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Rapid genotypic detection of *Bacillus anthracis* and the *Bacillus cereus* group by multiplex real-time PCR melting curve analysis

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Abstract

Bacillus anthracis has four plasmid possible virulence genotypes: pXO1$^+$/pXO2$^+$, pXO1$^+$/pXO2$^-$, pXO1$^-$/pXO2$^+$ or pXO1$^-$/pXO2$^-$. Due to the lack of a specific chromosomal marker for *B. anthracis*, differentiation of the pXO1$^-$/pXO2$^-$ form of *B. anthracis* from closely related *Bacillus cereus* group species is difficult. In this study, we evaluate the ability of *sspE*, pXO1 and pXO2 primers to discriminate individual *B. anthracis* and the *B. cereus* group genotypes using multiplex real-time PCR and melting curve analysis. Optimal conditions for successful multiplex assays have been established. Purified DNAs from 38 bacterial strains including 11 strains of *B. anthracis* and 18 *B. cereus* group strains were analyzed. Nine of the *B. cereus* group near-neighbor strains were shown by multilocus sequence typing to be phylogenetically proximate to the *B. anthracis* clade. We have demonstrated that the four plasmid genotypes of *B. anthracis* and *B. cereus* group near-neighbors were differentially and simultaneously discriminated by this assay.

Keywords: *Bacillus anthracis*; *Bacillus cereus* group; Multiplex detection; Real-time polymerase chain reaction; LightCycler

1. Introduction

The *Bacillus cereus* group is comprised of *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. weihenstephanensis* [1] and *B. pseudomycoides* [2]. *B. anthracis* and *B. cereus* are clinically important because the former is the causative agent of anthrax and an agent used in bioterrorism, and the latter causes food-borne gastroenteritis and opportunistic infections in immunocompromised patients [3–6]. Other members of this group have been reported to be potentially enteropathogenic [7–11]. The unambiguous detection of these organisms has been difficult due to their genetic and phenotypic similarity. A high level of genetic relatedness has been demonstrated by whole-genome DNA hybridization [12,13], 16S and 23S gene rRNA sequence analysis [14,15], sequence analysis of 16S–23S operons [16,17] and the *gyrB–gyrA* intergenic spacer region [16], pulsed-field gel electrophoresis analysis [16,18], multilocus enzyme electrophoresis [18–20], and restriction fragmentation pattern analysis of the genome [21]. All of these methods failed to discriminate among some members of the *B. cereus* group.

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Because of their extremely high genetic homology, some investigators have suggested that *B. anthracis*, *B. cereus* and *B. thuringiensis* should be classified as a single species [12,20].

B. anthracis is a highly fatal infectious agent in animals and humans and therefore its early and unambiguous diagnostic detection is essential for successful treatment and disease prevention. There have been many efforts to utilize rapid DNA-based detection methods, such as PCR [22–33], to replace time-consuming biochemical or culture-based diagnostic tests [34]. PCR-based methods can readily differentiate vaccine or fully virulent *B. anthracis* plasmid genotypes [22–24,26,35]. However, plasmid-cured *B. anthracis* [36–39], or near-neighbor species containing *B. anthracis* closely-related plasmids [40], are very difficult to distinguish from *B. anthracis*. In addition, plasmids or their virulence genes have been readily transferred within these groups by means of conjugation or transformation [41–50].

PCR methods developed for detection of the *B. anthracis* chromosome have suffered from lack of assay specificity; Ba813 [25,26,51], *vrrA* gene [27–29], gyrase B gene (*gyrB*) [30], SG-850 [31], the β subunit of RNA

Table 1
Bacterial strains used in this study and their DNA–based assay response (manuscript in preparation)

Species	Strain ID	Source[a]	Plasmid[b]		PCR for				SG–749[g]		*sspE* PCR[h]	
			pXO1	pXO2	Ba813[c]	*gyrB*[d]	*rpoB*[e]	*gyrA*[f]	PCR	Haplotype	71-bp	188-bp
B. anthracis	14578^T	ATCC	+	+	+	+	+	+	+	K	+	+
B. anthracis	CAU–1	Korea	+	+	+	+	+	+	+	K	+	+
B. anthracis	CAU–2	Korea	+	+	+	+	+	+	+	K	+	+
B. anthracis	CAU–3	Korea	+	+	+	+	+	+	+	K	+	+
B. anthracis	CN1	Korea	+	+	+	+	+	+	+	K	+	+
B. anthracis	CN2	Korea	+	+	+	+	+	+	+	K	+	+
B. anthracis	14185	ATCC	+	–	+	+	+	+	+	K	+	+
B. anthracis	14186	ATCC	+	–	+	+	+	+	+	K	+	+
B. anthracis	Sterne 34–F2	NVRQS	+	–	+	+	+	+	+	K	+	+
B. anthracis	BC	China	–	+	+	+	+	+	+	K	+	+
B. anthracis	Pasteur #2	NVRQS	–	–	+	+	+	+	+	K	+	+
B. thuringiensis	4BG1	BGSC	–	–	**+**	–	**+**	–	+	G	+	–
B. thuringiensis	4Y1	BGSC	–	–	**+**	–	**+**	–	+	G	+	–
Bacillus spp.	9594/3	Patra G.	–	–	**+**	–	**+**	–	+	G	+	–
B. thuringiensis	4AY1	BGSC	–	–	**+**	**+**	–	**+**	+	L	+	–
B. thuringiensis	4AJ1	BGSC	–	–	**+**	**+**	–	**+**	+	E	+	–
B. cereus	6E1	BGSC	–	–	**+**	**+**	–	–	+	M	+	–
Bacillus spp.	IB	Rogers J. E.	–	–	**+**	**+**	–	–	+	M	+	–
Bacillus spp.	III	Rogers J. E.	–	–	**+**	**+**	–	–	+	M	+	–
Bacillus spp.	003	Rogers J. E.	–	–	**+**	**+**	–	–	+	M	+	–
B. thuringiensis	4CC1	BGSC	–	–	**+**	–	–	–	+	**K**	+	–
B. cereus	6A7	BGSC	–	–	**+**	–	–	–	+	G	+	–
B. thuringiensis	4AB1	BGSC	–	–	**+**	–	–	–	+	E	+	–
B. thuringiensis	97–27	Hernandez E.	–	–	**+**	–	–	–	+	E	+	–
B. cereus	14579^T	ATCC	–	–	–	–	–	–	+	A	+	–
B. thuringiensis	2046^T	DSM	–	–	–	–	–	–	+	A	+	–
B. pseudomycoides	12442^T	DSM	–	–	–	–	–	–	+	J	+	–
B. weihenstephanensis	11821^T	DSM	–	–	–	–	–	–	+	G	+	–
B. mycoides	6462^T	ATCC	–	–	–	–	–	–	+	G	+	–
B. subtilis	6051^T	ATCC	–	–	–	–	–	–	–	NA[i]	–	–
B. megaterium	14581^T	ATCC	–	–	–	–	–	–	–	NA	–	–
B. licheniformis	14580^T	ATCC	–	–	–	–	–	–	–	NA	–	–
B. circulans	4513^T	ATCC	–	–	–	–	–	–	–	NA	–	–
Escherichia coli	4157	ATCC	–	–	–	–	–	–	–	NA	–	–
Enterbacter aerogenes	13048^T	ATCC	–	–	–	–	–	–	–	NA	–	–
Salmonella choleraesuis	13076^T	ATCC	–	–	–	–	–	–	–	NA	–	–
Pseudomonas aeruginosa	10145^T	ATCC	–	–	–	–	–	–	–	NA	–	–
Clostridium sporogenes	3584^T	ATCC	–	–	–	–	–	–	–	NA	–	–

[a] ATCC, American Type Culture Collection, Manassas, VA, USA; NVRQS, National Veterinary Research and Quarantine Service, Anyang-si, Kyeonggi-do, South Korea; BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, The Ohio State University, Columbus, OH, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

[b] Plasmids were detected by PCR (51). +, detected; –, not detected.

[c] Determined by PCR (25). +, detected; –, not detected; **+**, false positive reaction.

[d] Determined by PCR using primers BA1 and BA2r (30). +, detected; –, not detected; **+**, false positive reaction.

[e] Determined by PCR and sequencing (32). +, detected; –, not detected; **+**, false positive reaction.

[f] Determined by PCR and sequencing (33). +, detected; –, not detected; **+**, false positive reaction.

[g] Determined by PCR and its haplotype was determined by *Alu*I restriction digestion of the PCR product (31). +, detected; –, not detected; false positive reaction is indicated by boldface **K**.

[h] Determined by PCR (manuscript in preparation). +, detected; –, not detected.