# Detection of *Bacillus anthracis* DNA by LightCycler PCR

Constance A. Bell,[1]† James R. Uhl,[1] Ted L. Hadfield,[2] John C. David,[2]
Richard F. Meyer,[3] Thomas F. Smith,[1]
and Franklin R. Cockerill III[1,4]*

*Division of Clinical Microbiology[1] and Division of Infectious Diseases,[4] Mayo Clinic, Rochester,
Minnesota 55905; Armed Forces Institute of Pathology, Washington, D.C. 20306[2]; and
Centers for Disease Control and Prevention, Atlanta, Georgia 30333[3]*

Anthrax is a zoonotic disease that is also well recognized as a potential agent of bioterrorism. Routine culture and biochemical testing methods are useful for the identification of *Bacillus anthracis*, but a definitive identification may take 24 to 48 h or longer and may require that specimens be referred to another laboratory. Virulent isolates of *B. anthracis* contain two plasmids (pX01 and pX02) with unique targets that allow the rapid and specific identification of *B. anthracis* by PCR. We developed a rapid-cycle real-time PCR detection assay for *B. anthracis* that utilizes the LightCycler instrument (LightCycler *Bacillus anthracis* kit; Roche Applied Science, Indianapolis, Ind.). PCR primers and probes were designed to identify gene sequences specific for both the protective antigen (plasmid pX01) and the encapsulation B protein (plasmid pX02). The assays (amplification and probe confirmation) can be completed in less than 1 h. The gene encoding the protective antigen (*pagA*) was detected in 29 of 29 virulent *B. anthracis* strains, and the gene encoding the capsular protein B (*capB*) was detected in 28 of 29 of the same strains. Three avirulent strains containing only pX01 or pX02, and therefore only *pagA* or *pagB* genes, could be detected and differentiated from virulent strains. The assays were specific for *B. anthracis*: the results were negative for 57 bacterial strains representing a broad range of organisms, including *Bacillus* species other than *anthracis* (*n* = 31) and other non-*Bacillus* species (*n* = 26). The analytical sensitivity demonstrated with target DNA cloned into control plasmids was 1 copy per µl of sample. The LightCycler *Bacillus anthracis* assay appears to be a suitable method for rapid identification of cultured isolates of *B. anthracis*. Additional clinical studies are required to determine the usefulness of this test for the rapid identification of *B. anthracis* directly from human specimens.

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*Bacillus anthracis* is a spore-forming gram-positive bacillus that causes the disease anthrax in animals and humans. Infection in humans is extremely rare in the developed world and is generally due to contact with infected animals or contaminated animal products. Humans may contract the disease via three routes: cutaneous inoculation via a cut or abrasion, ingestion of contaminated meat, or inhalation of spores. In naturally acquired infections, the cutaneous mode of transmission is by far the most common, accounting for approximately 95% of infections in the United States (6). Death is uncommon in cases of cutaneous anthrax, but not uncommon in gastrointestinal or inhalation anthrax (7, 11, 13, 20, 21).

*B. anthracis* is easily cultivated in its vegetative (bacillary) form with conventional growth media. Sporulation can be achieved by using nutrient-deficient media. Because of their relatively small size (less than 5 µm), spores may be easily inhaled into the lower respiratory tract in humans (11, 20). This ease of cultivation, coupled with the hardy nature of its spores and the high mortality associated with inhalation anthrax, makes *B. anthracis* a potential biological warfare agent. Concern about the use of anthrax as an agent of bioterrorism and a weapon of mass destruction has increased due to the recent deliberate dissemination of anthrax spores in United States and the resulting inhalational and cuta-

neous infections. To ensure that patients infected with *B. anthracis* are treated as rapidly as possible, tests that rapidly identify the organism in culture, clinical specimens, and environmental samples are essential.

Culture represents the "gold standard" for identification of *B. anthracis*. A definitive identification of isolated colonies as *B. anthracis* may take 24 to 48 h or more and requires specialized testing, including direct fluorescent antibody (DFA) staining of capsule and cell wall polysaccharide and lysis of colonies by gamma phage. These confirmatory tests are not generally available in clinical microbiology laboratories in the United States, except at higher-level public health laboratories, which are part of the Laboratory Response Network established by the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. (17).

*B. anthracis* and other members of the *Bacillus cereus* group exhibit an extremely high degree of genomic homology (1, 9), making differentiation by PCR challenging. However, virulent isolates of *B. anthracis* contain two plasmids, pX01 and pX02, with unique virulence gene targets that allow the rapid identification of *B. anthracis* by PCR. Several conventional PCR assays have been described for the identification of *B. anthracis* (2–4, 16, 18, 19). These assays are time-consuming and labor-intensive, requiring a separate step after PCR for confirmation of product. These assays have been constructed to identify a gene target on a single plasmid (4, 16, 19) or both plasmids (2, 18) and use conventional PCR and product detection formats.

* Corresponding author. Mailing address: Mayo Clinic, Division of Microbiology, 200 1st St. SW, Rochester, MN 55905. Phone: (507) 284-2901. Fax: (507) 284-4272. E-mail: cockerill.franklin@mayo.edu.
† Present address: Tripler Army Medical Center, Honolulu, Hawaii.

Rapid-cycle real-time PCR represents a significant advance in PCR testing (5). Real-time PCR instruments have built-in thermocyclers and fluorimeters, which permit rapid PCR cycling and simultaneous detection of amplification product. Furthermore, PCR and product detection are performed in the same closed reaction vessel, which considerably lessens the chance for contamination. A first-generation real-time PCR platform, which uses the fluorogenic probe-based assay Taq-Man 5′ nuclease assay (Applied Biosystems, Foster City, Calif.), has been developed to detect a variety of biothreat agents, including anthrax (10). In our experience, this type of assay requires approximately 2 h to complete, and unlike newer real-time PCR platforms, information on product detection is not available to the technologist until all PCR cycles are completed (5).

Second-generation real-time PCR assays offer faster thermocycling, and information on product detection is available to the technologist with each PCR cycle. Assays using second-generation real-time PCR instrumentation (LightCycler; Roche Applied Science, Indianapolis, Ind.) have been developed, which detect the *capB* gene (plasmid pX02) (14) or both the *capB* and the *pagA* genes (plasmid pX01) (15). The first of these assays uses SYBR Gold and the second uses SYBR Green as fluorescent indicators of the amplification product. However, SYBR Gold and SYBR Green are dyes that will intercalate between any double-stranded DNA structure, and therefore specific product detection cannot be ensured.

Plasmids pX01 and pX02 have been completely sequenced, although the functions of many of the genes on these plasmids are not yet known. However, three proteins encoded by genes on plasmid pX01 have long been recognized as important contributors to *B. anthracis* pathogenicity. The three proteins are the protective antigen (*pagA* gene), edema factor (*cya* gene), and lethal factor (*lef* gene), and together the genes coding for these proteins encode the toxin complex. The protective antigen combines with both lethal factor and edema factor to produce binary toxins, and it is the protective antigen that allows the toxin complexes to bind to host cells for toxin delivery (8). The *capB* gene is one of three genes (*capA*, *capB*, and *capC*) on plasmid pX02 that are necessary for biosynthesis of the protective polypeptide capsule (8).

A rapid assay that identifies gene targets on both virulence plasmids (pX01 and pX02) is preferred, because *B. anthracis* strains can lack either plasmid. Vaccine strains, with reduced virulence in humans, may be used in biocrime hoaxes. These strains lack either virulence plasmid, and so identification of these strains in the setting of a potential biocrime is important to prevent undue anxiety and inappropriate treatment of exposed individuals. Likewise, rapid identification of virulent *B. anthracis* strains is especially important, since systemic disease may not be detected early enough by conventional techniques to allow sufficient time to begin effective antibiotic therapy. The high mortality associated with inhalation anthrax is thought to be due in large part to a delay in diagnosis (20). Recent cases of inhalation anthrax in the United States have demonstrated that mortality rates may be greatly reduced and patients can make a full recovery provided the infection is promptly diagnosed and appropriate antibiotic treatment is initiated in a timely manner (12).

This study presents the development and evaluation of a

TABLE 1. *B. anthracis* isolates tested for presence of *pagA* (plasmid pX01) and *capB* (plasmid pX02)

| Isolate | pX01/pX02 | LightCycler assay result | |
|---|---|---|---|
| | | *pagA* | *capB* |
| Ames | pX01⁺/pX02⁺ | + | + |
| BC3132 | pX01⁻/pX02⁺ | − | + |
| delta-Ames | pX01⁻/pX02⁺ | − | + |
| GT3 | pX01⁺/pX02⁺ | + | + |
| GT10 | pX01⁺/pX02⁺ | + | + |
| GT15 | pX01⁺/pX02⁺ | + | + |
| GT20 | pX01⁺/pX02⁺ | + | + |
| GT23 | pX01⁺/pX02⁺ | + | + |
| GT25 | pX01⁺/pX02⁺ | + | −[a] |
| GT28 | pX01⁺/pX02⁺ | + | + |
| GT29 | pX01⁺/pX02⁺ | + | + |
| GT30 | pX01⁺/pX02⁺ | + | + |
| GT34 | pX01⁺/pX02⁺ | + | + |
| GT35 | pX01⁺/pX02⁺ | + | + |
| GT38 | pX01⁺/pX02⁺ | + | + |
| GT41 | pX01⁺/pX02⁺ | + | + |
| GT45 | pX01⁺/pX02⁺ | + | + |
| GT51 | pX01⁺/pX02⁺ | + | + |
| GT55 | pX01⁺/pX02⁺ | + | + |
| GT57 | pX01⁺/pX02⁺ | + | + |
| GT62 | pX01⁺/pX02⁺ | + | + |
| GT68 | pX01⁺/pX02⁺ | + | + |
| GT69 | pX01⁺/pX02⁺ | + | + |
| GT77 | pX01⁺/pX02⁺ | + | + |
| GT80 | pX01⁺/pX02⁺ | + | + |
| GT85 | pX01⁺/pX02⁺ | + | + |
| GT87 | pX01⁺/pX02⁺ | + | + |
| Mayo 1 | pX01⁺/pX02⁺ | + | + |
| SD96-1-355 | pX01⁺/pX02⁺ | + | + |
| Sterne | pX01⁺/pX02⁻ | + | − |
| Vollum | pX01⁺/pX02⁺ | + | + |
| Zimbabwe | pX01⁺/pX02⁺ | + | + |

[a] Initial genotyping of this strain at the AFIP showed the presence of the *capA* gene. Subsequent testing in the present study did not reveal the presence of the *capB* gene, suggesting that the plasmid pX02 was lost by the organism. This was confirmed, because PCR assays for all encapsulation genes (*capA*, *capB*, and *capC*) were negative.

rapid-cycle real-time PCR assay, which uses the LightCycler instrument and the LightCycler *Bacillus anthracis* kit (Roche Applied Science) for the identification of *B. anthracis*. The assay identifies the protective antigen (*pagA*) gene from plasmid pX01 and the encapsulation B (*capB*) gene from plasmid pX02. LightCycler technology permits rapid PCR cycling with real-time fluorescent resonance energy (FRET) probe detection of product. Amplification and probe confirmation of product can be performed in the same closed reaction vessel and completed in less than 1 h. The assay was validated for use as a rapid method for identification of cultured isolates of *B. anthracis*. A bank of archived strains of *B. anthracis*, *Bacillus* spp. other than *B. anthracis*, and non-*Bacillus* bacteria were evaluated by this method.

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## MATERIALS AND METHODS

**Bacterial strains.** Thirty-one strains of *B. anthracis*, 31 strains of *Bacillus* spp. other than *B. anthracis*, and 26 strains of bacteria from genera other than *Bacillus*

TABLE 2. Other organisms tested for the presence of *pagA* (plasmid pX01) and *capB* (plasmid pX02)

| Organism | No. of isolates tested | LightCycler assay result | |
|---|---|---|---|
| | | *pagA* | *capB* |
| *Bacillus cereus* | 8 | − | − |
| *Bacillus circulans* | 2 | − | − |
| *Bacillus globigii* | 1 | − | − |
| *Bacillus laterosporus* | 1 | − | − |
| *Bacillus megaterium* | 3 | − | − |
| *Bacillus mycoides* | 1 | − | − |
| *Bacillus niacini* | 1 | − | − |
| *Bacillus pumilus* | 3 | − | − |
| *Bacillus silvestris* | 1 | − | − |
| *Bacillus sphaericus* | 1 | − | − |
| *Bacillus subtilis* | 4 | − | − |
| *Bacillus thuringiensis* | 4 | − | − |
| *Bacillus thuringiensis* serovar israeliensis | 1 | − | − |
| *Brucella abortus* | 1 | − | − |
| *Brucella bovis* | 1 | − | − |
| *Brucella melitensis* | 1 | − | − |
| *Burkholderia mallei* | 1 | − | − |
| *Burkholderia pseudomallei* | 1 | − | − |
| *Clostridium botulinum* | 4 | − | − |
| *Clostridium perfringens* | 2 | − | − |
| *Corynebacterium diphtheriae* | 1 | − | − |
| *Corynebacterium jeikeium* | 1 | − | − |
| *Deinococcus radiodurans* | 1 | − | − |
| *Francisella tularensis* | 1 | − | − |
| *Listeria innocua* | 1 | − | − |
| *Paenibacillus (Bacillus) lautus* | 3 | − | − |
| *Paenibacillus (Bacillus) macerans* | 1 | − | − |
| *Staphylococcus aureus* | 2 | − | − |
| Coagulase-negative *Staphylococcus* | 1 | − | − |
| *Vibrio cholerae* | 1 | − | − |
| *Yersinia pestis* | 2 | − | − |

and including other potential bioterrorism agents were evaluated (Tables 1 and 2). Phenotypic culture-based identification and, in some cases, genotypic identification of these bacterial strains were performed previously either at the Mayo Clinic or the Armed Forces Institute of Pathology (AFIP). Genotyping of strains by determination of the presence of either the *pagA* gene (plasmid pX01) or the *capA* gene (plasmid pX02) was previously performed at the AFIP for *B. anthracis* strains (Table 1). Fresh isolates of archived strains were used for nucleic acid extraction and subsequent evaluation by the LightCycler assay.

**Cultivation of bacterial strains.** *B. anthracis* strains were cultured on sheep blood agar plates and nutrient agar plates at 37°C. All *B. anthracis* strains were nonhemolytic, catalase-positive, nonmotile, gram-positive bacilli and were confirmed as *B. anthracis* by a DFA technique (CDC). Bacteria other than *B. anthracis* were identified by using the appropriate media, culturing conditions, and biochemical determinations.

**DNA template preparation.** Template or "target" DNA for use in the PCRs was isolated from bacterial strains with the High Pure PCR template preparation kit (Roche Applied Science) or the MagNA Pure LC DNA isolation kit (Roche Applied Science) according to the manufacturer's directions.

**PCR primers and probes.** Oligonucleotide primers for PCR were designed to amplify the desired gene products from *pagA* (GenBank accession no. M22589) and *capB* (GenBank accession no. M24150). FRET probes for each of these gene PCR products were labeled with fluorescein and LC (LightCycler)-Red640. These PCR primers and FRET probes for *pagA* and *capB* are available as analyte-specific reagents through Roche Applied Science (LightCycler *Bacillus anthracis* detection kit; catalog no. 33 03 411).

**Control DNA.** The positive control DNAs for *pagA* and *capB* are plasmid constructs containing a portion of these target genes. These controls are available as part of the LightCycler *Bacillus anthracis* detection kit.

**LightCycler PCR.** PCR amplification and product detection for all bacterial strains were performed with the Roche LightCycler *Bacillus anthracis* detection kit and the LightCycler instrument.

The assays to detect *pagA* and *capB* were performed in separate cuvettes with FRET probe detection. For the assay, 5 μl of extracted sample or positive control DNA was added to 15 μl of the PCR mix in each reaction cuvette. The reaction conditions are identical for both gene targets, so the *pagA* and *capB* tests may be performed with the same run. Amplification was performed for 1 cycle as follows: 95°C for 10 min followed by 45 cycles of 95°C for 10 s (denaturation), 55°C for 15 s (annealing), and 72°C for 12 s (primer extension).

**Melting curve analysis.** Following the completion of the PCR amplification reaction, a melting curve analysis was performed (50 to 85°C) to confirm the product identification. The paired FRET hybridization probes are designed to anneal with unique segments of either the *pagA* or *capB* gene and provide characteristic $T_m$ values.

**Analytical sensitivity.** Dilutions of DNA were prepared and assayed to determine the analytical sensitivity of the assay. Dilutions that yielded 5 to 1,000,000 copies of the target DNA per reaction were tested.

## RESULTS

**Analytical sensitivity.** Five copies of the *pagA* and the *capB* target sequences per reaction (1 copy per μl of sample) were detectable. Representative cycling curves for samples tested for *pagA* are shown in Fig. 1.

**Melting curve analysis.** Each amplification product for *pagA* and *capB* demonstrated a specific and characteristic melting curve. The $T_m$s for the products generated were 61.5°C for *pagA* and 67.0°C for *capB*. Representative melting curves for samples tested for *pagA* are shown in Fig. 1.

**Evaluation of bacterial strains.** Twenty-nine of the 32 *B. anthracis* strains evaluated in this study were considered virulent strains. These strains were shown previously to contain both the *pagA* and *capA* genes, indicating that both the pX01 and pX02 plasmids were present (Table 1). The LightCycler *Bacillus anthracis* assay detected the *pagA* gene target in all 29 of these *B. anthracis* strains, while the *capB* gene was detected in 28 of 29 of these strains (Table 1). The *capB* assay was repeated from the same DNA preparation for the one strain (GT25) for which the *capB* gene was not initially detected, and again the *capB* gene was not detected. To determine if a gene deletion had occurred in *B. anthracis* isolate GT25, seven PCR primer pairs were selected to amplify 325- to 492-bp fragments of the contiguous encapsulation protein genes, *capA* (1,236 bp), *capC* (450 bp), and *capB* (1,395 bp). Amplification of DNA was obtained from all seven PCRs with DNA extracted from *B. anthracis* strain BC3132 (pX02+), but not with DNA from isolate GT25. To determine if the DNA extracted from isolate GT25 is amplifiable, the *pagA* gene assay was repeated, and again the *pagA* gene was amplified. It was concluded that the *B. anthracis* GT25 isolate evaluated in the present study had lost all of the encapsulation (*capA*, *capC*, and *capB*) genes, implying that plasmid pX02 was also lost.

*B. anthracis* delta-Ames and BC3132 lack plasmid pX01, and therefore the *pagA* gene. The LightCycler assay detected the *capB* gene and not the *pagA* gene in these two strains. *B. anthracis* Sterne lacks plasmid pX02, and as expected, the LightCycler assay detected *pagA*, but not *capB*, in this strain.

Thirty-one strains of *Bacillus* spp. (other than *B. anthracis*) and 26 strains of other bacteria (genera other than *Bacillus* and including other potential agents of bioterrorism) were negative for both *capB* and *pagA* by the LightCycler assay (Table 2).
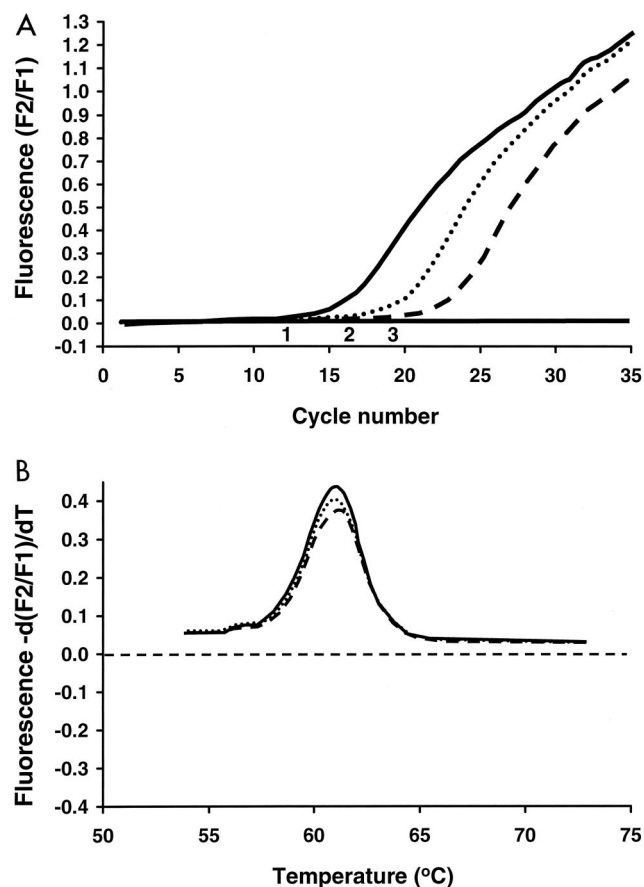
FIG. 1. (A) Representative LightCycler assay for detection of the *pagA* gene by using FRET probes. Shown are the results from three samples. The solid line indicates a DNA preparation from a *B. anthracis* strain with a relatively high concentration of target DNA, the dotted line indicates a positive plasmid control, and the dashed line indicates a DNA preparation from another *B. anthracis* strain with a relatively low concentration of target DNA. (B) Confirmatory analysis (quality control step) by evaluation of melting curves. In this case, the negative derivative of fluorescence, −d(F2/F1), over the derivative of time, dT, is compared to temperature. F1 refers to the F1 channel of the LightCycler that detects the 530-nm light emitted by the fluorescein fluorophore. F2 refers to the F2 channel of the LightCycler that detects the 640-nm light emitted by the LC (LightCycler)-Red640 acceptor fluorophore. The same melting curve occurs for the two samples and the positive control shown in panel A, indicating that DNA with identical sequences has been amplified for each of the three samples.

## DISCUSSION

Conventional culture-based methods are considered the gold standard for identification of *B. anthracis*. These manual testing methods are time-consuming, and confirmatory testing frequently requires referral of isolates to reference laboratories. The rapid-cycle real-time PCR assay described herein appears to be a useful test for confirming *B. anthracis* isolated by culture. The results of our study showed that the LightCycler *Bacillus anthracis* assay correctly identified all 31 *B. anthracis* strains tested. Twenty-eight of these strains carried both the *pagA* and *capB* genes and were confirmed as virulent *B. anthracis* strains. One strain was previously shown to carry both

the *pagA* gene and the *capA* gene and therefore was classified as a virulent strain. Subsequent analysis of this strain in the present study demonstrated the absence of the *capB* gene. Further evaluation of this strain indicated a loss of all three encapsulation genes (*capA, capB*, and *capC*), suggesting that plasmid pX02 was no longer present. Three strains lacked either of these genes and were correctly identified as avirulent *B. anthracis* strains. The specificity of the assay was also demonstrated. None of the 31 *Bacillus* species other than *B. anthracis* strains and none of the other 26 organisms representing genera other than *Bacillus* had positive results for either *pagA* or *capB*.

Methods currently used for presumptive identification of *B. anthracis* and which can be performed in most clinical laboratories include cultivation on sheep blood agar, Gram stain and catalase, and motility testing. *B. anthracis* organisms are large spore-forming gram-positive bacilli that are catalase positive and amotile and do not produce hemolysis on blood agar. To confirm organisms with these characteristics as *B. anthracis*, it is recommended that they be tested for lysis by gamma phage and that direct fluorescent antibody (DFA) testing for capsule antigen and cell wall polysaccharide be performed. The reliability of gamma phage and DFA testing is unknown, and currently in the United States, these tests can only be performed at higher-level laboratories that are part of the Laboratory Response Network (17).

PCR-based nucleic acid amplification methods have been recognized as valuable diagnostic tools for some time. However, conventional PCR testing has not been widely implemented in clinical laboratories, in part due to its requirement for time-consuming and labor-intensive steps, which include specimen preparation, amplification, and product confirmation. Moreover, there have been concerns about amplification product (amplicon) carryover contamination. The latter three concerns are essentially eliminated by rapid, real-time PCR testing methods, in which the amplification reaction and confirmation of product occur rapidly and automatically in a closed reaction vessel. Specimen preparation has also been streamlined by automated extraction platforms, such as the MagNAPure instrument (Roche Applied Science), which was used to extract DNA from the majority of *B. anthracis* strains evaluated in the present study. Other potential advantages of real-time PCR testing based methods compared to culture-based methods include enhanced sensitivity and shortened analytical turnaround times for the direct detection of infectious agents from human specimens. Using real-time PCR and FRET technology, we have noted significantly greater sensitivities and reduced analytical times compared with culture for the direct detection of group A streptococci, *Bordetella pertussis*, herpes zoster virus, herpes simplex virus, and cytomegalovirus from patient samples (5).

An analytical turnaround time of less than 1 h post-specimen processing is frequently possible with the LightCycler *Bacillus anthracis* assay. Extraction of DNA from *B. anthracis* isolates can be accomplished in approximately 1 h with the MagNA-Pure instrument and Mag NA Pure LC DNA isolation kit and in about 30 min with the High Pure PCR template preparation kit. Therefore, the total time for specimen processing and analysis is 1.5 to 2 h. Because the current application is described for identification of cultured isolates of *B. anthracis*, an

additional 18 to 24 h may be required for bacterial growth before the LightCycler test can be performed. We are currently evaluating the utility of this assay for detection of *B. anthracis* directly from patient samples. Preliminary work performed in our laboratory indicates that one copy of target DNA (either *capB* or *pagA*) can be detected by this method in 1 μl of spiked human blood. This is in the same range as that observed in the analytical sensitivity studies conducted for the present evaluation. Clinical studies are required to assess whether this sensitivity will be validated for direct detection of *B. anthracis* in human specimens. These studies may be difficult in view of the very low incidence of human anthrax infections. Presuming that this assay can be applied directly to human specimens, the analytical turnaround time should be considerably less than that required for conventional culture-based tests, which minimally require 24 h to complete.

Even with more widespread utilization of this real-time PCR testing for *B. anthracis*, there will be a continued need for culture. For example, the standard genetic fingerprinting method for bacteria is pulsed-field gel electrophoresis, and this method involves fragmentation of genomic DNA obtained from culture. Furthermore, antimicrobial susceptibility testing of *B. anthracis* requires that the organism be cultured.

The diagnostic potential of PCR for the direct detection of *B. anthracis* from human specimens has been confirmed in a report detailing the first 10 cases of inhalation anthrax associated with the recent deliberate release of anthrax in the United States (12). For 2 of the 10 patients reported in this study, all cultures of sterile fluids, including blood and pleural fluid, were negative for *B. anthracis*. Both of these patients received antimicrobial agents effective against *B. anthracis* early in their illness, which may have resulted in negative cultures. One of these patients had a positive PCR result for a blood sample; immunohistochemistry stains for *B. anthracis* cell wall and capsule antigen gave positive results for a pleural fluid sample. The second patient had a positive PCR result for *B. anthracis* for two pleural fluid samples; one of these pleural fluids was tested and was positive by immunohistochemical staining. For many patients in the study, it was noted that blood cultures rapidly became sterile after initiation of antibiotic therapy. All of these findings support the contention that laboratories that rely solely on conventional culture-based techniques may underdiagnose anthrax. A user-friendly real-time PCR assay like the LightCycler assay may provide a useful adjunct to culture for laboratories attempting to detect *B. anthracis*.

For PCR assays, we believe that gene targets on both virulence plasmids of *B. anthracis* (pX01 and pX02) should be amplified. This is because both of these plasmids must be present for an isolate to be virulent. Such PCR assays may therefore have the potential to differentiate virulent from avirulent (vaccine) strains. The targeting of chromosomal nucleic acid sequences for *B. anthracis* identification is discouraged due to the extensive chromosomal homology that exists among the organisms of the *B. cereus* genogroup. In fact *B. anthracis*, *B. cereus*, and *B. thuringiensis* may even belong to the same species (9).

Plasmids pX01 and pX02 may spread horizontally (9), and *B. anthracis* isolates may be "cured" of a plasmid in the laboratory (8). Indeed, Pasteur generated the first live, attenuated bacterial vaccine by growing *B. anthracis* at an elevated temperature.

This experiment resulted in the loss of the plasmid pX02 and therefore the loss of the antiphagocytic capsule genes that are located on this plasmid (8). Naturally occurring isolates lacking plasmid pX02 have been identified (22), and vaccine isolates without either plasmid pX01 or pX02 have been produced. These nonvirulent isolates could be used as hoax agents, necessitating the targeting of genes in both plasmids for the molecular detection of *B. anthracis*. The LightCycler assay presented herein detects both *pagA* and *capB* genes and therefore may help to distinguish virulent from avirulent or vaccine strains. However, in the setting of clinical disease, identification of only one gene target (*capB* or *pagA*) may indicate spontaneous loss of the respective plasmid with passage by culture in vitro.

In conclusion, the results of the present study indicate that the LightCycler *Bacillus anthracis* assay appears to be a suitable rapid method for identification of cultured isolates of *B. anthracis*. Further clinical studies are required to determine the utility of this test for the rapid identification of *B. anthracis* directly from human specimens.

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