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Multiplexed Detection of Anthrax-Related Toxin Genes

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Simultaneous analysis of three targets in three colors on any real-time polymerase chain reaction (PCR) instrument would increase the flexibility of real-time PCR. For the detection of *Bacillus* strains that can cause inhalation anthrax-related illness, this ability would be valuable because two plasmids confer virulence, and internal positive controls are needed to monitor the testing in cases lacking target-specific signals. Using a real-time PCR platform called MultiCode-RTx, multiple assays were developed that specifically monitor the presence of *Bacillus anthracis*-specific virulence plasmid-associated genes. In particular for use on LightCycler-1, two triplex RTx systems demonstrated high sensitivity with limits of detection nearing single-copy levels for both plasmids. Specificity was established using a combination of Ct values and correct amplicon melting temperatures. All reactions were further verified by detection of an internal positive control. For these two triplex RTx assays, the analytical detection limit was one to nine plasmid copy equivalents, 100% analytical specificity with a 95% confidence interval (CI) of 9%, and 100% analytical sensitivity with a CI of 2%. Although further testing using clinical or environmental samples will be required to assess diagnostic sensitivity and specificity, the RTx platform achieves similar results to those of probe-based real-time systems.
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Traditionally, *Bacillus anthracis* has been distinguished from other members of the *Bacillus cereus* group by time-consuming techniques such as colony morphology, penicillin susceptibility, γ-phage susceptibility, lack of hemolysis, and motility.[1] These methods are giving way to more rapid and quantifiable nucleic acid-based assays. Since the publication of the polymerase chain reaction (PCR) in 1985, applications involving this technology have revolutionized molecular medicine.[2] More recently, real-time PCR is becoming a preferred approach. This is mainly due to the intrinsic benefits of real-time PCR such as quick amplification and detection of target nucleic acids, quantitative accuracy, single-copy sensitivity, and a high level of specificity. Additionally, real-time PCR can be multiplexed to allow multiple target analysis in a single reaction. In the case of anthrax toxin gene detection, multiplexing is clearly beneficial because there are two virulence plasmids (pX01 and pX02) required for full virulence.

In a recent study by Hoffmaster et al,[3] high-coverage draft genome sequence of a *B. cereus* isolate (G9241) revealed the presence of a circular plasmid named pBCX01 with 99.6% similarity with the *B. anthracis* toxin-encoding plasmid pX01. In addition, this isolate was found to be 100% lethal in mice with symptoms similar to inhalation anthrax. The presence of a plasmid in a strain of *B. cereus* with a 99.6% homology to a toxin-encoding plasmid found in *B. anthracis* indicates that genetic diagnosis is more complicated than once thought.[3] Genes specifically associated with inhalation anthrax are located on two plasmids, pX01 and pX02.[4–6] The 182-kb pX01 plasmid harbors the structural genes for the anthrax toxin proteins (*cya* [edema factor], *lef* [lethal factor], and *pagA* [protective antigen]), as well as two *trans*-acting regulatory genes (*atxA* and *pagR*). The 96-kb pX02 plasmid carries three genes required for capsule synthesis (*capB*, *capC*, and *capA*), a gene associated with capsule degradation (*dep*), and a *trans*-acting regulatory gene (*acpA*). Another recent study by Pannucci et al[7] showed a high degree of sequence conservation between plasmid pX01 and the chromosome of some members of the *B. cereus* group, with several strains showing 80 to 98% homology. Therefore, a simplified multiplexed chemistry that specifically detects these plasmids or genes associated with these plasmids may prove to be as or more important than identification of the organism itself.

To this end, we developed two triplex assays using the MultiCode-RTx platform. MultiCode-RTx uses an expanded genetic base pair constructed from 2′-deoxy-5-methyl-isocytidine (iC) and 2′-deoxy-isoguanosine (iG).

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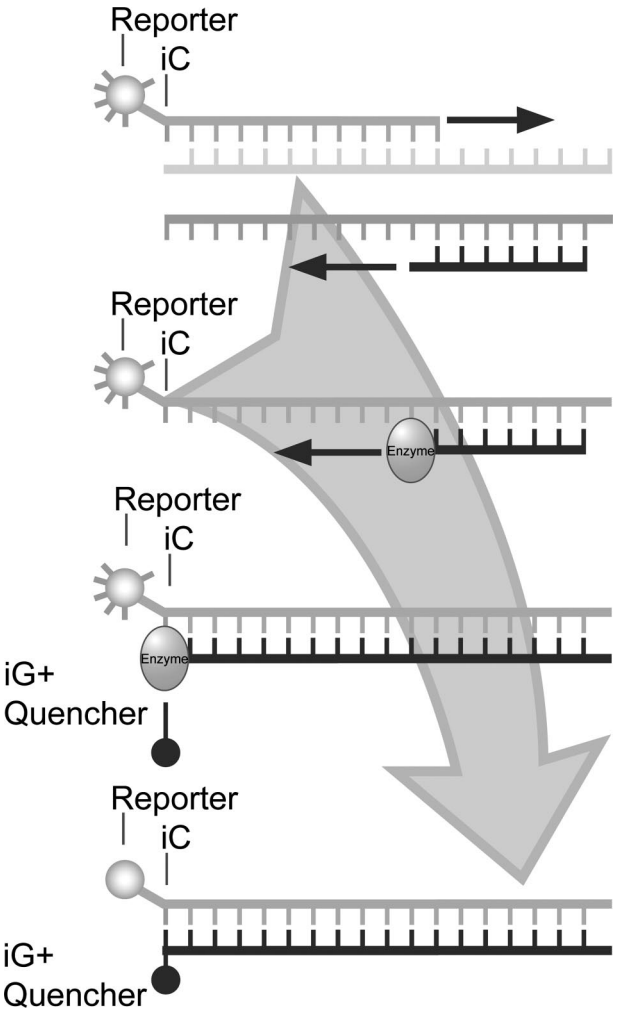

Figure 1. MultiCode-RTx system schematic. Targets are amplified with a standard reverse primer and a forward primer that contains a single iC nucleotide and a fluorescent reporter. Amplification is performed in the presence of dabcyl-diGTP. Site-specific incorporation places the quencher in close proximity to the reporter, leading to a decrease in fluorescence that can be observed during real-time PCR.[10]

In natural DNA, two complementary strands are joined by a sequence of Watson-Crick base pairs using the four standard nucleotides A, G, C, and T. However, the DNA alphabet need not be limited to the four standard nucleotides known in nature.[8,9] In fact, expanded nucleotide pairs have been chemically produced. In particular, the chemistries to produce phosphoramidite and triphosphate reagents of iC and iG have been optimized and are now commercially available. We previously reported this new chemistry (MultiCode-RTx) that uses iC and iG to incorporate site-specifically a quencher in close proximity to a fluorescent molecule during PCR[10] (Figure 1). Before running MultiCode-RTx, target-specific forward PCR primers carrying single iC bases near distinct 5′ fluorescent reporters and standard reverse primers are constructed using standard oligonucleotide chemical synthesis. Using a commercially available reaction mix containing iGTP-dabcyl, iC directs specific enzymatic incorporation of the iGTP-dabcyl in close proximity to each fluorophore. This incorporation reduces the fluorescence of reporters attached to the extended primers and is monitored using standard real-time PCR instrumentation. As the reaction proceeds, the instrument collects data (each target is analyzed using a distinct fluorophore and data collected in distinct channels). As more and more of the labeled primers are used up, the fluorescence signal specific for that primer goes down. As with all other real-time chemistries, standard curves constructed from Ct data from known concentrations of each target are used to determine concentrations within unknown samples. Additionally, the reaction can be analyzed for correct product formation after cycling is complete by melting the amplicons and determining their melting temperatures. This melt analysis can be used to verify that the anticipated amplicon was created.

Using this chemistry, we now report two 3-color LightCycler-1 multiplex real-time PCR assays. The first assay is specific for *pagA*:*capB*:internal positive control (IPC), and the second is specific for *cya:capB*:IPC, both target genes associated with inhalation anthrax. In this study, we show that the assays are able to differentiate specifically these targets from multiple other *Bacillus* species with limits of detection at or below previously published single-plex assays. We also demonstrate the chemistry using an instrument with a signal excitation laser and optics identical to the Idaho Technology Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.). The R.A.P.I.D. was acquired through the Joint Biological Agent Identification and Diagnostic System as the single Department of Defense-accepted platform for both identification and diagnostic confirmation of biological agents.

Materials and Methods

Bacterial Growth and Extraction

The bacterial strains analyzed in this study were acquired from the American Type Culture Collection (Manassas, VA), clinics, or entries from previous U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, Frederick, MD) collections. Either Bactozol kits (Molecular Research Center, Inc., Cincinnati, OH) or QIAamp DNA minikits (Qiagen, Valencia, CA) were used to extract DNA. Bactozol kits were used in accordance with the manufacturer's recommendations. QIAamp kits were used as follows. Cells were pelleted and resuspended in 180 μl of Dulbecco's phosphate-buffered saline (GibcoBRL, Rockville, MD). Twenty microliters of proteinase K and 200 μl of AL buffer (Qiagen) were added and mixed by vortexing. The mixture was incubated for 60 minutes at 55°C to lyse the cells. After incubation, 210 μl of 100% ethanol was added to the sample. The mixture was subject to RNase digestion, transferred to a QIAamp spin column, and centrifuged at 6000 $\times$ *g* for 2 minutes. Next, 500 μl of AW1 buffer (Qiagen) was added to the column, and the sample was centrifuged for 2 minutes at 6000 $\times$ *g*. After this centrifugation step, 500 μl of AW2 buffer (Qiagen) was added to the column, and the sample was centrifuged at 6000 $\times$ *g* for 2 minutes. Finally, 100 μl of

Table 1. Primers Used for Study

Oligo name	Sequence 5′→3′	Conc. in PCR (nmol/L)	Purpose	Software design
AS005	CAAACAGCCCAGTTACAATTACATTAG	200	*pagA* gene primer	Primer express
AS006	FAM-TXAATCCAGGAATCCTGCTCCATC	200	*pagA* gene primer	Primer express
AS007	CAGATAATGCATCGCTTGCTTTAG	200	*capB* gene primer	Primer express
AS008	HEX-TXGGATGAGCATTCAACATACCACG	200	*capB* gene primer	Primer express
MM698	HEX-XGATAATGCATCGCTTGCTTTAG	150	*capB* gene primer	Visual OMP
MM699	GCTGTTTCCTCATCAATCCC	150	*capB* gene primer	Visual OMP
PN 1143	FAM-TXCATGTCGGGGGCATATAAC	100	*cya* gene primer	Primer 3
PN 1144	TGCACCTGACCATAGAACG	100	*cya* gene primer	Primer 3
PN 1323	FAM-XCCTGCTCCATCTGATAATACTCTA	100	*pagA* gene primer	Visual OMP
PN 1324	AGCAGGCAAGGACAGTG	100	*pagA* gene primer	Visual OMP
PN 1139	Cy5-TXGCCTGCTGTGCTGTGT	100	IPC primer	Primer 3
PN 1140	TCGTGCGGTGCGTC	100	IPC primer	Primer 3
PN 1141	HEX-TXGCGCCGTAAAGAAGGTC	150	*capB* gene primer	Primer 3
PN 1142	CTACCCTGCGTTGCTCA	150	*capB* gene primer	Primer 3

Conc., concentration; X, 5-methyl-isocytosine.

AE buffer (Qiagen) preheated to 70°C was applied to the column, and the sample was centrifuged at 6000 × *g* for 1 minute to elute the DNA. The DNA concentration was determined by measuring the absorption of each sample at 260 nm with a DU series 7500 spectrometer (Beckman Instruments, Fullerton, CA).

Primers

All primer designations, sequence make-up, design software implemented, and concentrations used can be found in Table 1. Primer design packages used for this study were Primer Express (Applied Biosystems, Foster City, CA), Primer3,[11] and Visual OMP (DNA Software, Inc., Ann Arbor, MI). Primers AS005 through 008 were initially designed for Taqman use. Incorporation of the iC (X) nucleotides during synthesis was accomplished using standard coupling conditions.[12] All synthetic DNAs were quantitated by using extinction coefficients corresponding to the nucleotide makeup and examining initial stocks by OD 260. The DNAs were diluted to appropriate working concentrations in 10 mmol/L 3-(*N*-morpholino) propanesulfonic acid and 0.1 mmol/L ethylenediamine tetraacetic acid. BLASTN (*http://www.ncbi.nlm.nih.gov/BLAST/*) searches were performed for all primers and probes to eliminate priming to sequences other than those specified. All oligonucleotides were manufactured and purified by IDT (Coralville, IA). Both *cya*- and *pagA*-specific primer pair sets have a 100% match to *B. cereus* isolate G9241 pBCX01 plasmid DNA.[3] The *capB* primer pairs are not complementary to any known sequence within the G9241 isolate.

Real-Time PCR Amplification

PCR conditions included 1× ISOlution 1147 buffer (PN 1147; EraGen, Madison, WI) with addition of 2 mmol/L $MgCl_2$ to reach a final concentration of 4 mmol/L $MgCl_2$ per reaction, at a volume of 25 μl. PCR primers used and their concentrations can be found in Table 1. Titanium *Taq* DNA polymerase (Clontech, Palo Alto, CA) was used at 1× concentration. Cycling parameters for the two triplex assays were 2 minutes denaturation at 95°C followed by 45 cycles of 5 seconds at 95°C denaturation, 5 seconds of anneal at 55°C (*pagA*:*capB*:IPC) or 60°C (*cya:capB*:IPC), and 20 seconds at 72°C with optical read on the LightCycler-1 real-time thermal cycler (Roche Applied Science, Indianapolis, IN). Directly after cycling, thermal melts were run from 60 to 95°C with optical reading performed at 0.4°C increments.

Color Compensation

Color compensation is required for multicolor analysis on the LightCycler-1 instrument. We found that a single compensation file could be used to correct data sets acquired from multiple instruments. This is performed by analyzing the contribution of each single type of labeled DNA oligonucleotide to the signal obtained in each of the three detection channels of the LightCycler-1. The fluorophore set (6-carboxyfluorescein [FAM], hexachlorofluorescein [HEX], and cyanine 5 [Cy5]) that we used is not used by the standard color compensation reagents supplied by the instrument manufacturer. To compensate our data, we used solutions of oligonucleotides labeled with these dyes in 1× ISOlution 1147 without enzyme at the following concentrations: 300 nmol/L FAM, 1000 nmol/L HEX, and 1000 nmol/L Cy5. The instrument manufacturers compensation instructions were then followed to obtain compensation data capable of correcting for the spectral properties of our dye set.

Testing Parameters

All developed assays included the detection of an IPC (DM155) that was added at a level of 1000 copies per reaction and detected with primers 1139 and 1140. The fluorescence change of IPC reaction was monitored in the F3 channel (690–730 nm) of the LightCycler-1 instrument. Performance of the IPC reaction was analyzed by determining the mean Ct, SD, and percent coefficient of variation (%CV) for 218 total reactions each for both of the final triplex assays.

Synthetic oligonucleotide targets corresponding to the anthrax toxin-specific plasmid-associated gene targets were used to develop our assays. Standard curves (Ct versus copy number) were constructed from runs using

10-fold dilution series of these synthetic targets from 3 to 3×10^5 copies per reaction. Analytical specificity (true negatives/true negatives plus false positives) and sensitivity testing (true positives/true positive plus false negatives) was conducted using 100 pg of total extracted DNA from 38 strains of *B. anthracis*, 34 strains of *B. cereus*, 13 strains of *Bacillus thuringiensis*, and 1 strain each of 4 other *Bacillus* sp., as well as a cross-reactivity panel consisting of 72 different strains of other bacterial species (supplemental table at *http://jmd.amjpathol.org/*). Some *B. anthracis* strains contained copies of only one of the two anthrax toxin-specific plasmids. Each 32-capillary LightCycler-1 run included at least one reaction in which a positive control of 1 pg of extracted *B. anthracis* Ames DNA was added and at least one reaction in which no target was added. The analytical limit of detection and limit of quantitation were determined by analyzing (in duplicate) serial 10-fold dilutions of extracted DNA from the Ames strain of *B. anthracis* starting at 1 pg and ending at 1 fg.

Analysis Software

Commercially available real-time thermal cyclers use software designed to analyze reactions in which fluorescence increases with PCR product accumulation. To analyze decreasing fluorescence results, analysis software was developed that imports RTx raw data and performs cycle threshold and melt curve analyses. Raw F1, F2, and F3 component fluorescence data for both amplification and melt programs were exported from the LightCycler-1 Analysis software (Version 5.32) as text files and analyzed with EraGen Real-time Run Importer and Analysis Desktop v0.9.8 α (EraGen).

Results

Initial Assay Development

Initial studies focused on four duplex assays. Each duplex assay contained one set of primers specific to a plasmid target with the other primer set specific to the IPC. Two assays were specific to plasmid pXO1, whereas the other two were specific to plasmid pXO2. The two assays specific to pXO1 targeted two sequences found in the *capB* gene region using primer pairs 1141/1142 or 007/008. The two assays specific to pX02 targeted either *cya* using primer pair 1143/1144 or *pagA* using primer pairs 005/006. Standard curves (log copy number versus Ct) constructed from assays using a series of synthetic target dilutions were linear down to three copies for all duplexed systems (data not shown). With more than 100 reactions performed, 20 copies of synthetic DNA matching the correct gene target regions were detected 100% of the time.

Because we set out to develop assays that could simultaneously detect both plasmids, we combined the primer sets from the duplex assays to create two triplex assays, *pagA:capB*:IPC and *cya*:*capB*:IPC, using primer sets 005 to 008 and 1141 to 1144, respectively. After the cycling parameters were optimized using synthetic targets, we tested their analytical specificity using the DNA extracted from our panel of organisms (supplemental table at *http://jmd.amjpathol.org/*). Well-characterized strains from the U.S. Army Medical Research Institute of Infectious Diseases repository were used for this study. Strain plasmid profile analysis included historical PCR analysis for multiple targets with a variety of real-time and standard PCR primers. In addition, the strains were characterized for capsule and protective antigen production. When tested samples contained DNA extracted from organisms other than *B. anthracis*, which should not have contained either plasmid, we observed product formation in the channels specific to the *pagA* and *capB* primer sets. When the products were melted, the unidentified products differed in melting temperature (T_m) from the positive controls and suggested template independent amplification. We then decided to redesign the assays to eliminate these artifacts.

Optimized Assay Development

Because the approach of mixing primer sets from separate assays was unsuccessful, we decided to construct new assays using new multiplex primer design software called Visual OMP. Because the *cya* primer set 1143/1144 was sensitive down to the single-copy level and did not cross-react with the other panel strains tested (data not shown), we decided to incorporate the set into one of the new assays. Visual OMP design parameters for both assays also included the IPC primer set. The new designs obtained, *pagA*:*capB*:IPC (containing primers 1323, 1324, 698, and 699) and *cya*:*capB*:IPC (containing primers 1143, 1144, 698, and 699), demonstrated similar limits of detection yet a noticeable improvement in sensitivity and specificity over the previously developed triplex assays. Like the previous triplexes, standard curves for the new systems were linear down to three copies with R^2 values greater than 0.99 (Figure 2).

Limit of Detection

We then made 10-fold serial dilutions of DNA extracted from *B. anthracis* Ames to determine the limit of detection for the optimized assays. The extracted DNA was tested in duplicate to determine the lowest detectable concentration. The results indicated that the *pagA*:*capB*:IPC system was able to detect 100 fg of total extracted DNA from all replicates in both channels. The *cya*:*capB*:IPC system was able to detect 10 fg of genomic DNA in duplicate runs in the *cya*-specific channel (Figure 3). Using the Ct values observed and fitting them into the standard curve equations determined above, we estimated the detection limit for the pX01 and pX02 plasmids to be two and one copies for the *pagA*:*capB*:IPC system and nine and two copies for the *cya*:*capB*:IPC system, respectively.

Analytical Specificity and Sensitivity

Unlike the original triplex systems, these new triplex systems were specific and sensitive for the target panel. For example, the *pagA*:*capB*:IPC demonstrated specificity for

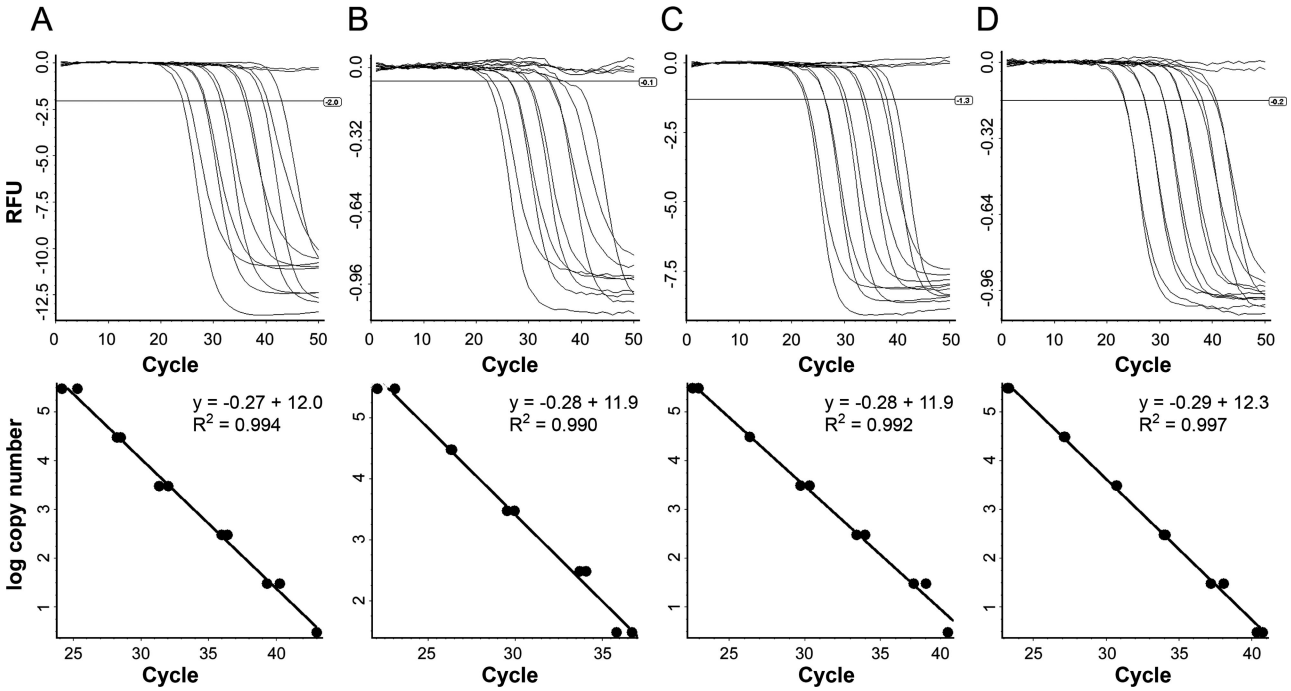

Figure 2. Real-time PCR detection and linear standard curve analysis of anthrax-related toxin genes. The two triplex MultiCode-RTx systems designed using Visual OMP, *pagA*:*capB*:IPC (**A**, *pagA*; **B**, *capB*) and *cya*:*capB*:IPC (**C**, *cya*; **D**, *capB*), were tested for linearity for both corresponding synthetic targets using 10-fold dilution series from 3 to 3 × 10^5 copies in duplicate on different days. **Top panels** show the decrease in fluorescence as relative fluorescence units (RFU) versus PCR cycles for all samples as they appear during the real time PCR reactions. As the fluorescence decrease passes the cycle threshold (Ct) line, a cycle number is tabulated and used to generate standard curves. Reactions containing larger target copy numbers yield a detectable decrease in RFU in fewer rounds of PCR corresponding to smaller Ct values. **Bottom panels** show linear curve analyses of log copy number versus Ct with best fit equation and R^2 values. Internal positive control is not shown.

strains that contain only one of the two virulence plasmids (pX01 or pX02). Of the seven strains containing only pX01, only the *pagA* primer-specific channel reported fluorescent change. Of the two strains containing only pX02, only the *capB* primer-specific channel reported fluorescent change. Two unrelated strains (*Yersinia frederiksenii* and *Salmonella choleraesius*) displayed weak signal change (Figure 4). When these wells were considered to be true false positives by Ct values alone, the assay showed ~97% specificity. However, by including the criteria of correct T_m values, software analysis indicated these to be true negatives. Triplicate re-testing for both the *Y. frederiksenii* and the *S. choleraesius* samples showed no detectable product formation. Therefore using dual criteria of Ct and correct T_m, the *pagA*:*capB*:IPC design was 100% specific. The total of 123 reactions testing panel DNAs from strains other than *B. anthracis* resulted in a 95% confidence interval (CI) of 2%. Additionally, the *pagA*:*capB*:IPC correctly detected all 38 *B. anthracis* strains, resulting in an analytical sensitivity of 100% with a CI of 9%. The *cya*:*capB*:IPC design also correctly detected all strains of *B. anthracis*, including those with single plasmids, again resulting in an analytical sensitivity of 100% with a CI of 9%. In addition, signal change was not observed when DNAs from our panel set were added, including no cross-reactivity to the *B. cereus* or *B. thuringiensis* strains tested. The common IPC sequence amplified almost identically in all assays with mean Ct values of 33.2 and 33.5 cycles for the *cya*:*capB*:IPC and *pagA*:*capB*:IPC, respectively. The SD of 0.5 cycles and 1.6%CV were identical for the two IPC reactions.

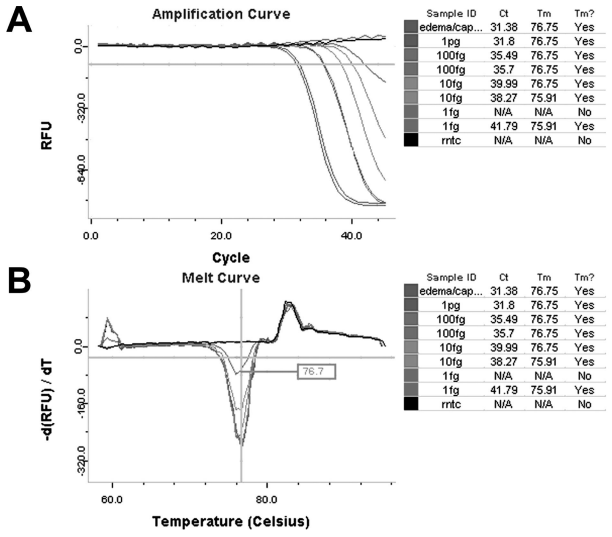

Figure 3. Limit of detection (LOD) for *B. anthracis* Ames genomic DNA. Representative data determining LOD using 10-fold dilution series from 1 pg to 1 fg of *B. anthracis* Ames total genomic DNA in duplicate. **A:** Real-time PCR data of decreasing relative fluorescence units (RFU) versus PCR cycle number. **B:** Postamplification thermal melt analysis of PCR products as the negative first derivative of RFU with respect to temperature (−d(RFU)/dT) versus temperature in °C. Data are from the *cya*:*capB*:IPC multiplex. LOD for *cya* primer set was 10 fg or ~2 copies.

Reproducibility

To determine the variation from run to run, we analyzed the data from eight positive control reactions over the

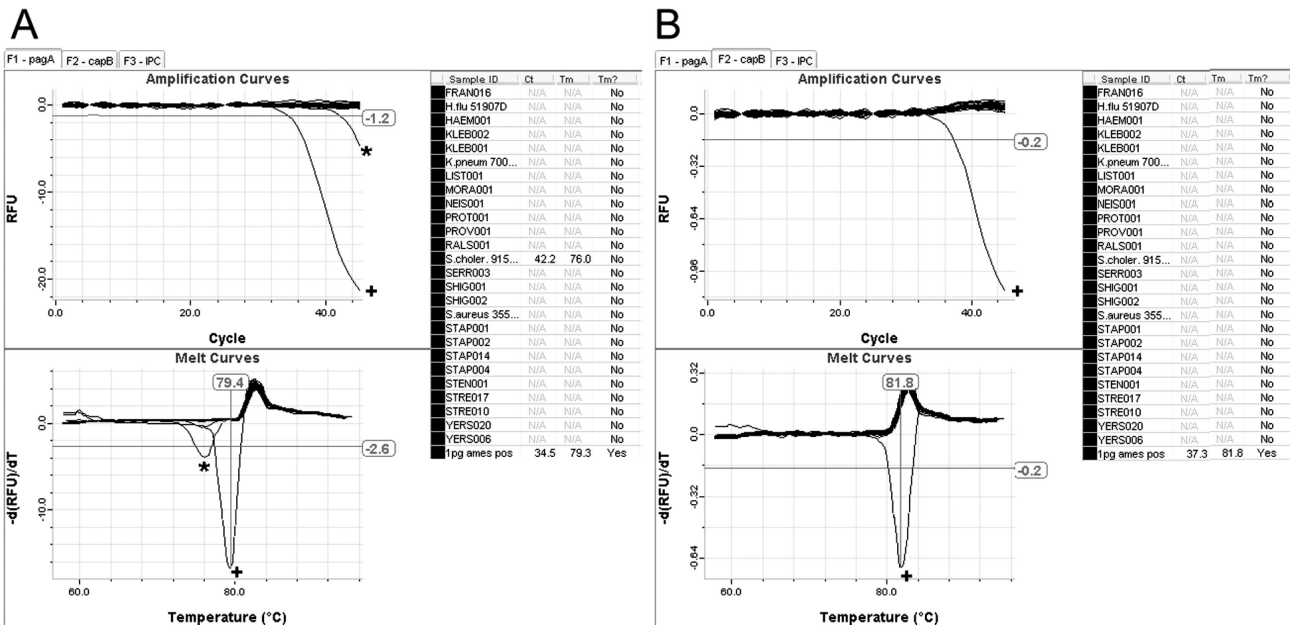

Figure 4. Detection of nonspecific amplification by T_m. For both **A** and **B**, the **top amplification curves** show real-time PCR data in relative fluorescence units (RFU) versus PCR cycle number, and the **bottom panels** show postamplification melt data as the negative first derivative of RFU with respect to temperature (−d(RFU)/dT) versus temperature in °C. A representative data set using a series of unrelated organisms along with 1 pg of Ames DNA control was amplified by the *pagA*:*capB*:IPC triplex primer set. **A:** Real-time and amplicon melt data from the *pagA* read in F1 channel. **B:** Data from *capB* read in F2 channel. The *Salmonella choleraesius* 9150 sample (*) amplified weakly in the F1 channel with a Ct of 42.2 compared with 34.4 for the Ames control (+). The F1 Melt Tm for the *S. choleraesius* amplicon was significantly different at 76.0°C compared with 79.3°C for the control (**A, bottom**), indicating that the *S. choleraesius* amplification product observed during real-time PCR was nonspecific. Triplicate re-testing of the *S. choleraesius* sample gave no detectable amplification (data not shown).

course of 4 weeks for both triplex systems. A positive control reaction consisting of 1 pg of total extracted DNA from *B. anthracis* Ames was included in each LightCycler-1 carousel of 32 capillaries. Mean Ct and T_m, SD, and %CV values from these runs are presented in Table 2. Variation in Ct values was greater than that of T_m, with %CV ranging from 1.9 to 5.0% and 0.1 to 0.5%, respectively.

Plasmid Copy Number

The standard curves from the positive control data set shown in Figure 2 were used to estimate the copy number of each target by using the average Ct from the genomic DNA-positive controls. We estimate there are about 100 copies of the *pagA* and *capB* targets in 1 pg of genomic DNA, consistent with the observed limit of detection of 100 fg or about 10 copies for these targets. The *cya*-specific assay indicates a higher copy number of around 900 copies per picogram, which agrees with the *cya* limit of detection of 10 fg or about 9 copies.

Discussion

Since 2001, when letters containing highly processed anthrax spores from the Ames strain of *B. anthracis* were found addressed to members of Congress and the media, public health diagnostic labs around the United States have become equipped with real-time PCR instruments and associated testing kits used to assay for the presence of anthrax. Real-time PCR has been chosen as the prime screening method for rapid identification because of its intrinsic benefits such as enhanced sensitivity and shortened analytical turnaround times when compared with the more standard culturing techniques.

The two MultiCode-RTx triplex designs presented in this study may provide an alternative to the single-plex anthrax-specific assays now used at many public health labs. Clinical studies are required to validate our systems for direct detection of anthrax toxin-specific plasmid-associated genes in human specimens. These studies may be difficult in view of the very low incidence of human anthrax infections. Yet presuming that these assays can

Table 2. Positive Control Testing Results from the *paga*:*capb*:IPC and *cya*:*capb*:IPC MultiCode-RTx *B. anthracis* Assays

	pagA:*capB*:IPC				*cya*:*capB*:IPC			
	pagA - F1 - FAM		*capB* - F2 - HEX		*cya* - F1 - FAM		*capB* - F2 - HEX	
	Ct	T_m	Ct	T_m	Ct	T_m	Ct	T_m
Mean	37.4	79.2	38.3	81.8	31.8	76.2	35.4	80.1
SD	1.7	0.2	1.9	0.1	0.6	0.3	1.1	0.4
%CV	4.5	0.2	5.0	0.1	1.9	0.4	3.0	0.5

Eight reactions of each assay were run using 1 pg of *B. anthracis* Ames DNA. The mean Ct, T_m, SD, and %CV are tabulated.

be applied directly to human specimens or environmental testing, the specificity and sensitivity demonstrated analytically here should increase the level of accuracy to that obtained using the current single-plex assays. The final RTx triplex systems developed reliably detected 10 to 100 fg of total *B. anthracis*-extracted DNA. These amounts translated into a copy number limit of detection of one to nine anthrax toxin-specific plasmids. Although the exact number of virulence plasmids per *B. anthracis* cell can vary (as many as 243 copies of pX01 and 32 copies of pX02 per cell can exist), typically more than one copy of each per cell exists.[13,14] This fact would further improve the limit of detection of *B. anthracis* cells. Analytical specificity and sensitivity were comparable with reported single-plex real-time assays.[4,15]

The data presented also show for the first time simultaneous quantitative detection of three independent targets using three colors on the LightCycler-1. Previous usage of three-color detection on the LightCycler was used for genotyping via melt analysis.[16] Unlike this multicolor system and many other real-time PCR chemistries, RTx does not use probes.[17–19] There are perceived benefits to using probes in PCR real-time detection, with the most important being specificity. Yet, probe-based systems are clearly more difficult to design and are complicated by the inherent fact that single-stranded DNA targets form intramolecular structures that interfere with probe binding.[20] Many primer design software programs have been developed to compensate for this by focusing on the probe region and probe design, while relaxing primer restraints. When proper primer design software is used, we do not believe probes are needed for specificity, as our data demonstrate. There are other real-time PCR technologies that do not use probes.[21–23] Compared with these systems, RTx does not require incorporation of hairpins in the primer design nor does it require special base sequence make-up near the 3′ ends. This allows for easy use of previously designed primer pairs. The RTx technology also allows multiplexing to assay multiple targets or to include internal controls. Real-time multiplexing is not an option with SYBR Green, although postreaction melt analysis multiplexing can be implemented.[24] The current availability of RTx reagents and Visual OMP design software should allow others to compare multiplexed probe-based systems with the RTx probe-free system.

Finally for bioweapon detection, the use of the LightCycler-1 instrument is important because it is essentially the same instrument as the R.A.P.I.D. For this reason, our successful multiplex results using the LightCycler-1 suggest that the RTx system would work equally well on the R.A.P.I.D. system. This device was recently chosen by the U.S. Army Space and Missile Defense Command Joint Biological Agent Identification and Diagnostic System for biothreat sample processing. The R.A.P.I.D. is a specialty instrument for military field hospitals, first responders, and use in other rough environments. The ability to test for multiple targets and internal control targets simultaneously should allow increased throughput and more consistent and controllable results.

We have demonstrated a simple closed-tube real-time PCR method to specifically detect genes associated with anthrax. The methodology involves the use of an expanded genetic alphabet to site-specifically incorporate a reporter into an amplification product. This method has several benefits such as rapid development due to ease of design, postreaction melt analysis, multiplex capability, large and consistent change in fluorescence output, long-term storage (no probe breakdown possible), and the ability to confirm the reactions by internal controls. The use of expanded base methodology is not limited to PCR.[25–27] There are other detection methods that could benefit from this chemistry such as oligo ligation assay, restriction fragment length polymorphism, single-stranded conformational polymorphism, or molecular beacons. With such a fundamental paradigm shift, we envision that additional base pairs will change the way scientists build new testing systems.

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