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PCR-Based Detection of *Bacillus anthracis* in Formalin-Fixed Tissue from a Patient Receiving Ciprofloxacin

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We demonstrate that *Bacillus anthracis* may be detected from a formalin-fixed, paraffin-embedded biopsy specimen, even after the patient has received antibiotic treatment. Although traditional PCR methods may not be sufficiently sensitive for anthrax detection in such patients, cycle numbers can be increased or PCR can be repeated by using an aliquot from a previous PCR as the template.

Bacillus anthracis, the causative agent of anthrax, has been postulated to be a likely agent of biological warfare or terrorism because of its physical properties and its virulence factors (8). Nonetheless, anthrax had not been encountered in the United States as a weapon of terror until the fall of 2001 (2). Current reports have documented 22 cases of anthrax that have met the Centers for Disease Control and Prevention (CDC) case definition; 12 cases (7 confirmed and 5 suspected) were of the cutaneous form (3, 10). Due to variation in symptoms and findings in cases of cutaneous anthrax, a definitive diagnosis of infection requires identification of the organism within the tissue. However, many pathological specimens are routinely fixed in formalin and embedded in paraffin prior to histological examination. Such samples are easy to store, transport, and section for histological staining. Tissue architecture and proteins are well maintained with this method. Nonetheless, extraction of nucleic acids is difficult, often yielding only degraded DNA (4). Obtaining usable bacterial DNA is made more difficult if a patient has already been started on antibiotic therapy.

We report our ability to amplify *B. anthracis* DNA from a formalin-fixed, paraffin-embedded skin biopsy specimen from a 34-year-old patient who had received ciprofloxacin for 3 days before the biopsy was performed. The major clinical features of this case have been previously reported (6; H. Yee, T. C. Gallagher, B. Strober, M. Pomerantz, M. Sanchez, S. Levine, M. J. Blaser, R. Hoffman, and B. A. Hanna, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. UL-7, 2001). In brief, the patient, an employee in the mailroom of a New York City newspaper, presented with a rapidly growing nodule on his left forearm. Within 24 h, the nodule developed a central black eschar (6). Notably, histological sections from the biopsy specimen obtained after 3 days of antibiotic treatment showed an edematous lesion (with pronounced superficial papillary dermal edema with near-vesicle formation) with diffuse interstitial dermal collagen fibrin deposition, diffuse scattered acute inflammation, vascular congestion, and vasculitis. A tissue Gram stain revealed scattered gram-positive, rod-shaped organisms, some of which were fragmented forms. Serological results for anthrax were negative, as were Gram-stained smears and cultures of the unfixed tissue. The CDC reported positive identification of anthrax in the tissue biopsy specimen by immunohistochemical staining.

Formalin-fixed, paraffin-embedded tissue samples obtained by punch biopsies of skin from three different patients were studied. One sample (sample B) was from the patient described above, who presented clinically with a rapidly growing nodule over the flexor surface of his left forearm (6). The other two samples (samples A and C) were from patients who were suspected of having anthrax. These patients were subsequently found not to be infected, and the samples were thus used as negative controls, while DNA from *B. anthracis* vaccine strains was used for positive controls. The laboratory was blind as to the source of each of the specimens. Only eight 5-μm-thick sections of each specimen were available for PCR. DNA was extracted from each of the samples by using the DNeasy tissue kit (Qiagen, Valencia, Calif.) protocol with the following exceptions: after rehydration, tissue was resuspended in 125 μl of enzymatic lysis buffer (20 mM Tris-Cl [pH 8.0], 2 mM EDTA, 1.2% Triton X-100, 20 mg of lysozyme/ml) and incubated at 37°C for 30 min prior to digestion by proteinase K; 70 μl of buffer ATL (2.5% edetic acid–2.5% sodium dodecyl sulfate) was included with the proteinase K digestion, which was allowed to proceed overnight. To increase the DNA concentration, elution was performed with 35 μl rather than 200 μl of buffer. PCRs were performed by standard methods in a reaction volume of 50 μl containing 0.5 U of *Taq* (Qiagen), 1.5 mM $MgCl_2$, and 200 ng of each primer. The 152-bp signal sequence of the *B. anthracis* chromosomal gene *rpoB* (Ba813) was amplified by PCR with primers R1 (5′TTAATTCACTTGCAAC TGATGGG3′) and R2 (5′AACGATAGCTCCTACATTTGG AG3′) as described previously (13, 14). A 264-bp signal sequence of *capC*, located on pX02, was amplified by using primers 57 cap (5′ACTCGTTTTTAATCAGCCCG3′) and 58cap (5′TAACCCTTGTCTTTGAAT3′) as described previously (15).

Neither sample A nor sample C tested positive by any PCR. After 35 cycles of PCR, sample B tested negative for the

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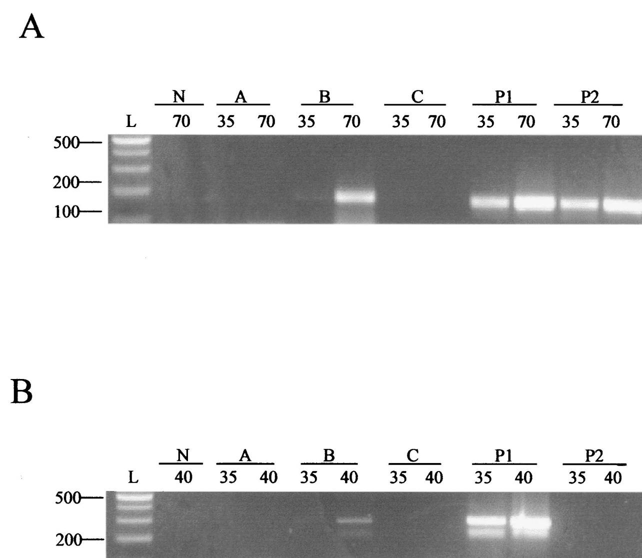

FIG. 1. Agarose gel electrophoresis of *B. anthracis* genes amplified by PCR. (A) Amplification with primers R1 and R2 of the 152-bp *rpoB* segment by PCR performed for 35 or 70 cycles (35 cycles plus 35 cycles). (B) Amplification with primers 57cap and 58cap of the 264-bp product from *capC* on pX02. Lanes: L, 100-bp ladder; N, negative control (no DNA template); A, B, C, DNA extracted from skin biopsy specimens from patients A, B, and C, respectively; P1, positive control (*B. anthracis* strain CAF10); P2, *B. anthracis* Sterne strain (lacking pX02) (5).

presence of *B. anthracis rpoB*. However, after an additional 35 PCR cycles, with the product of the first PCR used as the template, a clear band was seen (Fig. 1A). A positive PCR result with the *B. anthracis rpoB* primers is not equivalent to *B. anthracis* identification (12, 14, 16). However, *rpoB* still retains value as a chromosomal marker since *B. anthracis* can lose both its virulence plasmids (1, 11), although it is more likely to spontaneously lose pX02 (12). It was necessary to confirm identification of anthrax with a *B. anthracis*-specific marker. Sample B also tested positive for *capC*, located on pX02 (7, 17), one of three genes responsible for capsule expression. Although the appearance of the band obtained after 35 PCR cycles was faint, it was significantly increased in a 40-cycle reaction (Fig. 1B). Specific PCRs did not amplify the genes of the edema factor or the lethal factor when the appropriate primers were used (data not shown). One reason for the failure to amplify *pagA* from pX01 is that the expected product was 747 bp compared to 264 bp for *capC*, which is located on pX02, and to 152 bp for the segment of *rpoB* that is located on the chromosome; it is difficult to amplify larger products from DNA that is extracted from fixed tissue (19). Nonetheless, we believe that amplification of *capC* located on pX02 is sufficient because it is difficult for the organism to retain pX02 and lose pX01 (1, 11, 12). It should be noted that confirmation of all PCR products is usually important, and ordinarily Southern blotting or another probe format would accomplish that. However, our study was a retrospective confirmatory diagnosis for a skin biopsy specimen from a patient whose diagnosis of anthrax was already known, since the CDC had already confirmed the presence of *B. anthracis* by immunohistochemical staining. Most current protocols to detect the presence of *B. anthracis* take advantage of real-time PCR because of its closed system, which lessens the chance for contamination, and because of its confirmatory metrics (18). However, many laboratories do not yet possess this technology. Nonetheless, based on the results with our negative controls, we are confident that our visualized bands do not represent contamination.

These results contrast with those from a previous study (9) in which investigators successfully amplified genes of the protective antigen, lethal factor, and edema factor of *B. anthracis*. However, the patients from whom tissue was examined in that report all died of systemic anthrax (9) and it is therefore likely that a significantly higher quantity of *B. anthracis* DNA was present.

In this report, we demonstrate that *B. anthracis* may be detected and a diagnosis of anthrax may be confirmed by using only a very small amount of formalin-fixed, paraffin-embedded tissue, even after the patient has received antibiotic treatment. Although traditional PCR methods may not be sufficiently sensitive for anthrax detection in such patients, cycle numbers can be increased or PCR can be repeated with an aliquot from a previous PCR used as the template. With such techniques, formalin-fixed, paraffin-embedded clinical specimens can be examined to confirm the presence of anthrax, even long periods after the event and despite receipt of antibiotic treatments that lower or eliminate bacterial counts.

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