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Use of molecular beacons and multi-allelic real-time PCR for detection of and discrimination between virulent *Bacillus anthracis* and other *Bacillus* isolates

Andreas V. Hadjinicolaou [a,1], Victoria L. Demetriou [a,1], Johana Hezka [a], Wolfgang Beyer [b], Ted L. Hadfield [c], Leondios G. Kostrikis [a,*]

[a] Department of Biological Sciences, University of Cyprus, 75 Kallipoleos Avenue, P.O. Box 20537, 1678, Nicosia, Cyprus
[b] University of Hohenheim, Institute of Environmental and Animal Hygiene, Garbenstrasse 70, 70599, Stuttgart BW, Germany
[c] Midwest Research Institute, Florida Division, 1470 Treeland Blvd SE, 32909, Palm Bay, Florida, USA

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ABSTRACT

The awareness of the threat of *Bacillus anthracis*, the causative agent of the disease anthrax, as a biowarfare and bioterrorism weapon has revived the development of new technologies for rapid and accurate detection of virulent isolates in environmental and clinical samples. Here we explore the utility of molecular beacon real-time PCR technology for detection of virulent *Bacillus anthracis* strains. Molecular beacons are nucleic acid probes with high specificity, that act as switches by emitting fluorescence when bound to their nucleotide sequence targets by means of altering their conformation. In this study, five molecular beacons targeting *Bacillus anthracis capA*, *capB*, *capC*, *lef*, and *pag* alleles were designed and used in five uniplex assays. Another molecular beacon targeting the *Bacillus* group chromosomal *16s rRNA* allele was designed for use in a duplex assay with an internal PCR amplification control. The molecular beacons were used in a real-time PCR assay for the detection of and differentiation between virulent *B. anthracis* and other members of the *B. cereus* group at the molecular level. Various *B. anthracis* samples as well as other bacterial and human samples were used to demonstrate the sensitivity and specificity of this assay. Use of the molecular beacon real-time PCR technology should accelerate current efforts to swiftly detect *B. anthracis* strains and its virulence plasmids in clinical and environmental samples and may extend to the development of additional molecular beacon-based assays for the identification of other pathogenic agents or the identification of *B. anthracis* directly from clinical samples.

1. Introduction

Bacillus anthracis is a gram-positive, endospore-forming aerobe causing the acute mammalian disease anthrax. Being primarily a zoonosis, it can infect livestock and humans by cutaneous, gastro-intestinal and respiratory routes. Fatality rates can reach up to 100% for untreated inhalational anthrax, and so prompt diagnosis can have a major impact on the effectiveness of treatment. Since *B. anthracis* is easy to cultivate, the possibility of creating *in vitro* aerosols containing highly stable and resistant spores has made *B. anthracis* a very good weapon for bioterrorism (Jernigan et al., 2002).

Pathogenicity of *B. anthracis* depends on two virulence-determining factors; the poly-D-glutamyl capsule, and the anthrax toxin. Capsule production depends on genes *capA*, *capB* and *capC* on plasmid pX02. The anthrax toxin components are encoded by three toxin genes: *pag*, *cya* and *lef* on plasmid pX01 (Dixon et al., 1999). *B. anthracis* is the predominant organism that synthesises this capsular polymer, so the detection of capsular material can be used to distinguish it from its closest relatives. In this study, the genes *capA*, *capB*, *capC*, *pag* and *lef* are targeted for detection of plasmids pX01 and pX02 in *B. anthracis* strains.

Also targeted in this assay is the chromosomal *16s rRNA* gene, present in all bacteria. The members of the *B. cereus* are believed to have evolved from a common ancestor (Turnbull, 1999) and are indistinguishable when comparing the sequence of their *16s rRNA* gene. Therefore, this gene is used as a marker for detection of *Bacillus* spp. An assay that accurately distinguishes between *Bacillus anthracis* and other members of the *Bacillus* spp. would prove very useful (Helgason et al., 2000; Hu et al., 2006; Turnbull, 1999). Differentiation of *B. anthracis* from its close relatives has traditionally relied upon phenotypic characterisation (Dixon et al., 1999), and unambiguous identification requires multiple biochemical tests (Brown et al., 1958; Makino et al., 1993; Muller et al., 2004; Turnbull, 1999), usually taking 1–2 days and requiring referral to more specialised laboratories. Additional pre-enrichment steps may be required, slowing the

* Corresponding author. Laboratory of Biotechnology and Molecular Virology, Department of Biological Sciences, University of Cyprus, 75 Kallipoleos Avenue, P.O. Box 20537, 1678, Nicosia, Cyprus. Tel.: +357 22892885; fax: +357 22892893.
E-mail address: lkostrik@ucy.ac.cy (L.G. Kostrikis).
[1] These authors contributed equally to this study.

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identification process down even more. This and the increased awareness about biological weapons using *B. anthracis* have led to the necessity for improved and more rapid methods of identification.

Several conventional PCR and other microassays have been devised targeting various genes of *B. anthracis* (Beyer et al., 1995; Burton et al., 2005; Carl et al., 1992; Gierczynski et al., 2007; Henderson et al., 1994;

Table 1
Examination of DNA samples extracted from humans, *B. anthracis* and other organisms for the presence of *capA*, *capB*, *capC*, *pag*, *lef*, and *16s rRNA* alleles by the molecular-beacon-based real-time PCR assays.[a]

Organism	pX01/pX02 profile	pX01 alleles		pX02 alleles			Chromosomal allele
		pag	*lef*	*capA*	*capB*	*capC*	*16s rRNA*
B. anthracis field strain[b]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Turkey strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Ohio ACB strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Ames BA1015 strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis South Africa strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Pakistan SK-102 strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Ames BA1035 strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis PAK-1 strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis RA3 strain[c]	$pX01^+/pX02^+$	+	+	+	+	+	+
B. anthracis Sterne vaccine strain[b]	$pX01^+/pX02^-$	+	+	−	−	−	+
B. anthracis V770-NP-1R strain[c]	$pX01^+/pX02^-$	+	+	−	−	−	+
B. anthracis Sterne vaccine strain[c]	$pX01^+/pX02^-$	+	+	−	−	−	+
B. anthracis K3 strain[c]	$pX01^+/pX02^-$	+	+	−	−	−	+
B. anthracis delta Ames strain[b]	$pX01^-/pX02^+$	−	−	+	+	+	+
B. anthracis Pasteur strain[c]	$pX01^-/pX02^+$	−	−	+	+	+	+
B. anthracis CDC1014[b]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus 03BB102[c]	$pX01^+/pX02^+$	+	+	−	+	−	+
B. cereus G9241[c]	$pX01^+/pX02^-$	+	+	−	−	−	+
B. cereus 03BB108[c]	$pX01^-/pX02^+$	−	−	+	+	+	+
B. thuringiensis HD 1011[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis 97–27[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis HD 682[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis HD 571[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis Al-Hakdm[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis israelensis[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis kurstaki[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. thuringiensis morrisoni[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. megaterium MRI#122856[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. mycoides MRI#122855[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. coagulans MRI#122854[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. subtilis MERCK 110649[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus S-28[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus E33L[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus D-17[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus ATCC 4342[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus FMI[c]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus NCTC 7464[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus NCTC 11145[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
B. cereus ATCC 11778[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
S. enterica Enteritidis1030/17[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Enteritidis CVS-4809/2[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Enteritidis CVS-4421/1[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Enteritidis CVS-141/1–5[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Typhimurium CVS-4074/1[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Typhimurium CVS-4981/1[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Typhimurium CVS-4255/1[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Typhimurium CVS-131/2[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Bredeney 1030/1[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Infantis 1030/4[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Anatum 1030/5[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Hadar 1030/6[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Newport 1030/7[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. enterica Virchow 1030/11[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
E. coli ATCC 25922[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
E. coli O157 ATCC 35150[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
L. innocua NCTC 11288[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
L. invanovii NCTC 11846[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
L. invanovii ATCC 19119[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
L. monocytogenes NCTC 11994[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
E. feacalis ACTC29212[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
E. aerogenes ATCC 13048[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
S. aureus NCTC 1803[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
S. aureus ATCC 25923[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
M. luteus ATCC 9341[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
P. vulgaris ATCC 13315[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
P. aeroginosa ATCC 27853[d]	$pX01^-/pX02^-$	−	−	−	−	−	−
R. equi NCTC 1621[d]	$pX01^-/pX02^-$	−	−	−	−	−	+
Human (PBMC sample)[e]	$pX01^-/pX02^-$	−	−	−	−	−	−

Table 2
Sequences of DNA oligonucleotides used as molecular beacons and primers used in the real-time PCR assay.

Name	Oligonucleotide and molecular beacon sequence (5′–3′)[a]	Target allele[b]	Nucleotide position[c]	GenBank Accession no.	Reference
MBcapA	FAM-GCCTCGTGACGTCCCATCATAATGTCACCAACGAGGC-Dabcyl	*capA*	2144–2168	M24150	This study
2113 (F)	CGTGAGAACGAAAAATTGACGATG	*capA*	2113–2136	M24150	This study
2199 (R)	TGTACCGTAACGATTAACAATCTC	*capA*	2176–2199	M24150	This study
MBcapB	FAM-GCCGAGAGCCTCTTTAACTACCCTGCGTTGCTCGGC-Dabcyl	*capB*	561–584	M24150	This study
530 (F)	GCGCCGTAAAGAAGGTCCTAAT	*capB*	530–551	M24150	This study
619 (R)	CATTCACAAATAAGTGCTTCTGCTTC	*capB*	594–619	M24150	This study
MBcapC	FAM-GCCTGCAGGTAAAATACCTGTTCTTTCTGTAGCAGGC-Dabcyl	*capC*	1549–1573	M24150	This study
1518 (F)	TAGTATTAGGAGTTACACTGAGCC	*capC*	1518–1541	M24150	This study
1606 (R)	GAGTGCTAAATAACCAGGTACAAC	*capC*	1578–1606	M24150	This study
MBlef	FAM-GCGGAGCTTTCTCAAGTAGCTTTTCTGCTGCCTCCGC-Dabcyl	*lef*	955–979	M30210	This study
920 (F)	TAGAAGTAAAAGGGGAGGAAGCTG	*lef*	920–943	M30210	This study
1023 (R)	CTTTCCTCCAATTGCTTTATACATCTC	*lef*	997–1023	M30210	This study
MBpag	FAM-CAGCGGAAGTAGCAAATGTATATTCATCACTCCGCTG-Dabcyl	*pag*	356–372	AF306783	This study
316 (F)	CAATCTGCTATTTGGTCAGGATTTATC	*pag*	316–342	AF306783	This study
417 (R)	TTGGTCATCTACCCACATTGTTAC	*pag*	394–417	AF306783	This study
MB16srRNA	FAM-CAGCCGTTACCTCACCAACTAGCTAATGCGACGGCTG-Dabcyl	*16s rRNA*	240–264	EF062509	This study
211 (F)	TTCGGCTGTCACTTATGGATG	*16s rRNA*	211–231	EF062509	This study
294 (R)	TCGGCTACGCATCGTTGCCTTG	*16s rRNA*	271–294	EF062509	This study
MBIAC[d]	ROX-CGAGCCGCTACTCAGCAGAGGCTCCCTCGGGCTCG-Dabcyl				Hadjinicolaou et al., 2009
302 (F)	TTGGCGATAGCCTGGCGGTG				Hadjinicolaou et al., 2009
437 (R)	TGTTTACCGGGCATACCATCCAGAG				Hadjinicolaou et al., 2009

[a] FAM, fluorescein; Dabcyl, 4-4(4′dimethylamino phenylazo)benzoic acid; ROX, 6′-carboxy-X-rhodamine; underlined sequences indicate the sequences complementary to each other that form the stem structures of molecular beacons.
[b] *capA*, *capB* and *capC* correspond to alleles encoding the encapsulation proteins CapA, CapB and CapC; *lef,* lethal factor; *pag,* protective antigen; *16s rRNA,* small subunit (16S) ribosomal RNA gene.
[c] For molecular beacons, the nucleotide positions correspond to the target recognition sequences (non-underlined sequences).
[d] IAC, artificial internal amplification control; IAC primers amplify the IAC target DNA and MBIAC is a molecular beacon which recognizes the IAC (Hadjinicolaou et al., 2009).

Little et al., 1990; Mabry et al., 2006; Makino et al., 1993). Real-time PCR assays may offer an improved means of identification due to speed, sensitivity and specificity (Bell et al., 2002; Bode et al., 2004; Drago et al., 2002; Ellerbrok et al., 2002; Park et al., 2007; Ryu et al., 2003; Sohni et al., 2008a,b; Varma-Basil et al., 2004). Current rapid detection methods are reviewed by Edwards et al. (2006). Molecular beacons (Marras et al., 2006; Tyagi and Kramer, 1996) and real-time PCR technology can be combined to devise an assay for simultaneous high-throughput detection and quantification of the target.

In this study, we describe a molecular beacon-based real-time PCR assay detecting the *capA, capB, capC, pag, lef* and *16s rRNA* genes in a broad range of samples, with the aim of devising a molecular method for detecting *Bacillus anthracis* that is faster and more accurate than traditional methods and with the ability to detect the presence of the virulence plasmids and be used in routine diagnostic tests.

2. Methods

2.1. Bacterial strains and human genomic DNA

Seventeen *Bacillus anthracis* strains with all possible combinations of plasmid profiles were used in this study. Twenty-four different strains of *Bacillus* spp., 28 non-*Bacillus* bacterial DNA samples and 10 human DNA specimens were also included. All *Bacillus* strains used were previously identified by standard conventional biochemical. Human genomic DNA samples were obtained from the peripheral blood mononuclear cells (PBMC) isolated from consenting individuals from Cyprus (L.G. Kostrikis, unpublished data). The validity of the human DNA was tested by investigating the presence of the CCR5 gene by PCR (data not shown). Details of the samples tested are shown in Table 1.

2.2. Bacterial cultures and DNA preparation

The strains used in this study were cultured by adding 1 ml of bacteria in glycerol stock solution to 200 ml TSB and incubated for 4 h at 37 °C. Penicillin was added (50 units/ml), followed by incubation for a further 30 min, to help break down the bacterial cell wall. Cell pellets were collected after centrifugation of 10–20 ml aliquots of the broth at 3250 rcf for 10 min. Cells were lysed in a buffer containing proteinase K (DNeasy buffer, Qiagen, Valencia, CA) and the lysates were purified using magnetic beads using the MagAttract DNA Mini M48 kit (Qiagen, Hilden, Germany). Aliquots of the suspension were made and the pellets were collected for DNA extraction which was performed using the BioRobot M48 (Qiagen, Hilden, Germany) or the EasyDNA kit (Invitrogen, Carlsbad, CA). All *B. anthracis* DNA samples were filtered through a 0.2 μm filter prior to testing in the molecular assays.

2.3. Plasmid profiling

For the *Bacillus* strains involved in this study, the presence or absence of plasmids pX01 and pX02 was confirmed by real-time PCR using material from a single colony, grown on blood agar. This PCR-based

Notes to Table 1:
[a] (+) symbol indicates detectable real-time PCR amplification signal (C_T values <40) ranging from 16 to 35 depending on the original template DNA concentration; (−), undetectable signal ($C_T \geq 40$) indicating no template DNA.
[b] DNA samples donated from W. Beyer, University of Hohenheim, Institute of Environmental and Animal Hygiene, Stuttgart, Germany for this study. Strain designations are written after the name of each sample.
[c] DNA samples donated from T. L. Hadfield, Midwest Research Institute, Palm Bay, Florida, USA for this study. Strain designations are written after the name of each sample.
[d] Bacterial samples donated from the National Reference Laboratory for *Salmonella*, Department of Veterinary Services, Nicosia, Cyprus for a previous study (Hadjinicolaou et al., 2009). Strain designations are written after the name of each sample.
[e] Samples of human genomic DNA extracted from peripheral blood mononuclear cells (PBMC) isolated from 10 consenting individuals from Cyprus (L. G. Kostrikis, unpublished data).

Table 3
Sequences of DNA oligonucleotides used as target recognition sequences for the real-time PCR assay.

Name	Oligonucleotide sequence (5′–3′)	Target Allele[a]	Nucleotide Position	Amplicon Length (nts)	GenBank Accession no.	Reference
TCapA	CGTGAGAACGAAAAATTGACGATCGACGATGGTTGGTCACATTATGATGCGGACGTCACGTAAAAGAGATTGTTAATCGTTACGGTACA	*capA*	2113–2199	87	M24150	This study
TCapB	GCGCCGTAAACGAAGGTCCTAATATCGGTGAGCAACGCAGGGTAGTTAAAGAGGCTGCTGATTTACGAAGCAGAAGCACTTATTTGTGAATG	*capB*	530–619	90	M24150	This study
TCapC	TAGTATTACGCAGTTACACTCAGCCTTATTTTTACAGAAACAACGTATTTTACCTGCAGGTTTAGTTGTACCTGGTTATTTAGCACTC	*capC*	1518–1606	89	M24150	This study
Tlef	TAGAAGTAAAAGGGAGCAAGCTGTTAAAAAGAGGCAGCAGAAAGTACCATCTGATGTTTAGAGATGTATAAAAGCAATTGGAGGAAAG	*lef*	920–1023	104	M30210	This study
Tpag	CAATCTGCTATTTGGTCAGGATTTATCAAAGTTAAGAAGAGTGATGAATATACATTTGCTACTTCCGCTGATAATCATGTAACAATGTGGGTAGATGACCAA	*pag*	316–417	102	AF306783	This study
T16srRNA	TTCGGCTGTCACTTATGGATCGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA	*16 s rRNA*	211–294	84	EF062509	This study
TIAC [a]	TTGGCGATAGCCTGCGCGGTGGCTGTATCGACGATCATCTGCTACTAGCTCGAGGGAGCCTCTGCTGAGTAGCGACACTGATC GCCCTCGACTAGCTCGGTACATCTCTGGATGGTATGCCCGGTAAACA	N/A	N/A	129	N/A	Hadjinicolaou et al., 2009

[a] IAC, artificial internal amplification control (Hadjinicolaou et al., 2009).

plasmid profiling followed the protocols found in the new WHO anthrax guidelines (http://www.who.int/csr/resources/publications/AnthraxGuidelines2008), and the plasmid profiles of all samples tested are listed in Table 1.

2.4. Internal PCR amplification control

The internal amplification control (IAC) used in the PCR is a synthetic target, bearing no homology to sequences available in the NCBI library, designed in a previous study (Hadjinicolaou et al., 2009) and is amplified by primers 302 and 437 (Tables 2 and 3).

2.5. Primer and molecular beacon design

Molecular beacons, targeting the *Bacillus anthracis capA, capB, capC, pag, lef* and *Bacillus* group *16s rRNA* genes were designed in this study based on nucleotide sequences (Table 3) from the NCBI library (http://www.ncbi.nlm.nih.gov) using BLAST (Altschul et al., 1990) and standard protocols for molecular beacons (Bonnet et al., 1999; Kostrikis et al., 1998; Tyagi and Kramer, 1996; Vet and Marras, 2005) (Table 2). The molecular beacons, target oligonucleotides and primers were synthesised by MWG-Biotech AG Ltd (Ebersberg, Germany) and Midland Certified Reagent Company, Inc. (Texas, USA). All molecular beacons were labelled with DABCYL, 4′-(4′-dimethylaminophenylazo)benzoic acid at the 3′ end and with the FAM (Fluorescein) at the 5′ end, except for MBIAC, which is labelled with ROX (6′-carboxy-X-rhodamine). In addition to the central 24–30 nucleotide-long probe sequence, each molecular beacon had 4–5 of the 12 bases of its two arms complementary to the target sequence, for stronger and more stable hybridization.

2.6. Thermal transitions for molecular beacons and beacon-target complexes

To assess the thermodynamic characteristics, the quality and the purity of the molecular beacons, a melting curve analysis was performed by monitoring the fluorescence thermal transition profiles using the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, California, USA). The cycling parameters were as follows: 1 cycle for 2 min at 95 °C followed by 50 cycles each consisting of the data collection step for 30 s and a second step for 10 s, starting at 80 °C and applying auto-incrementation of −1 °C per half-minute cycle until 31 °C were reached. The reaction consisted of a 25 μl solution containing 12.5 μl Platinum ® Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, CA), 1 μl (5 pmol/μl or 8 pmol/μl in the case of MBcapB) of the beacon probe with or without 100 pmol of a perfectly complementary single-stranded oligonucleotide target (Table 3), with distilled water making up the remaining volume. Changes in fluorescence were measured at 490 nm and the data collected at each temperature interval were plotted to form these thermal denaturation profiles and determine the optimal annealing temperature for the real-time PCR reactions (Fig. 1).

2.7. Standard curves

The *B. anthracis*-specific control DNA targets used were synthetic oligonucleotides (MGW-Biotech AG, Ebersberg, Germany) containing the target recognition sequence for each beacon (Table 3). The concentrations of the oligos were quantified by UV absorbance spectrophotometry at 260 nm using a NanoDrop UV spectrophotometer (NanoDrop Technologies, USA) and the solutions were subsequently diluted to concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10 molecules/5μl to be used as target standards. Uniplex real-time PCR reactions were performed in triplicate on these dilutions of the targets to create standard curves and calculate the limit of detection as done previously (Hadjinicolaou et al., 2009).

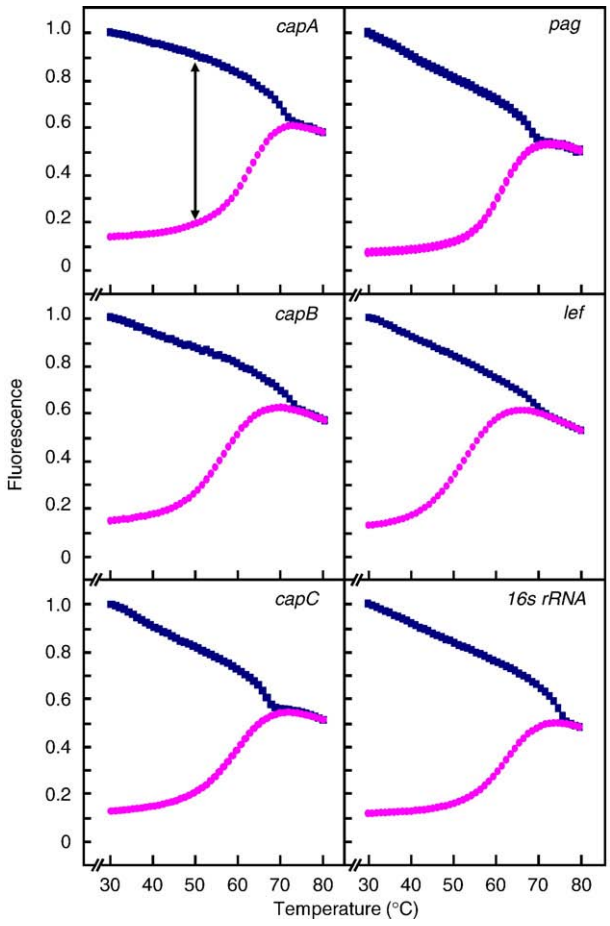

Fig. 1. Normalised fluoresence thermal transitions of molecular beacon (pink circles) and beacon-target complexes (blue squares) constructed for the detection of five *B. anthracis*- (*capA, capB, capC, pag,* and *lef*) specific genes and one chromosomal *Bacillus*-specific (*16s rRNA*) gene. The DNA sequences of the molecular beacons and synthetic oligonucleotides corresponding to each gene target are presented in Tables 1 and 2. For each molecular beacon, the thermal stabilities differ between the perfectly complementary molecular-beacon-target double-stranded DNA hybrids and this influences the extent of fluorescence emission at different temperatures. The temperature selected (50 °C) for hybridisation in the standard PCR reactions allows optimal resolution of the fluorescence signal.

2.8. Molecular beacon-based real-time PCR analysis

To detect the *Bacillus* group *16s rRNA* gene and discriminate between *Bacillus* and non-*Bacillus* strains a molecular-beacon duplex real-time PCR assay was used that included the components needed for amplification and detection of IAC and the *16s rRNA* gene fragment. To detect the *Bacillus anthracis capA, capB, capC, pag* and *lef* genes, five molecular-beacon uniplex real-time PCR assays were carried out by using the general method of detecting single sequences with nucleotide-specific molecular beacons (Tyagi and Kramer, 1996) and real-time PCR (Heid et al., 1996). Details of molecular beacons and PCR primers are shown in Table 2. The PCR primers and the target recognition sequences of the molecular beacons were designed to have similar hybridization temperatures and all amplicons have similar lengths (Table 3). The real-time PCR assays were performed on a 7900HT real-time PCR System (Applied Biosystems, Foster City, California, USA) and fluorescence was recorded at 490 nm. For the duplex assay each 25 μl reaction contained 5.0 μl sample DNA, 0.5 μl IAC standard (10^6 copies/5 μl), 1.0 μl distilled water, 12.5 μl Platinum Quantitative PCR SuperMix-UDG, 1.0 μl MBIAC, 1.0 μl MB16srRNA and 1.0 μl (20 pmol/μl) of each of the primers needed for the PCR amplification of TIAC and the *16s rRNA* gene target. For the uniplex assays, each 25 μl reaction contained 5.0 μl DNA, 4.5 μl distilled water, 12.5 μl Platinum Quantitative PCR SuperMix-UDG, 1.0 μl of one of the five molecular beacons and 1.0 μl (20 pmol/μl) each of the forward and reverse primers. In non-template controls, the DNA was replaced with sterile water. The thermal cycling program was the same for both uniplex and duplex reactions. It consisted of denaturation (94° for 10 min), followed by forty cycles of amplification (94 °C denaturation for 15 s, 50 °C annealing and data collection for 30 s, and 72 °C polymerization for 30 s). During the data collection stage of each cycle, the fluorescence emission in each sample was automatically recorded at 490 nm.

2.9. Phylogenetic evaluation of Bacillus group 16s rRNA gene-specific molecular beacon

Genbank accession numbers for 39 reference *16s rRNA* gene sequences used in phylogenetic analysis of the *Bacillus* group *16s rRNA* gene-specific molecular beacon are as follows: X07714, M34130, J01859, E05133, Z76672, AB073312, AF453251, AF228918, AF228919, AF130983, U90316, Z49264, AF512997, U00679, X75943, L37605, D83367, X66100, L37603, Y15856, AF107307, X66101, L37601, D83368, AB009937, L37599, X55059, AF290552, AJ294817, AB021196, AB065370, AF074970, AF233579, D16266, AJ310100, AB021189, D16272, D16273, X60629. Sequence alignment was performed with the Clustal X program (Larkin et al., 2007). The aligned sequences were manually edited to retain only the regions which have homologues to all other aligned positions, retaining a final length of 1246 nucleotides. A maximum likelihood phylogenetic tree was constructed based on the Tamura-Nei substitution model (Tamura and Nei, 1993) using the quartet puzzling method in Tree-Puzzle (Schmidt et al., 2002). Similarity plots between *Bacillus* strains were performed using SimPlot implementing an 80-nucleotide-long sliding window moving along the gene in 20-nucleotide steps (Ray, 1998). Pairwise evolutionary distances for the 84 nucleotide-long *16s rRNA* gene amplicon were calculated with the DNADIST program in PHYLIP (Felsenstein, 2001) using the Kimura 2-parameter correction (Kimura, 1980) and the neighbour-joining method (Saitou and Nei, 1987).

3. Results

3.1. Thermal transitions of molecular beacons and beacon-target complexes

Normalised fluorescence signal for both the beacon and the beacon-target hybrid was plotted against temperature to give a thermal denaturation profile for each beacon (Fig. 1). At low temperatures, complementary beacon-target hybrids give out a bright fluorescence signal. At high temperatures the hybrids dissociate and there is a marked decrease in fluorescence. In the absence of target, the beacons unravelled at high temperatures and exhibited a melting temperature above 60 °C in all cases. In the temperature interval 31 to 55 °C, the probe-target hybrids elicited significantly stronger fluorescence than the probe alone, allowing the detection of target sequence. 50 °C was chosen as an optimal annealing temperature for subsequent real-time PCR runs. At this temperature the difference in fluorescence signal between beacon alone and beacon-target hybrids is large, in the absence of target any fluorescence detected is background level and the temperature is high enough to prevent less energetically favourable hybrids from forming. In the process of carrying out the melting curve analysis for all beacons, different concentrations were tested, in order to find a concentration at which the fluorescence signal was neither too low nor saturated. The concentrations at which the particular beacons exhibited the desired amount of fluorescence signal were: MBIAC, 50 pmol/μl; MBcapA, 5 pmol/μl; MBcapB, 8 pmol/μl; MBcapC, 5 pmol/μl; MBlef, 5 pmol/μl; MBpag, 5 pmol/μl; and MB16srRNA, 5 pmol/μl;. Finally, these thermal

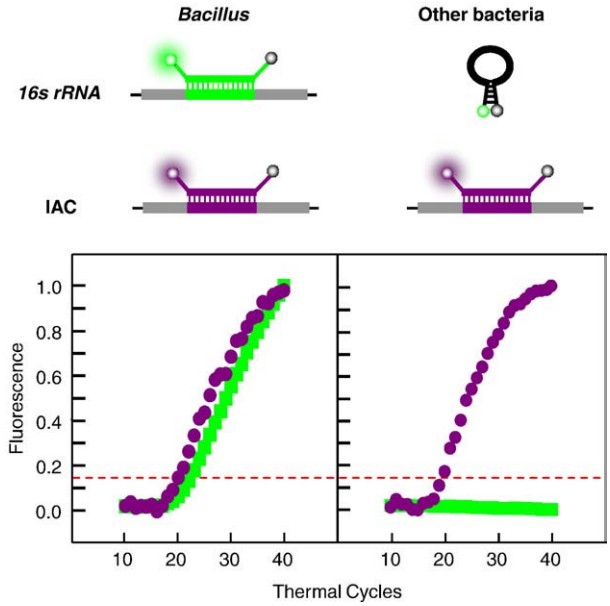

Fig. 2. Representative real-time PCR results as established by the duplex reaction described in Materials and Methods. The plots show average normalised linear PCR amplification of representative samples shown for demonstration of typical results obtained from *Bacillus* and non-*Bacillus* bacteria. With DNA from non-*Bacillus* bacterial samples, only the IAC-specific, ROX-labelled molecular beacon hybridises to the IAC targets, generating violet fluorescence, whereas the *16s rRNA* gene-specific, FAM-labelled molecular beacon retains its stem-and-loop structure and cannot produce a green fluorescent signal. With *Bacillus* DNA present, both molecular beacons hybridise to their respective targets and generate both green and violet fluorescence. The dashed line on the plots represents the normalised threshold for detection of fluorescence, the baseline above which fluorescence increases significantly on PCR amplification and detection of the target sequence.

denaturation profiles illustrate the quality of the molecular beacons and their efficiency in hybridising with the appropriate target sequence.

3.2. Standard curves and limit of detection

The copy numbers of control DNA target oligonucleotides used for plotting the standard curves ranged from 10 to 10^6 copies per reaction. The threshold cycle (C_T) at which significant DNA amplification was observed for each target sequence gave a linear relationship for standard curves along a 6-log range. The reactions worked well for all target standard concentrations tested and the lower limit of detection for the assay was found to be 10 copies of target per reaction. Based on the standard curves and the limit of detection of this assay, negative results were defined as those exhibiting no PCR amplification before 40 cycles.

3.3. Detection of B. anthracis alleles by molecular beacon-based real-time PCR

All samples were investigated initially by a duplex real-time PCR reaction to detect the *16s rRNA* gene. The IAC was amplified and detected by MBIAC in all reactions. PCR amplification and detection of both the IAC and *16s rRNA* gene was clear for all 39 *Bacillus* isolates, whereas only PCR amplification of the IAC was detected in non-*Bacillus* samples, apart from seven samples, where the *16s rRNA* gene target amplification was also detected (Table 1). Positive results had C_T values ranging from 16 to 35 cycles. Normalised PCR amplification plots from *Bacillus* and non-*Bacillus* bacteria for this reaction are seen in Fig. 2.

Following the duplex reaction which discriminated *Bacillus* strains from the majority of non-*Bacillus* strains, five uniplex reactions were carried out to detect the *pag*, *lef*, *capA*, *capB*, and *capC* genes. Despite the discrimination based on the first step duplex assay, all samples were tested with all five uniplex reactions to ensure the specificity of the gene targets and molecular beacons chosen. Stock DNA was diluted 100-fold for use in the uniplex and duplex assays, resulting C_T values within the range on the standard curves (data not shown). Normalised PCR amplification plots from *B. anthracis* strains with different plasmid profiles and a non-*anthracis* member of the *Bacillus* genus are seen in Fig. 3. For detection of the pX02 plasmid genes *capA*, *capB* and *capC*, 11 of the 16 *B. anthracis* strains tested positive for all three pX02 genes, confirming the genotypic plasmid profile analysis. Positive results (>10 copies of DNA per reaction) had C_T values ranging from 19 to 35. All 61 non-*Bacillus anthracis* samples tested negative (C_T value>40), apart from *B. cereus* strain 03BB102, which was positive for *capB*, and *B. cereus* strain 03BB108, which was positive for all three pX02 genes (Table 1). For the detection of the pX01 genes *lef* and *pag*, 13 out of 16 *B. anthracis* samples gave positive PCR results for both targets, in agreement with the plasmid profiling results. Positive PCR results for these targets gave C_T values in the range 21–32. All non- *B. anthracis* samples showed no PCR amplification of these targets, except *B. cereus* strains 03BB102 and G9241, which were positive for both targets (Table 1).

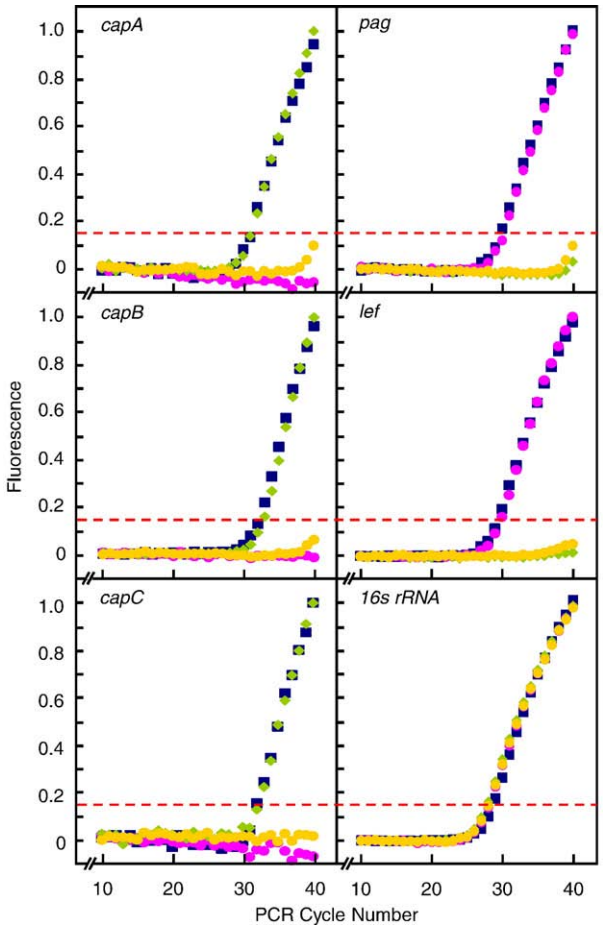

Fig. 3. Normalised real-time fluorescence detection of three different *Bacillus* strains in six real-time PCR assays using *Bacillus anthracis*– (*capA, capB, capC, pag, lef*) and *Bacillus*-specific (*16 rRNA* gene) molecular beacons. The symbols in the plots denote the sample represented in each curve: *B. anthracis* (field isolate, pX01+/pX02+) (blue squares), *B. anthracis* (vaccine strain, pX01+/pX02−) (pink circles), *B. anthracis* (delta ames strain, pX01−/pX02+) (green rhombi), *B. cereus* (yellow circles). The dotted horizontal lines define the threshold fluorescence level for determining the threshold cycle (C_T) values.

3.4. Selectivity of the real-time assay

The selectivity and accuracy of the test is measured by calculating specificity and sensitivity. Specificity is the probability that the PCR will give true negative results and is calculated by: true negative/(true negative + false positive). Sensitivity shows the strength of the assay in recognising its target, calculated by: true positive/(true positive + false negative). For the *16s rRNA* gene target, all 41 *Bacillus* samples investigated were positive giving a sensitivity of 100%. The specificity was 82% since seven of the 38 non-*Bacillus* specimens tested gave positive results. For the *capA, capB, capC, lef* and *pag* genes, all reactions had 100% sensitivity and specificity. All 12 strains of *B. anthracis* possessing the *capA, capB* and *capC* genes on plasmid pX02 had positive PCR reactions. The same was true for the 14 strains of *B. anthracis* possessing the *lef* and *pag* genes on plasmid pX01. Any strains not possessing these genes according to the initial genotypic analysis tested negative. Only one strain, *B. cereus* 03BB102, which was reported to posses a pX02-like plasmid or components of the pX02 plasmid, was negative for *capA* and *capC*, but positive for *capB*.

3.5. Phylogenetic evaluation of the 16s rRNA gene target for the Bacillus genus

Phylogenetic analysis of the *16s rRNA* gene (Fig. 4) indicated a strong evolutionary separation between the gram-positive and gram-negative bacteria. Moreover, a distinct cluster was formed for each one of the *Staphyloccocus* and *Geobacillus* genus, while *Bacillus* and *Staphylococcus* genera are shown to have a closer relation. To clarify the partial similarity among the genera a similarity plot was constructed using Simplot with a sliding window size of 80 nucleotides (Ray, 1998). In the region spanning the the amplified PCR sequence, a substantially higher similarity was shown between the *Bacillus* and *Geobacillus* genera. The average evolutionary distances of this region between *B.anthracis* strains and *B.cereus* strains, *Bacillus-Geobacillus* strains, *Staphylococcus* strains and Gram-negative strains were 0.000, 0.058, 0.100 and 0.374 respectively. Even though the amplified region is quite small for informative phylogenetic analysis, there is substantial evidence that *Bacillus* and *Geobacillus* strains are genetically close in this region.

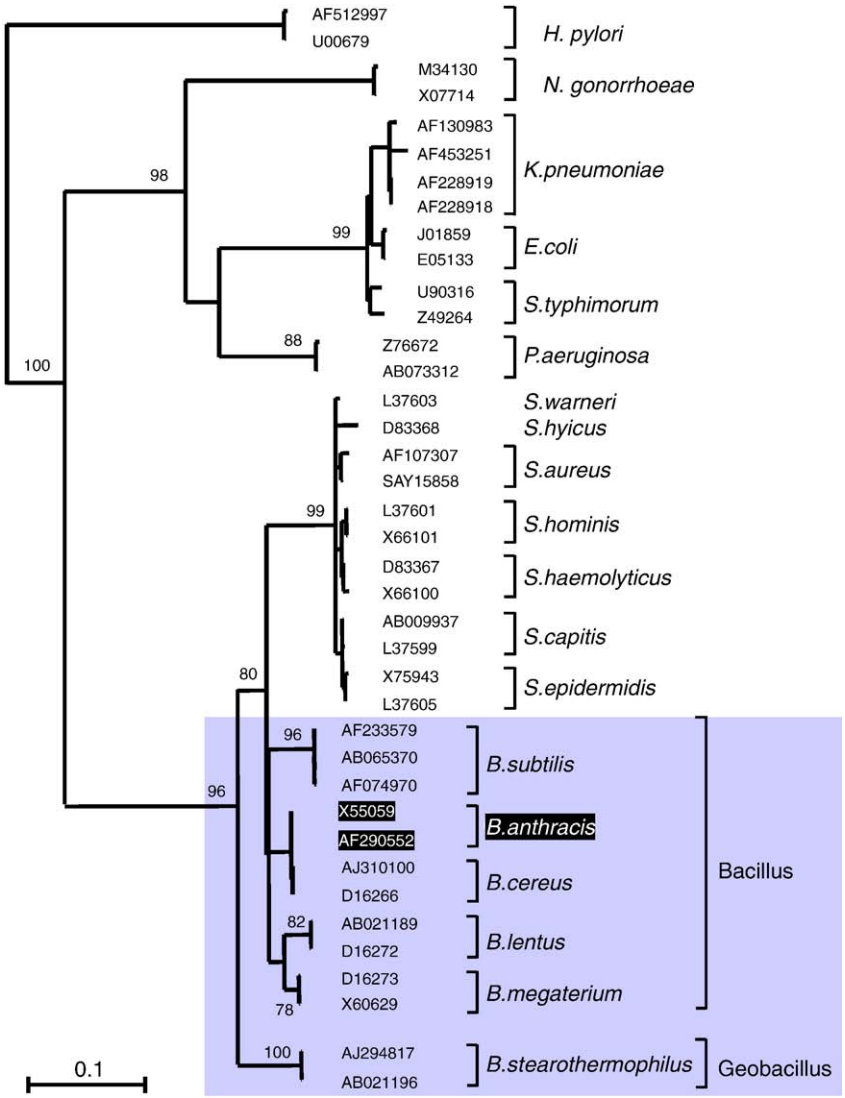

Fig. 4. Phylogenetic tree of 39 representative reference *16s rRNA* gene sequences from Gram-negative and positive bacteria including two *B. anthracis* strains. The divergence between any two sequences is obtained by summing the horizontal branch length, using the scale at the lower left. The numbers indicated at several main nodes are quartet puzzling support values (Schmidt, 2002). The GenBank accession number indicates each organism and the brackets on the right of the tree denote the names of the organisms and two determined *Bacillus* geni. The shaded region shows the tree region which contains the organisms with the highest homology to the *Bacillus 16s rRNA* gene-specific molecular beacon target.

4. Discussion

A variety of bacterial samples was used in this study to test the specificity and sensitivity of the assay in the detection of the genus *Bacillus* and more specifically *B. anthracis*. A range of *B. anthracis* subspecies with all possible combinations of plasmid profiles was included to test the accuracy of the detection. The study includes a significant number of *B. anthracis* samples but also other *Bacillus* and non-*Bacillus* bacteria. Six genes were targeted: *lef* and *pag* on plasmid pX01, *capA, capB* and *capC* on plasmid pX02, and the chromosomal *16s rRNA* gene. Many genes or gene combinations have been exploited in previous studies to target *B. anthracis* (Bell et al., 2002; Drago et al., 2002; Ellerbrok et al., 2002; Gierczynski et al., 2007; Makino et al., 1993; Ryu et al., 2003; Sohni et al., 2008a,b; Varma-Basil et al., 2004), but no previous study has aimed to detect all six genes targeted in this study. This variation in gene location increases the chance of correctly detecting and identifying *B. anthracis* specifically and any member of the *Bacillus* genus simultaneously. Plasmids and individual fragments of gene clusters can be lost or gained by bacteria. This is especially relevant to the *pag* and *lef* genes present on different loci of plasmid pX01 unlike the capsule genes that together form an operon on plasmid pX02 (Koehler, 2002). In light of this information, detecting a single gene present on a specific plasmid does not ensure the presence of the other genes as well, as demonstrated by the *B. cereus* 03BB102 isolate. In the case of *B. anthracis* this is extremely important in terms of evaluating the potential virulence of each strain. This is the reason why each gene was targeted individually with a different molecular beacon probe in this study.

The results from the detection of the chromosomal *16s rRNA* gene demonstrate that this reaction can distinguish *Bacillus* spp. and filter out most non-*Bacillus* species from a wide range of samples. This step of the assay is highly sensitive, correctly identifying all *Bacillus* strains included in the experiment, and highly specific, but not 100%, as it also picks up certain non-*Bacillus* samples (Table 1). However, the ability to filter out 82% of non-*Bacillus* strains makes this step very advantageous, and it is important to note that any non-*Bacillus* strains that are detected in this step are eliminated in the next steps of the assay. The non-*Bacillus* bacteria in which the *16s rRNA* gene target was detected, were confirmed as true positives by testing the homology of the target sequence in BLAST against sequences in the NCBI database. Altering the design of the molecular beacon and primers for this target to exclude these bacteria, however, would possibly compromise the sensitivity of the detection.

The assays for detection of the *capA, capB, capC, lef* and *pag* target genes all had extremely high sensitivity and specificity. The pX01/pX02 virulence plasmid profiles of all *B. anthracis* strains were confirmed, illustrating the quality of the molecular beacon and primer design. *B. anthracis* virulence genes were also detected in three specific *B. cereus* strains: 03BB102, 03BB108 and G9241 (Table 1). Strain 03BB102 was $pX01^+$ and also positive for *capB*, which is in plasmid pX02. Strain 03BB108 was found to be $pX01^-/pX02^+$. Finally, strain G9241 presented the virulence plasmid profile $pX01^+/pX02^-$. It is very uncommon for *B. cereus* isolates to carry *B. anthracis* virulence plasmids (Pannucci et al., 2002). However, all three of these strains were known to have these uncharacteristic plasmid profiles, as they have been previously reported (Hoffmaster et al., 2006; Hoffmaster et al., 2004). Hence, their profiles mistakenly make them appear to be *B. anthracis* isolates in this assay, and further confirmation would be required for discrimination between *B. anthracis* and these specific isolates. However, these are exceptional occurrences and the identification of the presence of pX01-like and/or pX02-like plasmid genes is clinically significant, as these isolates are pathogenic (Avashia et al., 2007; Hoffmaster et al., 2006; Hoffmaster et al., 2004). Our results on these strains are in agreement with previous findings (Hoffmaster et al., 2006), but there was one discrepancy, however, in the detection of the *capA* and *capC* genes in the 03BB102 strain, in which the presence of these genes has previously been demonstrated (Hoffmaster et al., 2006), but gave negative results in our study. Further investigation is needed to explain this, as this result alone does not indicate a fault in the assay design.

Another issue raised by these results is the discrimination between the avirulent *B. anthracis* strain CDC 1014, which does not possess either of the virulence plasmids, and non-*anthracis Bacillus* species. To distinguish between such strains, a chromosomal *anthracis*-specific marker could be used, as published previously (Bode et al., 2004; Ramisse et al., 1996). However, such strains are very rarely found in the environment and are not virulent, not posing a threat to public health and safety.

Strong fluorescence signals were observed in all positive PCR results in both the uniplex and the duplex assays, indicating the quality of the primer and beacon design. This assay is also promising as a quantitative method, potentially having the ability to detect low enough target DNA copy numbers to directly detect clinically relevant amounts of pathogens in environmental and clinical specimens. For a quantitative approach, serial dilutions of known concentrations of *B. anthracis* DNA could be used for the construction of standard curves, for a more representative measure of comparison.

Overall, the unique incorporation of six molecular beacons with real-time PCR and the utilisation of this combination to detect virulent *B. anthracis* isolates and other members of the *cereus* group of *Bacillus*, has not been met in previous publications although various groups have recently demonstrated efficient detection and quantification of *B. anthracis* using modern molecular techniques (Antwerpen et al., 2008; Kim et al., 2005; Moser et al., 2006; Olsen et al., 2007; Skottman et al., 2007; Sohni et al., 2008a,b; Van Ert et al., 2007; Varma-Basil et al., 2004), showing that there is still ongoing research into the molecular detection of *B. anthracis*. The results for detection of the *16s rRNA*, *lef*, *pag*, *capA*, *capB* and *capC* genes in this study demonstrate the primers and beacons for each reaction work well individually and that they amplify and detect their target sequence with very good specificity and sensitivity, illustrating the quality of the beacon and primer design.

This molecular beacon-PCR assay is useful for any laboratory in possession of a real-time PCR. It is a fast, reproducible, simple, specific and sensitive way to detect virulent and non-virulent *B. anthracis* strains. The design of the assay gives it potential to be used for quantification, and for probable detection of multiple members of the *Bacillus* genus. Also, more significantly, the sensitivity of the test and its low limit of detection, are promising factors for direct detection from real clinical and environmental samples which have low numbers of bacteria. Therefore, the assay has the flexibility to be a potentially powerful diagnostic tool.

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