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Identification of Capsule-Forming *Bacillus anthracis* Spores with the PCR and a Novel Dual-Probe Hybridization Format

TIMOTHY C. REIF,[1] MALCOLM JOHNS,[1] SURESH D. PILLAI,[2] AND MITCHELL CARL[1]*

Accelerated Product Development Program, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland 20889,[1] and Texas A & M University Research Center, El Paso, Texas 79927[2]

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Anthrax is a fatal infection of humans and livestock that is caused by the gram-positive bacterium *Bacillus anthracis*. The virulent strains of *B. anthracis* are encapsulated and toxigenic. In this paper we describe the development of a PCR technique for identifying spores of *B. anthracis*. Two 20-mer oligonucleotide primers specific for the *capB* region of 60-MDa plasmid pXO2 were used for amplification. The amplification products were detected by using biotin- and fluorescein-labeled probes in a novel dual-probe hybridization format. Using the combination of PCR amplification and dual-probe hybridization, we detected two copies of the bacterial genome. Because the PCR assay could detect a minimum of 100 unprocessed spores per PCR mixture, we attempted to facilitate the release of DNA by comparing the effect of limited spore germination with the effect of mechanical spore disruption prior to PCR amplification. The two methods were equally effective and allowed us to identify single spores of *B. anthracis* in PCR mixtures.

Bacillus anthracis is a gram-positive endospore-forming bacterium that is capable of producing fatal infections both in livestock and in humans (14). At the present time, humans are infected primarily through contact with products derived from contaminated animals which are infected after they come into contact with soil-borne spores. Humans can become infected, however, when contaminated soil is ingested or inhaled or comes in contact with an open wound (1). Virulent strains of *B. anthracis* are encapsulated and cause death in humans and animals by producing various toxins, including the lethal factor, the protective antigen, and the edema factor (9, 17). The poly-D-glutamic acid capsule and the toxins are encoded by genes present on two large plasmids, designated pXO2 and pXO1, respectively (5, 12). The virulence of encapsulated bacteria has been demonstrated with several species, including *Escherichia coli* and *Neisseria meningitidis* (3, 10). The capsule of *B. anthracis* is unique in that it is composed of a homopolypeptide (D-glutamic acid) (7). Previous studies (11) have shown that 60-MDa plasmid pXO2 is essential for capsule formation and that the *cap* region located on plasmid pXO2 encodes the capsular protein. Three cistrons (*capB*, *capC*, and *capA*) have been identified within the *cap* region (11).

There is a growing need for methods to detect *B. anthracis* spores and vegetative cells. The development of such methods should help prevent large-scale livestock destruction and protect humans who may come into contact with spores and vegetative cells.

We have previously described (4) the detection of *B. anthracis* spores by using PCR amplification of a 1.2-kb region of the edema factor gene. In this paper we describe the development of a second PCR assay in which a DNA fragment within the *capB* gene is targeted. Use of this PCR assay allowed us to detect single spores of *B. anthracis*.

* Corresponding author. Present address: HIV Division, Naval Medical Center of San Diego, San Diego, CA 92134-5000. Phone: (619) 532-6188. Fax: (619) 532-8798.

MATERIALS AND METHODS

Bacterial strains and DNA preparations. DNAs from several *B. anthracis* strains (strains Ames, Vollum, New Hampshire, and Sterne) were prepared as previously described (16) and were kindly provided by Dario Leslie, Chemical and Biological Defence Establishment, Porton Down, Salisbury, United Kingdom. Each DNA was ethanol precipitated (16), vacuum dried, and resuspended in sterile distilled water. The amounts of DNAs were determined by spectrophotometry or fluorimetry with a DNA minifluorometer (Hoefer, San Francisco, Calif.).

For controls, DNAs were extracted (16) from various bacterial species, including *Clostridium botulinum* type A and E strains ATCC 25763 and ATCC 9564, *Yersinia pestis* ATCC 19428, *Vibrio cholerae* ATCC 14035, *Bacillus megaterium* ATCC 14581, *Bacillus licheniformis* ATCC 12759, *Bacillus thuringiensis* ATCC 10792, *Bacillus cereus* ATCC 14579, *Bacillus globigii* ATCC 9372, and *Bacillus subtilis* ATCC 6051 (all obtained from the American Type Culture Collection, Rockville, Md.), as well as *Salmonella typhi*, *Escherichia coli*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Arthrobacter globiformis*, and *Marinococcus halopoilus* (all kindly provided by Richard Haberberger, Bethesda Naval Hospital, Bethesda, Md.).

Spore preparation and processing. Spores of *B. anthracis* Vollum were obtained by seeding vegetative cells onto new sporulation medium (18) and incubating the preparation at 37°C for 72 h; microscopic examination revealed that approximately 90% sporulation had occurred by this time. The spores were washed off the agar surface with distilled water and heat shocked at 60°C for 1 h to kill any vegetative cells present. The spore preparation was then washed three times with distilled water and stored at 4°C. The numbers of *B. anthracis* spores were determined by direct colony counting, using nutrient agar.

Various methods for preparing the spores prior to PCR amplification were analyzed. Aliquots of spores were germinated in phosphate-buffered saline (PBS) supplemented with 300 mM L-alanine and 6 mM *ortho*-carbamyl-D-serine for 4.5 h and then boiled for 30 min. Alternatively, spores were physically disrupted with a mini-bead beater (Biospec Corp., Bar-

tersville, Okla.) for 10 min at the maximum speed. Each tube contained 1 ml of glass beads (diameter, 0.1 mm) and 1 ml of spore suspension. After physical disruption, the supernatants were removed and used directly as templates for PCR amplification.

PCR amplification. Two oligonucleotide primers, BACA1FI (ACAACTGGTACATCTGCGCG; corresponding to nucleotides 470 to 489) and BACA6RI (GATGAGGGATCAT TCGCTGC; corresponding to nucleotides 1073 to 1092), were constructed on the basis of the sequence of the capsular antigen gene (*capB*) of *B. anthracis* (11). BACA1FI and BACA6RI (Synthecell Corp., Gaithersburg, Md.) were designed to amplify a 622-bp fragment. PCR amplification of genomic DNA was initially performed by using a GeneAmp reagent kit, AmpliTaq polymerase (Perkin Elmer Cetus Corp., Norwalk, Conn.), and a model 480 DNA thermal cycler (Perkin Elmer Cetus Corp.). Template DNA was denatured at 94°C for 4 min, and this was followed by 1 min of annealing at 37°C and extension for 1 min at 72°C. This first step was then followed by 25 cycles consisting of denaturation at 94°C for 1.5 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. At the end of 25 cycles, the reaction mixture was kept at 72°C for 7 min to complete synthesis of all of the extended strands.

Subsequently, PCR amplification of genomic DNA and spore preparations was accomplished by using 50-μl reaction mixtures. In the thermal cycle an initial 2-min denaturation step at 95°C was followed by 35 cycles consisting of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. The final step consisted of extension at 72°C for 10 min. Amplification experiments were performed by using *Taq* polymerase, deoxynucleoside triphosphates (Boehringer Mannheim, Indianapolis, Ind.), and a model 9600 DNA thermal cycler (Perkin Elmer Cetus Corp.).

Detection of PCR-amplified products. The amplified products were detected by ethidium bromide staining of DNA separated by polyacrylamide gel electrophoresis (PAGE), as well as a novel dual-probe hybridization format in which we used a biosensor capable of detecting minute changes in pH (Molecular Devices, Menlo Park, Calif.) (13). Electrophoresis was performed by using 15 to 20 μl of PCR product on a 4 to 12% polyacrylamide gel (NOVEX, San Diego, Calif.) and a constant voltage of 100 V for 1 to 2 h. The gels were stained with ethidium bromide (1 μg/μl) for 5 to 10 min and were photographed by using a UV transilluminator and type 665 film (Polaroid Corp., Cambridge, Mass.).

In the dual-probe hybridization format we relied on simultaneous hybridization of both a biotin-labeled probe and a fluorescein-labeled probe to target regions within the PCR-amplified product (13). The 5′ biotin-labeled probe, PACABL1 (CTGACGAGGAGCAACCGATTAAGCGCCG TA; corresponding to nucleotides 508 to 537 [11]), and the 5′ fluorescein-labeled probe, PACAFL1 (CTTGCTTTAGCGG TAGCAGAGGCTCTTGGG; corresponding to nucleotides 930 to 959 [11]) (Applied Biosystems, Foster City, Calif.), were designed on the basis of the internal sequence of the 622-bp amplified fragment. A total of 20 μl of PCR product was added along with 25 μl of hybridization buffer (30 mM sodium phosphate, 450 mM NaCl, 3 mM EDTA, 0.25% Triton X-100; pH 7.4) containing 120 pg of each probe. This PCR product-probe solution was incubated at 100°C for 5 min during a denaturation step and then incubated at 55°C for 15 min during the hybridization step. A 100-μl portion of a streptavidin solution (20 μg/ml) was immediately added to the hybridization mixture, and the resulting preparation was then filtered through a biotinylated membrane (Molecular Devices). Polyclonal anti-fluorescein antibody conjugated to urease was then incubated for 30 min with the streptavidin-captured probe-PCR product complex. Excess anti-fluorescein antibody was then washed off, and the membrane-bound urease-containing complex was exposed to urea in a pH-sensitive silicon sensor (6, 8). The change in pH resulting from the hydrolysis of urea was recorded in microvolts per second. All samples were analyzed in triplicate, and statistical significance was determined by using the Student *t* test.

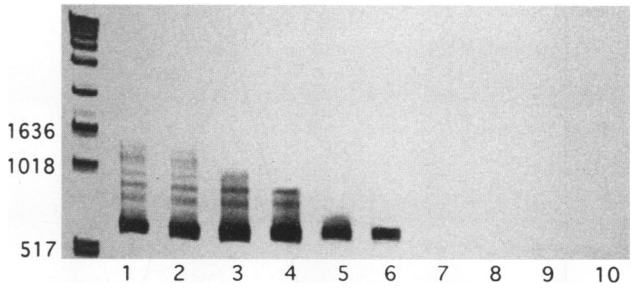

FIG. 1. Sensitivity of the PCR assay as determined by PAGE and ethidium bromide staining. Lanes 1 through 9 contained the PCR products obtained when the starting template copy numbers were 2.7×10^7, 2.7×10^6, 2.7×10^5, 2.7×10^4, 2.7×10^3, 2.7×10^2, 2.7×10^1, 2.7×10^0, and 2.7×10^{-1}, respectively. Lane 10 shows the results of the PCR assay when no DNA template was present.

RESULTS

Specificity and sensitivity of the PCR assay. We attempted to amplify the targeted 622-bp fragment from 1-μg samples of DNAs isolated from *B. anthracis* Ames, Vollum, New Hampshire, and Sterne, as well as from various control bacteria (see above), by using 100-μl reaction mixtures. The expected 622-bp fragment was successfully amplified from all of the anthrax strains except strain Sterne, which lacks plasmid pXO2 containing the *capB* gene (data not shown). We were not able to amplify the targeted 622-bp fragment when we used DNA templates obtained from *C. botulinum* types A and E, *V. cholerae*, *B. megaterium*, *B. licheniformis*, *B. thuringiensis*, *B. cereus*, *B. globigii*, *B. subtilis*, *Salmonella typhi*, *E. coli*, *Shigella sonnei*, *P. aeruginosa*, *K. pneumoniae*, *A. globiformis*, and *M. halopoilus* (data not shown). Although a PCR product of the correct size was amplified from *Y. pestis*, probes PACABL1 and PACAFL1 hybridized to the PCR products amplified from the anthrax strains but not to the product amplified from *Y. pestis* (data not shown).

Serial 10-fold dilutions of *B. anthracis* Ames DNA were prepared, and aliquots containing known quantities of DNA corresponding to 1.4×10^{-2} to 1.4×10^7 genomic copies were then used in the PCR assay for amplification of the 622-bp fragment. The numbers of genomic copies used were estimates based on the following assumptions: a single copy of plasmid pXO2 was present in each vegetative cell (16), the average size of plasmid pXO2 was 90 kb, and the average size of the bacterial genome was 30×10^6 bp (2). When we used ethidium bromide staining of PCR products after PAGE, we consistently detected at least 1.4×10^2 copies (Fig. 1). When we used the biosensor assay to detect PCR products (Fig. 2), we consistently detected at least 2.7 copies ($P < 0.05$, Student's *t* test) when the averages for three replicates were compared with the averages for three negative controls containing no DNA. We noted that the signals obtained with PCR dilutions increased significantly ($P < 0.05$, Student's *t* test) when the initial number

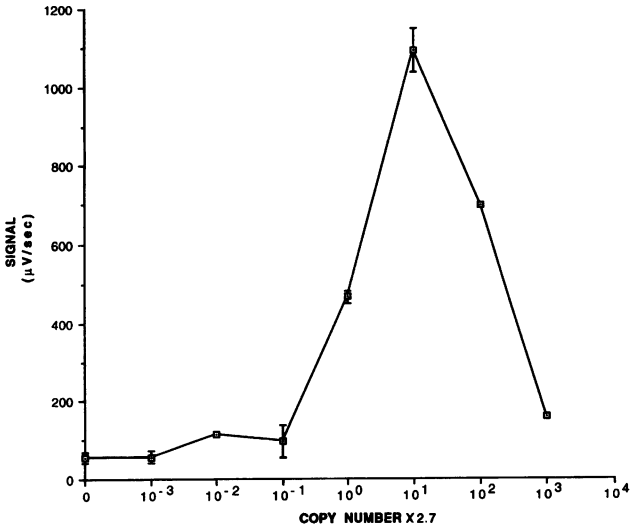

FIG. 2. Sensitivity of the modified PCR assay as determined by the dual-probe hybridization assay. The values are the means and standard errors of the values obtained for three replicates.

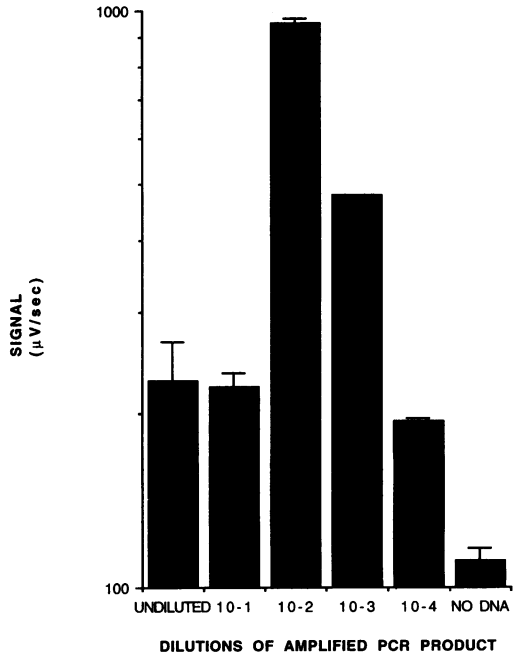

FIG. 3. Effect of diluting the amplified PCR product on the intensity of the signal obtained from the dual-probe hybridization assay. The values are the means and standard errors of the values obtained for three replicates. The starting template copy number was 2.7×10^7.

of template copies was increased from 2.7 to 27. The signals obtained with PCR dilutions containing 270 to 2,700 template copies were significantly weaker ($P < 0.05$, Student's t test), however, than the signals obtained with the PCR dilutions containing 27 copies. We hypothesized that the apparent decrease in signals at higher concentrations might have been due to competition between the second strand of the PCR product and the oligonucleotide probes. To prove this, in a separate experiment, we examined serial dilutions of the product of a PCR mixture that initially contained 1.4×10^7 template copies (Fig. 3). Our hypothesis was supported when we observed a significant increase in signals up to a dilution of 1/1,000 compared with the initial undiluted PCR product ($P < 0.05$, Student's t test).

Identification of spores of *B. anthracis*. We attempted to amplify DNA from *B. anthracis* spores by adding serial 10-fold dilutions of unprocessed spores directly to PCR mixtures (Table 1) and were able to detect as few as 10^2 unprocessed spores per PCR mixture. Supernatants were obtained from these 10-fold dilutions by passing spore preparations through 0.2-μm-pore-size filters, and all of the supernatants which corresponded to spore concentrations of 10^3 spores per reaction mixture or greater amplified the targeted fragment. Therefore, it appeared that the presence of spores in the PCR mixtures resulted in an increase in sensitivity of only 1 log above the sensitivity seen with the corresponding supernatants. Release of DNA from unprocessed spores was, therefore, relatively inefficient, and the major sources of DNA in the spore preparations were clearly extracellular.

In order to facilitate the release of DNA from spores, we compared the effect of limited spore germination with the effect of mechanical spore disruption prior to PCR amplification. Germination of spores in PBS supplemented with 300 mM L-alanine and 6 mM *ortho*-carbamyl-D-serine for 4.5 h followed by boiling for 30 min allowed us to detect 1 spore per PCR mixture (Table 2). Identical results were obtained when the spores were mechanically disrupted with a mini-bead beater for 10 min (Table 2). The two procedures, therefore, appeared to be equally effective in causing the release of DNA from *B. anthracis* spores.

DISCUSSION

Identification of pathogenic microorganisms is sometimes problematic, especially when cultivation is difficult or hazardous. In addition, numerous reports (15) have shown that viable but nonculturable microorganisms often constitute a significant portion of the environmental microbial flora. Under these circumstances, detection methods which rely on cultivation of organisms can result in underestimation of the actual risk posed by infectious microorganisms. PCR amplification of infectious agents can overcome some of the limitations of the conventional cultural methods used for isolation and detection.

In this study we developed a PCR assay to detect *B. anthracis* DNA by amplifying a 622-bp region of plasmid pXO2. Virulent *B. anthracis* has been shown to harbor both this plasmid and plasmid pXO1, which contains various toxin genes described

TABLE 1. PCR amplification of untreated *B. anthracis* spores and corresponding supernatants

No. of spores	Biosensor signal (μV/s)	
	Spores	Supernatants
10^3	287.7 ± 13.1[a]	283.4 ± 41.9[a]
10^2	249.3 ± 31.0[a]	156.7 ± 40.6
10	134.2 ± 31.6	111.8 ± 15.1
1	82.6 ± 4.8	98.6 ± 21.5
10^{-1}	78.7 ± 2.3	107.0 ± 14.7
10^{-2}	80.0 ± 3.1	89.6 ± 3.2
Negative control[b]	112.2	112.2
No DNA	90.0 ± 3.3	90.0 ± 3.3

[a] This value was significantly greater than the corresponding value for the control containing no DNA ($P < 0.05$, Student's t test).

[b] PCR mixtures containing 0.5 ng of *B. subtilis* genomic DNA as the template.

TABLE 2. Identification of germinated spores of *B. anthracis* by using the PCR

No. of spores	Biosensor signal (μV/s)	
	Germinated spores	Mechnically disrupted spores
10^3	345.3 ± 12.3[a]	ND[b]
10^2	229.5 ± 31.5[a]	ND
10	191.1 ± 12.3[a]	293.6 ± 32.2
1	170.0 ± 11.8[a]	356.2 ± 40.3
10^{-1}	83.5 ± 0.5	110.6 ± 23.4
10^{-2}	104.7 ± 13.0	106.6 ± 7.7
Negative control[c]	92.8	69.6
No DNA	85.6 ± 1.2	72.0 ± 2.5

[a] This value was significantly greater than the corresponding value for the control containing no DNA ($P < 0.05$, Student's t test).
[b] ND, not determined.
[c] PCR mixture containing 0.5 ng of *B. subtilis* genomic DNA as the template.

previously. Therefore, any attempt to identify virulent *B. anthracis* must target genes located on both of these plasmids. When we used primers specific to the *capB* region, as few as 140 copies of the target region could be amplified by using the appropriate amplification conditions followed by PAGE and ethidium bromide staining. When we used the dual-probe hybridization format, it became evident that the primers were capable of amplifying the targeted 622-bp fragment from a single genomic copy of *B. anthracis*. This level of sensitivity corresponded to our previous levels of detection sensitivity, and no nested PCR was necessary (4). The levels of sensitivity described above are estimates since we assumed that each spore or vegetative cell contains a single copy of plasmid pXO2 (16), that the average size of the plasmid is 90 kb (11), and that the average size of the bacterial genome is 30×10^6 bp (2). At the present time, we cannot explain why we were able to consistently amplify a DNA fragment of the proper size when *Y. pestis* DNA was used as the template in the PCR. We hypothesize that *Y. pestis* contains a gene which is partially homologous to the *capB* gene of *B. anthracis* since we could not demonstrate hybridization of the two *capB*-specific probes to the PCR product amplified from *Y. pestis* DNA.

The dual-probe hybridization format used in this study increased the sensitivity of the PCR assay above the sensitivity which was obtained when PAGE and ethidium bromide staining were used and also ensured specificity since no electrical output occurred unless two probes hybridized to the target. Furthermore, the dual-probe hybridization format requires less time (45 min) than conventional hybridization protocols. We clearly demonstrated that at high PCR product concentrations, probes appear to be inhibited from hybridizing to the target. This inhibition may be due to competition from the second strand which is finally overcome when the PCR product is diluted prior to probe hybridization.

We believe that the combination of the PCR assay described in this paper and the assay described previously (4) should permit rapid identification of all virulent strains of *B. anthracis*.

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