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# Direct Detection of *Bacillus anthracis* DNA in Animals by Polymerase Chain Reaction

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***Bacillus anthracis* is a soil pathogen capable of causing anthrax. To establish a method for specifically detecting *B. anthracis* for practical applications, such as for the inspection of slaughterhouses, the *cap* region, which is essential for encapsulation in *B. anthracis*, was used in a DNA hybridization study by polymerase chain reaction (PCR). Oligonucleotide primers were designed to amplify a 288-bp DNA fragment within the *capA* gene by PCR. The amplified DNA sequence specifically hybridized to the DNA of *B. anthracis* but not to that of other bacterial strains tested. Since this PCR-based method efficiently and specifically detected the *capA* sequence of bacteria in blood and spleen samples of mice within 8 h after the administration of live *B. anthracis*, this PCR system could be used for practical applications. By using lysis methods in preparing the samples for PCR, it was possible to amplify the 288-bp DNA segment from samples containing very few bacteria, as few as only 1 sporeforming unit, indicating that the PCR detection method developed in this study will permit the monitoring of *B. anthracis* contamination in the environment.**

*Bacillus anthracis*, the causative agent of anthrax, was originally isolated from soil. Anthrax is an important zoonosis of domestic and wild animals but occurs as a skin infection in humans exposed to diseased animals. It is well established that *B. anthracis* has two major virulence factors, a capsule and a tripartite protein exotoxin (1, 12, 14–16). Although outbreaks of anthrax have become very rare in most countries, anthrax is still one of the most hazardous diseases in many areas where the soil is contaminated with *B. anthracis* spores. From the viewpoint of public health, humans and animals exposed to the risk of anthrax require a monitoring system to detect bacterial contamination in livestock or in the environment, but a reliable monitoring system has still not been developed.

Several conventional methods, including Gram staining, capsule staining (10), immunological tests (10), infection experiments (10) with guinea pigs or mice, and gamma phage sensitivity tests (phage typing [3]), have been established and used to check slaughterhouse livestock for *B. anthracis*. Among these, the most reliable for the diagnosis of anthrax is immunological tests performed in conjunction with Gram and capsule staining. However, it has been shown that there are some problems in specificity with these methods; bacteria other than *B. anthracis*, such as *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus subtilis* also give positive reactions in these tests (4, 8). While the gamma phage sensitivity test is specific for *B. anthracis*, it takes a few days to complete and requires a large number of bacteria. Hence, it is urgent and necessary from the viewpoint of public health to establish a highly sensitive, reliable, rapid, simple, and widely applicable method for detecting *B. anthracis* in livestock.

The polymerase chain reaction (PCR) technique is a method for amplifying nucleic acids by repeating cycles of temperature changes to promote template denaturation, oligonucleotide primer hybridization, and polymerase-mediated polymerization (11). If oligonucleotide primers specific for *B. anthracis* are available for PCR, a method to specifically distinguish this bacterium from many other foreign microbes or viruses and detect it directly in human, animal, and soil samples may be established (13). To obtain such specific oligonucleotide sequences, we tried to choose a DNA segment coding for a characteristic phenotype unique to *B. anthracis*, such as a virulence factor necessary for pathogenesis. Moreover, because more than $10^5$-fold amplification over the input quantity of nucleic acid is usually feasible, PCR is well suited for identifying a small number of DNA sequences, such as those found in infectious materials in the early phase of infection (5).

We previously cloned the *cap* region that is essential for encapsulation from the 96.5-kb virulence plasmid of *B. anthracis* and characterized its genetic organization. *B. licheniformis*, *B. megaterium*, and *B. subtilis* all express capsule-like substances that are immunologically cross-reactive with that of *B. anthracis* but show no homology with the *B. anthracis cap* region at the nucleotide sequence level, suggesting that the *cap* region is highly specific for *B. anthracis* (7, 8). We have therefore chosen the *cap* region to evaluate for potentially useful specific oligonucleotide sequences for PCR.

## MATERIALS AND METHODS

**Animals, bacterial strains, and plasmid used in this study.** Specific-pathogen-free mice (BALB/c), 4 to 8 weeks old, were supplied by the Animal Medicine Section in our institute. All bacterial strains used in this study are listed in Table 1. Plasmid pCAP1, which contains the complete *cap* region essential for encapsulation (7, 8), was used in isolating a DNA probe.

**DNA preparation.** Plasmid DNAs were isolated by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (9). To isolate total bacterial DNA, bacterial cells

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TABLE 1. Bacterial strains used in this study

| Organism type | Group[a] | Strain | Source or reference |
|---|---|---|---|
| Gram positive | | *Bacillus anthracis* Davis | 7 |
| | | *Bacillus anthracis* Pasteur II[b] | 18 |
| | A | *Bacillus cereus* IID872 | This laboratory |
| | | *Bacillus licheniformis* NIAH227 | 8 |
| | | *Bacillus megaterium* NIAH368 | 8 |
| | | *Bacillus subtilis* NIAH801 | 8 |
| | | *Bacillus thuringiensis* | This laboratory |
| | | *Clostridium botulinum* 003-9 | S. Kozaki[c] |
| | | *Clostridium difficile* 7626 | Gifu[d] |
| | | *Clostridium perfringens* 5256 | Gifu |
| | B | *Corynebacterium diphtheriae* 3182 | Gifu |
| | | *Enterococcus faecalis* 8357 | Gifu |
| | | *Erysiperothrix rhusiopathiae* 10089 | Gifu |
| | | *Lactococcus lactis* 8591 | Gifu |
| | | *Listeria monocytogenes* EGD | This laboratory |
| | | *Mycobacterium tuberculosis* 27874 | Gifu |
| | | *Staphylococcus aureus* | This laboratory |
| | | *Streptococcus pneumoniae* | This laboratory |
| Gram negative | C | *Actinobacillus pleuropneumoniae* Ng1 | This laboratory |
| | | *Aeromonas* sp. ATCC 9071 | This laboratory |
| | | *Campylobacter fetus* 8746 | Gifu |
| | | *Campylobacter jejuni* 91-569 | S. Kaneko[e] |
| | | Enterohemorrhagic *Escherichia coli* | This laboratory |
| | | Enteropathogenic *Escherichia coli* | This laboratory |
| | | Enterotoxigenic *Escherichia coli* | This laboratory |
| | | *Klebsiella pneumoniae* IID5209 | This laboratory |
| | D | *Legionella pneumophila* 10260 | Gifu |
| | | *Pasteurella multocida* 87-37 | S. Kaneko |
| | | *Proteus vulgaris* IID874 | This laboratory |
| | | *Pseudomonas acidovorans* 11501 | Gifu |
| | | *Pseudomonas aeruginosa* P13 | This laboratory |
| | | *Salmonella choleraesuis* SB242 | This laboratory |
| | | *Salmonella enteritidis* | This laboratory |
| | E | *Salmonella typhimurium* LT2 | This laboratory |
| | | *Serratia marcescens* IID5218 | This laboratory |
| | | *Shigella flexneri* YSH6000 | 6 |
| | | *Vibrio cholerae* IID936 | This laboratory |
| | | *Vibrio parahaemolyticus* 91-572 | S. Kaneko |
| | | *Yersinia enterocolitica* | This laboratory |
| | | *Yersinia pseudotuberculosis* | This laboratory |

[a] See Fig. 3.
[b] The strain designated Pasteur II by Japanese researchers is attenuated for virulence but still contains both the toxin and capsule plasmids and therefore differs from ATCC 4229, also designated a Pasteur vaccine strain, because the latter contains only pXO2.
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were suspended in TES buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl [pH 8.0]) containing lysozyme (10 mg/ml), and sodium dodecyl sulfate (SDS) and proteinase K were added. After incubation at 55°C, the DNAs were treated with RNase, extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

**DNA amplification and gel electrophoresis.** A 288-bp *capA* DNA sequence from *B. anthracis* was amplified by the PCR method in a reaction mixture (100 μl) containing 10 mM Tris-HCl (pH 8.3); 60 mM KCl; 1.5 mM $MgCl_2$; 1 mg of gelatin; 200 mM each dATP, dTTP, dCTP, and dGTP; 50 pmol of oligonucleotide primers; 2.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.); and 1 μl of various DNA solutions (containing about 1 μg of DNA for spleen samples and 0.1 to 0.01 μg of DNA for other samples). Denaturation was done at 95°C for 1.0 min, annealing at 65°C for 2 min, and extension at 72°C for 1.5 min; amplification was repeated for 30 cycles. The amplified products were subjected to electrophoresis in 6% polyacrylamide gels and stained with ethidium bromide.

**Preparation of *B. anthracis* spore suspension.** Since *B. anthracis* Davis is an asporogenous strain (18), avirulent *B. anthracis* Pasteur strain II was used in preparing spore suspensions. Spore suspensions were prepared as previously described by Uchida et al. (18). Spore suspensions were diluted in saline and then plated onto nutrient agar plates to calculate the number of sporeforming units (SFU) present.

Appropriate dilutions were mixed with blood and spleen samples for use in PCR reactions as specifically described for each experiment.

**Preparation of blood samples. (i) Boiling method.** One hundred microliters of heparinized blood was boiled for 15 min and then centrifuged at 10,000 × *g* for 10 min at 4°C.

**(ii) Lysis method.** One hundred microliters of heparinized blood was mixed with 100 μl of 2× TNE (200 mM Tris-HCl, 20 mM EDTA, 200 mM NaCl [pH 8.0]) containing proteinase K (200 μg/ml), and then 20 μl of 20% SDS was added. After 60 min of incubation at 55°C, DNA samples were extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in 100 μl of TE buffer.

**Preparation of spleen samples. (i) Boiling method.** Spleen tissue was mixed with an equal volume of sterile water and crushed well, boiled for 15 min, and centrifuged at 10,000 × *g* for 10 min. One microliter of the resulting supernatant was used directly for PCR.

**(ii) Lysis method.** One hundred milligrams of spleen tissue was mixed with 100 μl of sterile water and crushed until an emulsion was formed. To this spleen suspension, 200 μl of 2× TEN containing proteinase K (200 μg/ml) was added, and then 50 μl of 20% SDS and 50 μl of 20% Sarkosyl were added. This spleen suspension containing bacteria was incubated at 55°C until it cleared (less than 3 h). The lysates were then extracted three times with phenol-chloroform, precipitated with ethanol, and dissolved in 500 μl of TE buffer. One microliter of the resulting suspension was used for PCR.

**Other techniques.** Southern and dot hybridization were done by the methods described by Maniatis et al. (9). For DNA labeling, a random primer labeling kit was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Restriction enzymes were purchased from Toyobo Inc.

## RESULTS

**Specificity of the *cap* region for various bacterial DNAs.** We previously determined the complete nucleotide sequence of the *cap* region, its genetic organization, and its gene products (7, 8). The results showed that the *cap* region consisted of three genes, designated *capA*, *capB*, and *capC* (Fig. 1). Capsular polypeptides immunologically cross-reactive with that of *B. anthracis* were found in *B. licheniformis*, *B. megaterium*, and *B. subtilis*, but no nucleotide sequence homologous to the *cap* region was found in any of these bacteria (8). Thus, we investigated whether the *cap* region was suitable for a PCR-based *B. anthracis* identification method. First, total DNAs isolated from the bacterial strains listed in Table 1 were digested with *Eco*RI, subjected to agarose gel electrophoresis (Fig. 2A), and transferred to a nitrocellulose membrane filter. The uniqueness of the *capA* gene to *B. anthracis* was examined by Southern hybridization with a 210-bp *Aat*I-*Bam*HI fragment from *capA* as a DNA probe (Fig. 1). This *capA* probe strongly hybridized only to the *Eco*RI-digested total DNA from *B. anthracis*. A 1.4-kb band, corresponding to the 1.4-kb *Eco*RI fragment in the *capA* gene (Fig. 1), hybridized with the probe (Fig. 2B, leftmost lane). In contrast, none of the other bacterial DNAs digested with *Eco*RI hybridized with this probe (Fig. 2B, all other lanes). These results confirmed our previous data (8) and prompted us to make oligonucleotide primers for PCR from the *capA* gene.

**Specificity of oligonucleotide primers.** Because the *cap* region has a G+C content of approximately 30% overall (8), two short portions of *capA* containing 50% and 62% G+C

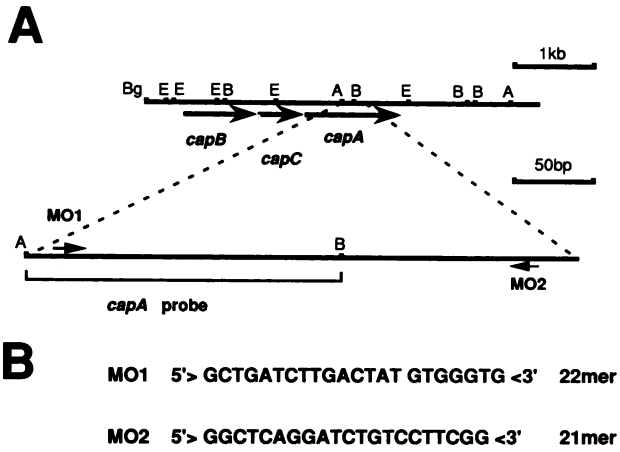


FIG. 1. Restriction map of the *cap* region (A) and sequences of the oligonucleotide primers used for PCR (B). The top line shows the restriction map of the *cap* region on a large plasmid, pTE702, which is essential for encapsulation in *B. anthracis* Davis (7). Oligonucleotides MO1 and MO2 were synthesized according to the DNA sequence of the *cap* region (8). A, *Aat*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI.

were chosen and designated MO1 and MO2, respectively (Fig. 1B). With various concentrations of $MgCl_2$ in the reaction mixture and at various annealing temperatures, PCR with purified total DNA from *B. anthracis* was performed and optimum conditions for PCR were determined as described in Materials and Methods. Under those conditions, only a single band corresponding to a 288-bp DNA segment was amplified (Fig. 3, lane 3). To test whether this PCR system was specific for *B. anthracis*, PCR was performed for all of the strains shown in Table 1. No amplified DNA was seen in any of the mixtures without *B. anthracis* DNA (Fig. 3, lanes 5, 7, 9, 11, and 13), whereas a single 288-bp band that hybridized with the DNA derived from *B. anthracis* DNA samples (Fig. 3, lane 3) was amplified in the DNA mixtures containing *B. anthracis* DNA (Fig. 3, lanes 4, 6, 8, 10, and 12), demonstrating that PCR with the MO1 and MO2 primers gives rise to a DNA amplification specific to *B. anthracis*.

**Animal model experiments.** Since mice are used in virulence assays for *B. anthracis*, we used mice as an animal model to optimize the conditions for PCR. The limitations for detection of bacteria by PCR were examined by using blood and spleen samples mixed with various amounts of *B. anthracis* spores. To prepare samples for PCR, both the boiling and lysis methods were compared for simplicity and the total time required. PCR efficiently amplified a 288-bp DNA band from blood and spleen samples which contained approximately $10^6$ SFU prepared by the boiling method (Fig. 4, lanes 2 and 8), but not from boiled samples containing less than $10^3$ SFU (Fig. 4, lanes 4, 9, and 10). On the other hand, a 288-bp band was efficiently amplified in blood and spleen samples containing approximately $10^3$ SFU prepared by the lysis method (Fig. 4, lanes 6 and 12), and a faint band could still be seen even from a lysis method spleen sample containing approximately 1 SFU (Fig. 4, lane 7). Those results suggested that although it was possible to use DNA prepared by both the boiling and lysis methods for PCR, the lysis method was more efficient, as bacterial DNA could be detected from samples containing less than $10^3$ SFU,

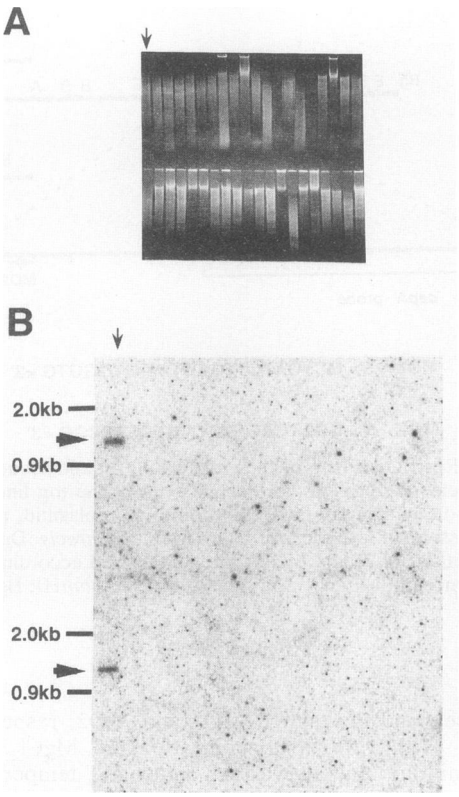


FIG. 2. Southern hybridization of total DNAs from various gram-positive and gram-negative bacteria with a *capA* probe. All bacterial strains used are listed in Table 1. The arrow in each panel shows the lane loaded with *B. anthracis* total DNA. DNA samples from the other bacterial strains were loaded in the other lanes, and the DNA samples were loaded in the order that they are listed in Table 1. All DNA samples were digested with *Eco*RI. (A) Ethidium bromide-stained 0.7% agarose gel. (B) Southern hybridization autoradiogram. A 1.4-kb *Eco*RI fragment that hybridized with the *capA* probe is indicated by the arrowheads, and size markers are shown on the left.

whereas DNA prepared by the boiling method required at least $10^3$ SFU for detection.

Next, we intraperitoneally challenged mice with aliquots of bacterial culture. Their spleens and blood samples were taken at 24 or 48 h after the challenge. The number of bacteria in both samples was calculated, and they were also subjected to PCR as described in Materials and Methods. The blood and spleen samples contained $7 \times 10^6$ CFU/ml and $1.8 \times 10^8$ CFU/g, respectively. Under the PCR conditions used, a 288-bp *capA* segment was amplified from all samples regardless of the extraction procedure used (Fig. 5), but samples prepared by the lysis method were more efficiently amplified than those prepared by the boiling method (compare lanes 4 and 6 in Fig. 5).

## DISCUSSION

In this study, we established a PCR system that detects *B. anthracis* animal samples without bacterial growth by using oligonucleotide primers within the *cap* region, which is essential for encapsulation in *B. anthracis*. This PCR system was found to be highly specific for *B. anthracis*, because the oligonucleotide primers used hybridized only to *B. anthracis*

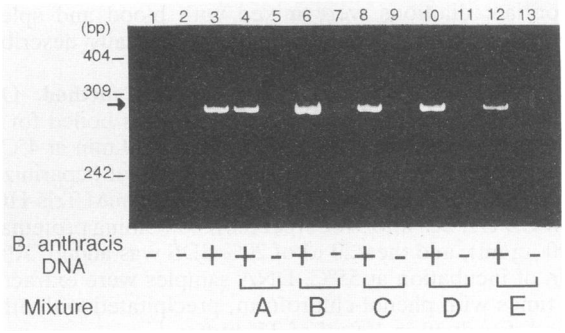


FIG. 3. PCR amplification of the *capA* DNA sequence with total DNAs from various bacterial strains. Mixtures A, B, C, D, and E indicate pooled DNA samples containing total DNAs isolated from all strains in groups A, B, C, D, and E shown in Table 1, respectively, with (+) and without (−) *B. anthracis* DNA. For each PCR, 0.25 μg of DNA from each sample was used. As a positive control, a reaction mixture containing 0.25 μg of *B. anthracis* DNA was used (lane 3). As negative controls, reaction mixtures without DNA samples (lane 1) or with *B. anthracis* DNA but without primers (lane 2) were prepared. The 288-bp *capA* band is indicated by the arrow, and size markers are shown on the left.

total DNA, giving rise to an amplified 288-bp DNA sequence (Fig. 3). Moreover, with this system it has become possible to make a diagnosis in only 1 day with very small numbers of bacteria. The system is also more reliable for the detection of *B. anthracis* than conventional methods (see the introduction).

The two methods used for sample preparation, boiling and lysis, were compared by PCR for the detection of various numbers of bacteria. The two methods needed a maximum of 4 and 8 h, respectively, to detect the specific amplified DNA band from livestock samples by PCR. The boiling method required less time and was simpler to use to prepare samples than the lysis method. The lysis method was, however, obviously a more sensitive bacterial detection method (for example, compare lanes 9 and 12 in Fig. 4). In cases of septicemia caused by *B. anthracis*, blood samples have frequently been shown to contain approximately $10^8$

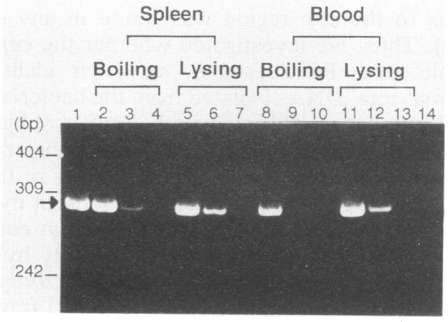


FIG. 4. PCR amplification of the *capA* DNA sequence in mouse samples. Lane 1, reaction mixture containing only purified *B. anthracis* DNA; lanes 2, 5, 8, and 11, reaction mixtures with samples containing $10^6$ SFU; lanes 3, 6, 9, and 12, reaction mixtures with samples containing approximately $10^3$ SFU; lanes 4, 7, 10, and 13, reaction mixtures with samples containing approximately 1 SFU; lane 14, reaction mixture without any DNA. The arrow indicates the 288-bp *capA* band, and size markers are shown on the left.

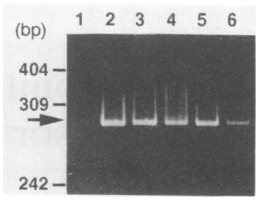


FIG. 5. PCR amplification of the *capA* DNA sequence in samples from mice infected with *B. anthracis*. Lane 1, reaction mixture without bacterial DNA; lane 2, reaction mixture containing 0.25 μg of purified DNA from *B. anthracis*; lanes 3 and 4, spleen DNA samples prepared for PCR by the lysis and boiling methods, respectively; lanes 5 and 6, blood DNA samples prepared for PCR by the lysis and boiling methods, respectively. The arrow indicates the 288-bp *capA* band, and size markers are shown on the left.

bacteria per ml, so the boiling method is still useful for practical inspections by PCR. However, since the blood and organs of cattle and pigs contain a small number of bacteria at the onset of anthrax, the lysis method should be more reliable for detection than the boiling method.

Since *B. anthracis* represents a soil bacterium that is able to survive in the soil in its spore form for a long time (2), surveillance of contamination in certain soils, such as in stock farms, is important. In this context, the PCR system with *capA* primers established in this study would facilitate the monitoring of bacterial contamination by detecting the 288-bp band from soil samples. Indeed, the sensitivity of the PCR system was shown to be high enough to detect very small numbers of *B. anthracis* (approximately 1 SFU per sample), so it may be applicable to soil samples.

Recently, Turnbull et al. (17) indicated that anthrax-like organisms lacking a capsule have been detected in samples taken from particularly harsh environments, such as sewage, and some of them have been identified as *B. anthracis* by using various oligonucleotides that hybridize to toxin genes. The study suggested that those $Cap^-$ bacteria were derived from fully virulent *B. anthracis* established elsewhere. Since both the capsule and the exotoxin are essential virulence factors for *B. anthracis*, the oligonucleotide primers designed in this study together with other primers specific for the toxin gene (17) will be useful for a more in-depth survey.

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