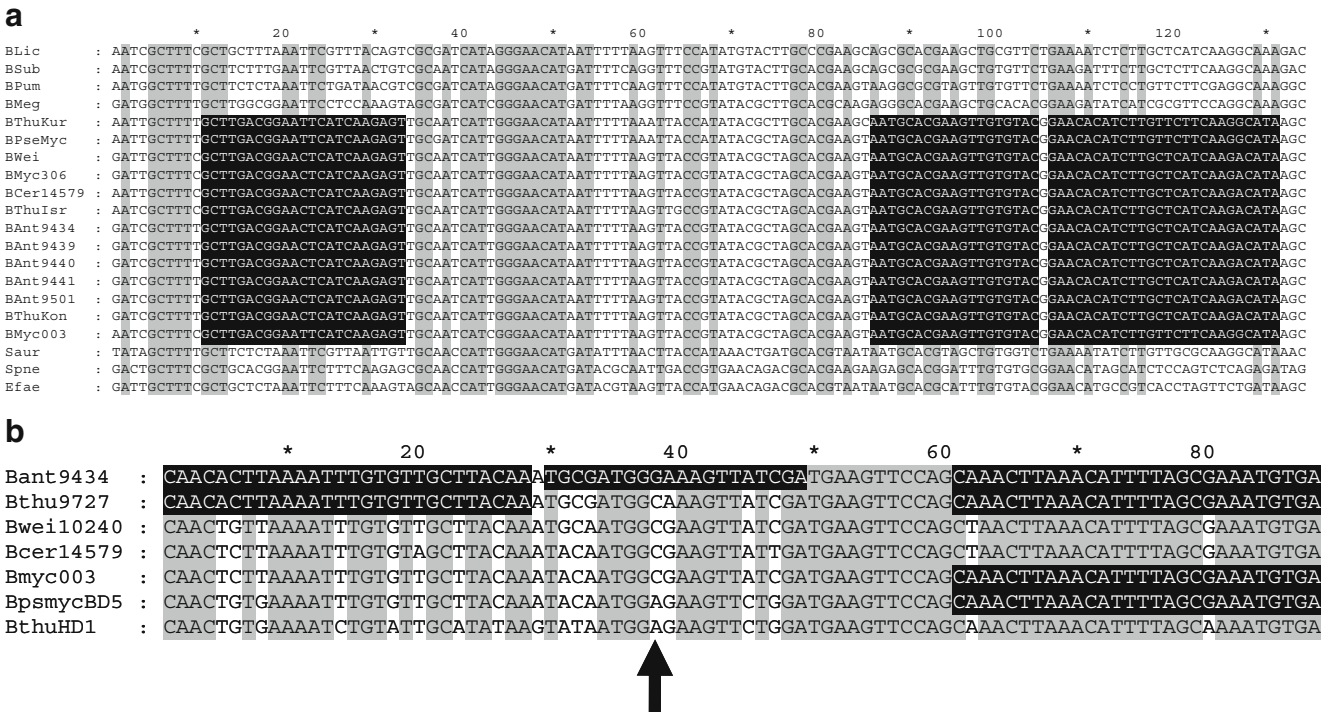

Fig. 1 **a** Multiple alignments of *pts*I sequences from *Bacillus cereus* group and other non-*B. cereus* as well as few other Gram-positive. *Dark boxes*, *from left to right* BC forward primer, BC probe, BC reverse primer. *BLic*, *Bacillus licheniformis* strain 352; *BSub*, *Bacillus subtilis* strain 299; *BPum*, *Bacillus pumilus* strain 592; *BMeg*, *Bacillus megaterium* strain 327; *BThuKur*, *Bacillus thuringiensis* serovar Kurstakii strain HD-1; *BPseMyc*, *Bacillus pseudomycoides* strain BD-05; *BWei*, *Bacillus weihenstephanensis* strain WS2480; *BMyc306*, *Bacillus mycoides* strain NRS-306; *BCer14579*, *Bacillus cereus* ATCC 14579; *BThuIsr*, *Bacillus thuringiensis* serovar Israelensis 4Q2-72; *BAnt9434, BAnt9439, BAnt9440, BAnt9441, BAnt9501*, *Bacillus anthracis* strains 9434, 9439, 9440, 9441, and 9501; *BThuKon*, *Bacillus thuringiensis* serovar Konkunkian strain 97-27; *BMyc003*, *Bacillus mycoides* strain MYC003; *Saur*, *Staphylococcus aureus* ATCC 35884; *Spne*, *Streptococcus pneumoniae* ATCC 33400; *Efae*, *Enterococcus faecalis* ATCC 29212. **b** Multiple alignments of *pur*A amplicons from *Bacillus* of the *cereus* group. *Dark boxes*, *from left to right* BA forward primer, BA specific probe, BA reverse primer. The *arrow* highlights the nucleotide C/A→G SNP in BA compared to other members of BC cluster. *Bant9434*, *Bacillus anthracis* strain CEB 9434; *Bthu9727*, *Bacillus thuringiensis* serovar Konkunkian strain CEB 97-27; *Bwei10240*, *Bacillus weihenstephanensis* strain UCL WS2480; *Bcer14579*, *Bacillus cereus* strain UCL ATCC14579; *Bmyc003*, *Bacillus mycoides* strain UCL MYC003; *BpsmycBD5*, *Bacillus pseudomycoides* strain UCL BD05; and *BthuHD1*, *Bacillus thuringiensis* serovar Kurstakii strain UCL HD-1

above. Extracted DNA was eluted in 50 µL of nuclease-free elution buffer and stored at −20 °C until used as template in PCR.

DNA quantification and serial dilutions

Real-time PCR and primers

The *pts*I and adenylosuccinate synthetase (*pur*A) genes (accession number AE017225) were selected from a panel of molecular targets, previously described for the development of an assay for identification of Gram-positive and Gram-negative bacteria (patent WO/2005/090596). The *pts*I gene codes for the Enzyme I of the phosphoenolpyruvate: sugar phosphotransferase system which catalyses the phosphorylation of Hpr protein (encoded by *pts*H gene) by phosphoenolpyruvate (Gonzy-Tréboul 1987). The *pur*A gene codes for the adenylsuccinate synthetase, an enzyme which catalyzes the Mg^{2+} dependent condensation of a molecule of inosine monophosphate with aspartate to form adenylosuccinate (Mehrotra et al. 2007). Sequences alignments of the *pts*I, performed as described by Thompson et al. (1994) revealed that this gene was highly conserved among strains in the *B. cereus* group (Fig. 1a). Regarding the *pur*A target, two *B. anthracis*-specific point mutations (SNPs) were identified in the gene: the C/A→G mutation at position 1,050 and the mutation G→A at position 1,051 of *B. anthracis*, when compared to all *B. cereus* group, excepted for *B. thuringiensis* serovar Konkukian 97-27, which only displayed the C/A→G at position 1,050 (Fig. 1b). The 1050 C/A→G mutation, (third position in the 350th codon of *pur*A protein) is neutral whereas the 1,051 G→A mutation (first position of the 351st codon of *pur*A protein) results an aminoacid substitution, (with glutamic acid in *B. cereus* being replaced by lysine in *B. anthracis*). excepted for *B. thuringiensis* serovar Konkunkian which does not harbor the 1,051 G→A mutation. The primers and the probe for the specific amplification and the detection of

Table 2 Primers and probes used in this study

Type	Target	Name	Sequence (5′→3′)	Position	Reference
Primer	*pur*A	PUR-ANT-FOR	CAACACTTAAAATTTGTGTTGCTTACAA	5208833–5208806	AE017225
Primer		PUR-ANT-REV	TCACATTTCGCTAAAATGTTTAAGTTTG	5208746–5208773	
Fluorogenic MGB-Probe		PUR-ANT-PRO	FAM-CGATGGGAAAGTTAT-NFQ-MGB	5208802–5208788	
Fluorogenic LNA-Probe		LNA-BA1	FAM-TCGATAACTTTCCCATCGCA-NFQ	5208785–5208804	
Non-fluorogenic C-probe		C-probe-BA	TGCGATGGMAAAGTTATCGATGAAGT	5208804–5208779	
Primer	*pts*I	PTS-BAC-FOR	GCTTGACGGAAYTCATCAAGAGT	3902805–3902827	AE017225
Primer		PTS-BAC-REV	TATGYCTTGAWGARCAAGATGTGTTC	3902925–3902900	
Fluorogenic MGB-Probe		PTS-BAC-PRO	VIC-ACAACTTCGTGCATT-NFQ-MGB	3902894–3902880	
LNA-Probe		LNA-BC2	YakimaYellow-GTACACAACTTCGTGCATT-NFQ	3902898–3902880	
Primer	pXO1	Lef-1-FOR	CATCGGTCTGGAAATAAAGGATGTA	150116–150092	AE011190
Primer		Lef-2-REV	GCACTACTTTCGCATCAATCCTTATA	150037–150062	
Fluorogenic MGB-Probe		pXO_1-LEF-PRO	FAM-CTTTTTCGGATTGCTTA-NFQ-MGB	150067–150083	
Fluorogenic LNA-Probe		LNA-pXO1	FAM-TCTTTTTCGGATTGCTTA-BHQ1	150066 - 150083	
Primer	pXO2	CapA-1-FOR	TGACGATGGTTGGTGACATTATG	54939–54917	AE011191
Primer		CapA-2-REV	AATCTGTACCGTAACGATTAACAATCTC	54871–54898	
Fluorogenic MGB-Probe		pXO_2-CAPa-PRO	VIC-TTTACGTGACGTCCCA-NFQ-MGB	54900–54915	
Fluorogenic LNA-Probe		LNA-pXO2	Yakima-Yellow-TTTACGTGACPTCCCATCA-BHQ1	54900–54918	

Letters M, W, Y and R correspond to nucleotides A/C, A/T, C/T, and A/G respectively, according to the IUPAC code. Underscored nucleotides correspond to locked nucleic acids

NFQ non-fluorescent quencher

B. anthracis were manually designed after alignment of all *B. cereus* group *pur*A gene sequences available. The *B. anthracis* probe was selected to encompass the specific C/A→G and G→A SNP on the *pur*A gene. Similarly, consensus primers and the probe for the amplification and the detection of all members of the *B. cereus* group were designed for the amplification of the *pts*I gene. In addition, the lethal factor gene (*lef*) of virulence plasmid pXO1 (accession number AE011190), and the capsule biosynthesis protein gene (*cap*) from virulence plasmid pXO2 (Accession number AE011191) were used as targets for specific amplification and detection of the pXO1 and pXO2 plasmids, respectively. Primers and probes were designed using the Primer Express™ Software (version 3.0, Applied Biosystems).

Table 3 BA/BC duplex Real-time PCR of 250 pg of target: Ct values (n=9) obtained with different probes used in this study

Type of probe	Probe conc. (nM)	C-probe conc. (nM)	*Bacillus anthracis* strain 9501		*Bacillus mycoïdes* strain KNC3-1		*Bacillus thuringiensis konkukian* strain 97-27	
			FAM	VIC[a]	FAM	VIC[a]	FAM	VIC[a]
MGB	100	0	23.48±0.04	26.51±0.03	40	24.76±0.18	25.87±0.45	24.51±0.13
		600	23.57±0.08	26.87±0.55	40	24.79±0.24	40	24.93±0.32
		150	23.22±0.16	26.62±0.45	40	24.81±0.12	31.97±1.37	24.57±0.07
		37.5	23.44±0.52	25.19±0.11	40	25.46±0.22	26.24±0.34	24.33±0.65
LNA	100	150	24.46±0.37	25.86±0.14	40	24.24±0.12	40	24.42±0.24
	50	150	24.88±0.30	26.05±0.34	40	24.43±0.05	40	24.47±0.08
		37.5	23.97±0.19	26.32±0.37	40	24.43±0.23	40	24.45±0.34
	25	37.5	24.52±0.16	26.24±0.11	40	24.38±0.24	40	24.67±0.07
		0	24.70±0.20	26.62±0.37	40	24.90±0.10	36±0.50	24.94±0.17

[a] VIC fluorophore was replaced by Yakima Yellow in LNA probes (same excitation and emission wavelengths)

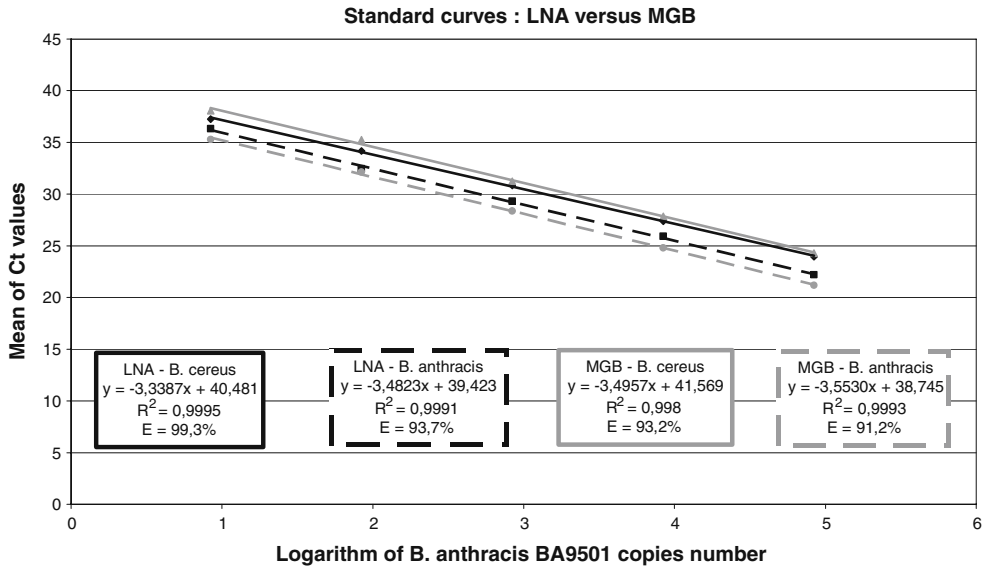

Fig. 2 Comparison of BA/BC MGB and LNA probes

Specificity of primers and probes was assessed *in silico* against the NCBI (GenBank) nucleotide sequence databases by using BLASTN (Altschul et al. 1997). Primers used in this study (Table 2) were purchased from Eurogentec (Ougrée, Belgium).

MGB probes

The minor groove binding (MGB) probes were selected for the assay since their use in the 5′Taq nuclease assays was demonstrated to have lower background fluorescence and improved specificity compared to non-MGB probes (Kutyavin et al. 2000).

The *B. anthracis pur*A, as well as the pXO1 MGB probes, were synthesized with the fluorescent reporter 6-carboxyfluorescein (FAM) covalently coupled to the 5′-end. At the 3′-end, MGB probes included a MGB group and a non-fluorescent quencher. The *B. cereus* consensus MGB probe and the capsule synthesis (*cap*A) gene probe were labeled with the VIC reporter at their 5′-end. MGB probes were purchased from Applied Biosystems (Foster City, USA; Table 2).

Locked nucleic acids probes

Locked nucleic acids (LNA) probes are modified nucleic acids in which the sugar has been conformationally locked, imparting unprecedented hybridization affinity towards DNA and RNA (Kumar et al. 1998). LNA containing oligonucleotides have been reported to improve

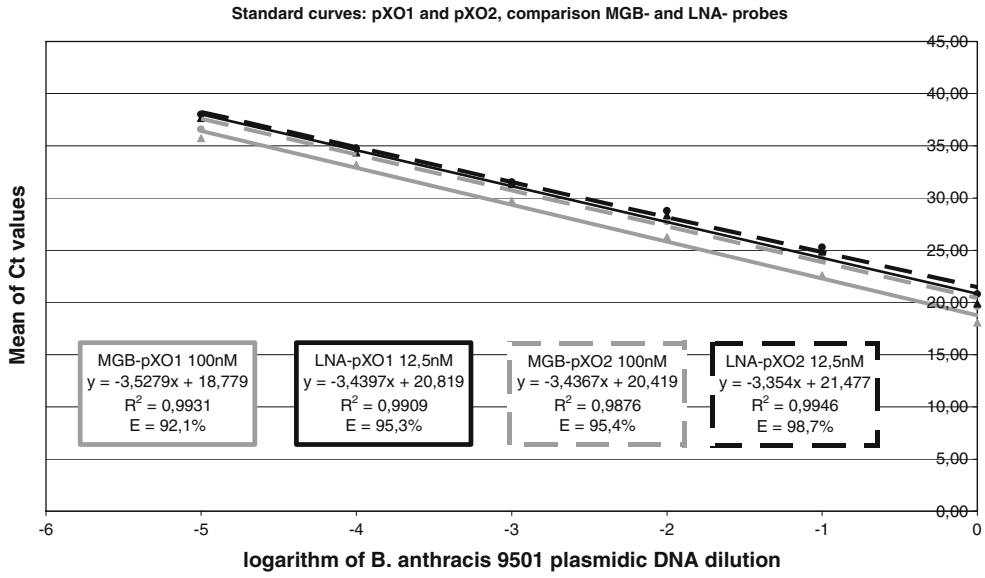

Fig. 3 Comparison of pXO1/pXO2 MGB and LNA probes

Table 4 Identification of BA in environmental samples using the two duplex Real-time PCR

Sample *N*°	Sample type	Spiking status	Chromosomal targets		Virulence plasmids		Final real-time PCR identification
			*pts*I	*pur*A	pXO1 *lef*A	pXO2 *cap*A	
1	Soil-01	Nihil	−	−	−	−	Negative
2	Soil-02	Nihil	−	−	−	−	Negative
3	Soil-03	BA (Ames strain) spores 2×10^5	+	+	+	+	BA pXO1+/pXO2+
4	Soil-04	Nihil	−	−	−	−	Negative
5	Soil-05	Nihil	−	−	−	−	Negative
6	Soil-06	Nihil	−	−	−	−	Negative
7	Soil-07	*B. thuringiensis* (powder)	+	−	−	−	BC group
8	Soil-08	BA (Ames strain) spores 2×10^7	+	+	+	+	BA pXO1+/pXO2+
9	Soil-09	Nihil	−	−	−	−	Negative
10	Soil-10	Nihil	−	−	−	−	Negative
11	Soil-11	Nihil	−	−	−	−	Negative
12	Soil-12	Nihil	−	−	−	−	Negative
13	Soil-13	BA (Ames strain) spores 2×10^4	+	+	+	+	BA pXO1+/pXO2+
14	Soil-14	Nihil	−	−	−	−	Negative
15	FM-01	Nihil	−	−	−	−	Negative
16	FM-02	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
17	FM-03	Nihil	−	−	−	−	Negative
18	FM-04	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
19	FM-05	Nihil	−	−	−	−	Negative
20	FM-06	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
21	FM-07	Nihil	−	−	−	−	Negative
22	FM-08	Nihil	−	−	−	−	Negative
23	LF-01	Nihil	−	−	−	−	Negative
24	LF-02	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
25	LF-03	Nihil	−	−	−	−	Negative
26	LF-04	Nihil	−	−	−	−	Negative
27	LF-05	Nihil	−	−	−	−	Negative
28	LF-06	Nihil	−	−	−	−	Negative
29	LF-07	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
30	LF-08	BA (Vollum strain) spores 3×10^7	+	+	+	+	BA pXO1+/pXO2+
31	LBLS-01	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
32	LBLS-02	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
33	LBLS-03	BA (Delta Sterne strain) spores	+	+	−	−	BA pXO1-/pXO2-
34	LBLS-04	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
35	LBLS-05	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
36	LBLS-06	Nihil	−	−	−	−	Negative
37	LBLS-07	Nihil	−	−	−	−	Negative
38	LBLS-08	Nihil	−	−	−	−	Negative
39	LBLS-09	Nihil	−	−	−	−	Negative
40	LBLS-10	Nihil	−	−	−	−	Negative
41	Powder[a]-01	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
42	Powder-02	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
43	Powder-03	Nihil	−	−	−	−	Negative
44	Powder-04	Nihil	−	−	−	−	Negative
45	Powder-05	Nihil	−	−	−	−	Negative
46	Powder-06	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-

Table 4 (continued)

Sample *N*°	Sample type	Spiking status	Chromosomal targets		Virulence plasmids		Final real-time PCR identification
			*pts*I	*pur*A	pXO1 *lef*A	pXO2 *cap*A	
47	Powder-07	*B. thuringiensis kurstaki*	+	−	−	−	BC pXO1-/pXO2-
48	Powder-08	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
49	Powder-09	BA (Sterne strain) spores	+	+	+	−	BA pXO1+/pXO2-
50	Powder-10	Nihil	−	−	−	−	Negative

FM filter membrane, *LF* liquid filtrate, *LBLS* liquid containing burned leaves or smog (burning fog oil, burning rubber, burning diesel, burning vegetation, or smoke gunshot)

[a] The powder samples each contain one of the following backgrounds: dipel pesticide powder (*B. thuringiensis kurstaki*), talcum powder, or creamer powder

mismatch discrimination in SNP genotyping studies (Mouritzen et al. 2003).

The *B. anthracis* specific *pur*A and pXO1 LNA probe were labeled with the FAM as reporter dye at the 5′-end and the black hole quencher 1 (BHQ1) as a quencher at the 3′-end. Similarly, the *B. cereus* group specific and pXO2 LNA probes were labeled with the combination of Yakima-Yellow as reporter at the 5′-end and BHQ1 as the quencher. LNA probes were purchased from Eurogentec (Ougrée, Belgium; Table 2).

Real-time PCR assays

Each duplex real-time PCR was carried out in 25 μL of a reaction mixture containing 2.5 μL of extracted DNA as template, 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems) containing dUTP and uracyl N-glycosylase (UNG), 300 nM of each primer and 100 nM of each MGB fluorogenic probe or 50 nM of each LNA fluorogenic probe. Real-time PCR amplification was performed with an ABI Prism 7700 sequence detector (Applied Biosystems) under the following conditions: incubation at 50 °C for 2 min to activate UNG, initial denaturation at 95 °C for 10 min, and then 40 cycles of amplification with denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was tested in triplicate and data were recorded as Cycle threshold (Ct) on a TaqMan 7900HT Sequence Detection System (Applied Biosystems), using the analytical software from the manufacturer.

Standard curves determination and detection limit

The DNA concentrations were quantified by using the Nanodrop® 3.0.1, ND-1000 Spectrophotometer, (Nanodrop® Technologies, Inc., Montchanin, USA). In order to define the limit of detection (LOD), a standard curve was constructed of (10×) serially diluted DNA of *B. anthracis* (BA9501) ranging from 2.5 ng to 2.5 fg which were used as a template for RT-PCR MGB and LNA assays. Ct values obtained were plotted against the logarithm of copies to assess the dynamic range. The efficiency of real-time PCR assays was calculated as described by Wong and Medrano (2005). The real-time PCR assay was subsequently performed on DNA extracted from 50 environmental masked samples spiked with inactivated *B. anthracis* and/or *B. cereus* spores. PCR inhibition was assessed as previously described (Lecouvet et al. 2004).

Results

Assays on the 41 *B. anthracis* strains resulted in specific PCR amplification by using both the MGB and LNA probes. However, one *B. cereus* group isolate, *B. thuringiensis* serovar Konkunkian 97-27, resulted in positive, although delayed fluorescence signal, by use of MGB probes (Table 3). This observation is consistent with data reported by others authors, with some *B. cereus* group strains and is probably caused by mispriming of the Taq DNA polymerase (Ellerbrok et al. 2002; Klee et al. 2006). We hypothesized that the addition to the mix already containing *pur*A primers and probe of a non-fluorogenic probe annealing to the *pur*A probe cognate sequence in *B. cereus* could compete favorably for annealing on the *B. cereus pur*A target and therefore block the annealing of the *B. anthracis pur*A probe and decrease drastically the subsequent generation of unspecific fluorescence generated by mispriming of *B. cereus*. The non-fluorescent *B. cereus pur*A probe (C-probe) consisted of a non-labeled oligonucleotide blocked with a phosphate in 3′-position to prevent DNA polymerization. The non-fluorescent (C-probe) was added to the *pur*A/*pts*I real-time PCR MGB mixture already containing *pur*A primers and *pur*A probe, and *pts*I primers and *pts*I probe (600 nM of C-probe added to MGB–probe mix) and this addition resulted in the quenching of the

fluorescence *B. anthracis* unspecific fluorescence generated during *B. thuringiensis* serovar Konkunkian 97-27 without any impact on the Ct values of the PCR. No C-probe was needed when using LNA-probes (Table 3). Standard curves of the real-time PCR displayed dynamic ranges on 4 log DNA dilutions (Figs. 2 and 3). The LOD giving a reliable and reproducing signal was found to be 25 fg, which corresponds approximately to four genomes of *B. anthracis* (Ct) which corresponds to Ct values of 35.85–38.33 (confidence interval at 95%). Accordingly, Ct values higher than 35.85 were considered arbitrarily as negative.

The primers and probe targeting the *phosphotransferase system I* gene did not amplify any DNA of non-*B. cereus* group bacteria. No difference in PCR amplification using MGB and LNA probes could be observed (Table 1 and Online Resource Table 1). The real-time PCR assay for detection of virulence plasmids pXO1 and pXO2 was negative for all species excepted in *B. anthracis* strains and were in agreement with previous data on the strains (Table 1 and Online Resource Table 1). The presence or absence of *B. anthracis* and/or *B. cereus* in all the 50 environmental samples was correctly detected (Table 4 and Online Resource Fig. 4). No PCR amplification signal for *pur*A, *pts*I, *lef* and *cap*A targets could be detected for any of the 146 Gram-positive non-*B. cereus* group and Gram-negative bacteria strains tested (Online Resource Table 1b).

Discussion

The aim of this study was to setup a rapid, sensitive, specific, and non-hazardous method in order to improve identification of *B. anthracis* in environmental samples in operational fields. Conventional culture methods are not suited for this purpose because they are slow and rely solely on phenotypic characteristics, which can sporadically also be found in other *Bacillus* strains. The past decade has witnessed a spectacular increase in publications of molecular methods for identification of anthrax (Beyer et al. 1995; Ramisse et al. 1996; Ellerbrok et al. 2002; Ryu et al. 2003; Easterday et al. 2005; Van Ert et al. 2007; Skottman et al. 2007; Olsen et al. 2007; Hadjinicolaou et al. 2009; Antwerpen et al. 2008), as these have been tipped to replace efficiently culture methods. Most of these methods have focused on the amplification of pXO1 and pXO2 plasmids virulence genes (Beyer et al. 1995; Ramisse et al. 1996; Ellerbrok et al. 2002; Cheun et al. 2003; Ryu et al. 2003). However, reports that these virulence plasmids are not *B. anthracis*-specific underscore the need for additional assays focusing on amplification of chromosomal targets (Hoffmaster et al. 2002; Easterday et al. 2005; Marston et al. 2006; Van Ert et al. 2007; Antwerpen et al. 2008) in addition to detection of virulence plasmids pXO1 and pXO2. Selection of a specific *B. anthracis* chromosomal target may reveal to be a difficult task, given the very high similarity between *B. anthracis* and *B. cereus* group genomes. Most assays using a chromosomal target often rely on *B. anthracis*–SNPs for discrimination with their *B. cereus* counterparts. So far, most of them have failed to discriminate unambiguously *B. anthracis* (Qi et al. 2001; Ellerbrok et al. 2002; Hoffmaster et al. 2002; Easterday et al. 2005; Klee et al. 2006; Van Ert et al. 2007). The new duplex real-time PCR developed in this study allowed total discrimination between *B. anthracis* and all *B. cereus* group strains tested. However, given the high sensitivity of the *B. anthracis* identification issue, one must be cautious and avoid relying on only one SNP. In order to increase the specificity of the assay, the use of multiple targets not dependent on a single locus is recommended. The use of multiple targets also decreases the risk of false-positive results from contamination, because each target is amplified in separate PCR reaction. The real-time PCR assay described here is a new tool which can be associated with other pre-existing assays such as the LRN assay (Qi et al. 2001) for a more reliable rapid identification of *B. anthracis* in environmental samples. Although LNA and MGB probes gave equivalent fluorescence signals, have therefore opted for LNA probes ahead of MGB probes in routine practice, as they are cheaper, have a low optimum concentration and display low background fluorescence and do not need addition of the C-probe in comparison with MGB probes.

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Conflict of interest None

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