Evaluation and Validation of a Real-Time Polymerase Chain Reaction Assay for Rapid Identification of *Bacillus anthracis*

To the Editor: During the 2001 anthrax outbreak, we evaluated and validated a highly sensitive and specific three-target (two plasmid and one chromosomally located target) 5′ nuclease assay (real-time polymerase chain reaction [PCR]) for detection and identification of *Bacillus anthracis*. This PCR assay was successfully used to rapidly test hundreds of suspect isolates as well as screen environmental samples for the presence of *B. anthracis* throughout the 2001 anthrax outbreak. For the first time in an outbreak setting, a PCR assay was used to detect *B. anthracis* directly from clinical specimens, consequently becoming a part of the laboratory confirmation of anthrax. In this letter, we describe the evaluation of this assay on a diverse panel of bacterial isolates including isolates obtained throughout the outbreak. A supplement, which includes data on the use of this assay on environmental and clinical specimens, is online (available from: URL: http://www.cdc.gov/ncid/EID/vol8no10/02-0393_sup.htm).

Identification of *B. anthracis* has traditionally been determined by using phenotypic differences between *B. anthracis* and the rest of the *B. cereus* group (i.e., lack of motility and hemolysis, susceptibility to penicillin, typical colony morphology, and susceptibility to lysis by gamma phage); however, these methods are slow and require at least 24 h for completion. The recent bioterrorism-associated outbreak and the ongoing threat emphasize the importance of rapid microbiologic diagnosis for the timely and adequate implementation of control and preventative measures.

For *B. anthracis*, the main targets for development of such assays, primarily PCR-based, have been and continue to be genes encoding its virulence factors: a tripartite exotoxin and an antiphagocytic capsule (1–4). The toxin genes (*pag*A, *lef*, and *cya*) are encoded on the 182-kb virulence plasmid, pXO1, while the genes required for capsule biosynthesis (*capB*, *capC*, and *capA*) are encoded on the 96-kb virulence plasmid, pXO2 (5–7). These plasmid-located virulence genes seem to be restricted to *B. anthracis*, giving the plasmid-based assays a high degree of specificity (8). However, strains of *B. anthracis* that lack these plasmids have been reported (4,9). Consequently, having an assay focus on a specific chromosomal target for detection of avirulent and plasmid-cured *B. anthracis*, as well as those that potentially could have been genetically engineered, is essential. Chromosomal markers, such as *vrrA* and Ba813, have been used to characterize *B. anthracis* (9–12) and to detect it in tissues of victims of the anthrax outbreak that occurred in 1979 in Sverdlovsk, former Soviet Union (12), but these markers are not restricted to *B. anthracis*. Recently, Qi et al. developed a fluorescence resonance energy transfer PCR assay that targets the *B. anthracis* chromosomally located *rpoB* gene. This assay appears to be the most specific described to date with only 1 of 175 non-*B. anthracis* bacilli reported as positive (13).

Over the past several years, activities in the area of bioterrorism preparedness in the United States have resulted in the establishment of an international Laboratory Response Network (LRN), which was instrumental in the identification of the agent used in the 2001 outbreak (14). One of the major initiatives of LRN has been development and validation of rapid and specific assays for identification of *B. anthracis* and other agents likely to be used in a bioterrorism event.

Primer and probe set BA1 targets a region of pX02, BA2 targets pXO1, and BA3 targets a region of the *B. anthracis* chromosome. Probes were labeled with 6-carboxy-fluorescein phosphoramidite and 5-carboxy-tetramethyl-rhodamine.

LRN PCR assays using the BA1, BA2, and BA3 primer and probe sets were performed with the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany), Smart Cycler (Cepheid, Sunnyvale, CA), or ABI Prism 7700 (Applied Biosystems, Foster City, CA) instruments. The LightCycler Faststart DNA master hybridization probes kit (Roche Diagnostics GmbH) reagents were used on all real-time platforms. Reactions comprised 1X reaction mix, 5 mM $MgCl_2$, 500 nM each primer, and 100 nM probe in a reaction volume of 20 µL (LightCycler) or 25 µL (Smart Cycler, ABI Prism 7700). Thermal cycler conditions consisted of an initial 10-min hold at 95°C followed by 40–45 cycles of 10 s (LightCycler) or 15 s (Smart Cycler, ABI Prism 7700) at 95°C and 30 s (LightCycler, Smart Cycler) or 60 s (ABI Prism 7700) at 60°C. Real-time data were collected during the 60°C extension step of each cycle. Amplification of the human β-actin gene was used as a real-time PCR control when used in clinical samples to ensure negative results were not from inhibition of the PCR reaction. This real-time PCR assay was considered positive when all three targets were positive (Figure).

A total of 542 isolates were tested. Eighty-one *B. anthracis* isolates were tested to evaluate sensitivity of the real-time PCR approach (Table). Seventy-five were selected to provide a test population representing diverse sources, genotypes, geographic origins, and dates of isolation. The isolates included those collected from animals, humans, and other sources (i.e., industrial sites associated with anthrax outbreaks); the isolates span at least 58 years (1939–1997). Fifty-three of the isolates were previously characterized by multiple-locus variable-number tandem repeat analysis (MLVA) (15) and were included to ensure a representative range of the 89 described MLVA genotypes to date.

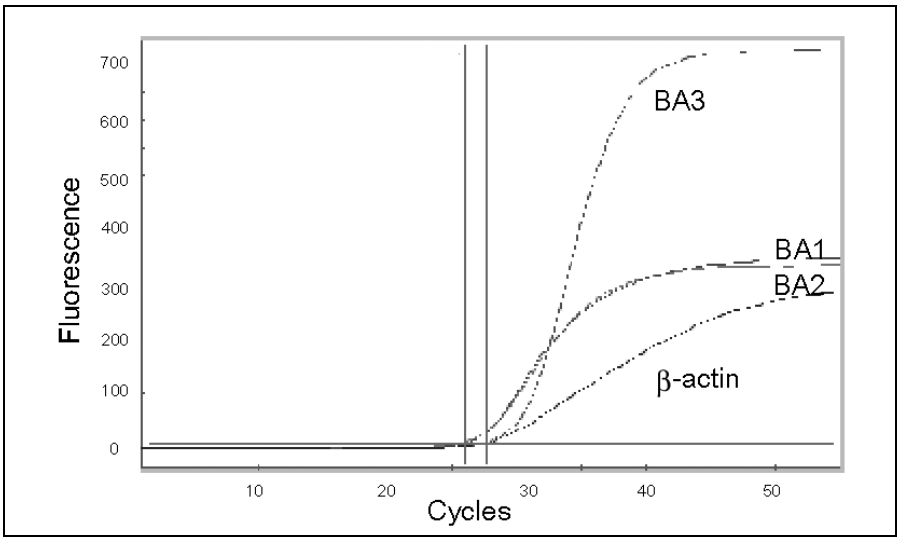

Figure. Real-time polymerase chain reaction graph of three *Bacillus anthracis* markers and β-actin control detected in a pleural fluid specimen from a patient with inhalational anthrax. The horizontal line indicates a threshold value; the vertical lines indicate cross-threshold values for each marker. BA1, primer and probe set targeting a region of pXO2; BA2, primer and probe set targeting a region of pXO1; BA3, primer/probe set targeting a region of *B. anthracis* chromosome.

Six *B. anthracis* type and standard strains included: five pXO1 cured strains (including the Pasteur strain) and one pXO2 cured strain (the veterinary vaccine strain Sterne). The *B. anthracis* New Hampshire strain (16) was used as a positive control for all real-time PCR assays. This isolate was originally cultured from a patient with inhalational anthrax in New Hampshire in 1957. This real-time PCR is designed to identify fully virulent (wild-type) *B. anthracis,* which will give positive results in all three markers. However, naturally occurring isolates have been found lacking either virulence plasmid, and a number of laboratory strains have been plasmid cured, as well. PCR results for these strains will reflect the lack of one or both of their plasmids.

A total of 317 *B. anthracis* isolates obtained during the bioterrorism-associated anthrax outbreak from October to December 2001 were also analyzed by PCR. These included 27 isolates from clinical specimens, 4 from powders and 286 isolates from environmental samples. MLVA was performed on 135 of these isolates; all were indistinguishable (17).

For evaluation of the assays' specificity we tested 56 archived members of the *Bacillus* genus: *B. subtilis* (9 strains, 5 clinical, 4 unknown), *B. cereus* (23 strains, 9 clinical, 14 environmental), *B. thuringiensis* (12 strains, 6 clinical, 3 insects, 3 unknown), *B. mycoides* (1 strain, unknown), *B. megaterium* (10 strains, 7 clinical, 3 unknown), and the environmental *Bacillus* spp. isolate, Ba813_11, which resulted in a previously reported false-positive result in the *B. anthracis*-specific PCR assay targeting *rpoB* (13). In addition, 88 isolates from environmental and clinical specimens, which were confirmed not to be *B. anthracis* by standard microbiologic methods were tested. These isolates were selected because of their lack of hemolysis and because they had a colony morphology similar to *B. anthracis* on blood agar plates.

Before testing, all strains were stored at –70°C in brain heart infusion broth (BHIB, Centers for Disease Control and Prevention [CDC], Atlanta, GA) or water containing 20% glycerol. Identification of all strains was confirmed by using standard microbiologic procedures and the LRN testing algorithm (14,18). Colony-lysis DNA preparations were used for all *Bacillus* spp. strains. Isolates were streaked onto trypticase soy agar containing 5% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated overnight at 37°C. A single colony was transferred and dispersed into 0.22 μM centrifugal filter units (Millipore, Bedford, MA) containing 200 μL 10 mM Tris-HCl (pH 8.0). The suspension was heated at 95°C for 20 min and then cooled to room temperature. The filter units were then centrifuged at 6,000 x *g* in a microfuge for 2 min and the filter discarded. The resulting lysate was stored at –20°C until use.

The lower limit of detection of each assay was tested by using five *B. anthracis* strains: Ames (2000 031656), Pakistan-sheep (20000316 48), French-bovine (2000031651), Sterne (2000031075), and Pasteur (20 00031759). DNA was extracted from vegetative cells by first pre-treating cell pellets with lysozyme and lysostaphin and then using the MasterPure DNA Purification kit (Epicentre, Madison, WI), following the manufacturer's protocol for cell samples. *B. anthracis* spores were quantitated microscopically and tested directly in the real-time PCR assay without DNA extraction. Vegetative-cell DNA was tested at concentrations ranging from 10 ng to 400 fg DNA per reaction. Spores were tested at concentrations ranging from 100,000 spores to 1 spore per reaction. All reactions were performed in duplicate on the LightCycler, Smart Cycler, and ABI Prism 7700 instruments.

All 75 wild-type (fully virulent) *B. anthracis* isolates tested were positive for all three targets resulting in 100% sensitivity (95% confidence interval [CI] 95% to 100%). Strains cured of pXO1 or pXO2 produced negative results for the loci specific to these plasmids (Table). In addition, all 317 *B. anthracis* isolates from the 2001 outbreak were also positive for all three PCR targets (Table).

None of the 56 archived non–*B. anthracis* isolates, representing five other *Bacillus* species was positive for any of the three LRN PCR targets,

Table. Origin, designations, and results of real-time polymerase chain reaction assay for *Bacillus anthracis* strains

B. anthracis	No. analyzed	Temporal range and geographic origin	MLVA genotypes represented[a]	No. positive/total		
				Ba1[b]	Ba2[b]	Ba3[b]
Human isolates	30	1943–1996 Africa, Asia, Australia, Europe, North America	3, 4, 22, 23, 28, 32, 34, 35, 36, 37, 41, 43, 44, 45, 50, 66, 68	30/30	30/30	30/30
Animal isolates	29	1939–1997 Africa, Asia, Australia, Europe, North America, South America	3, 10, 20, 26, 29, 30, 35, 38, 40, 45, 48, 49, 51, 55, 57, 78, 80, 81, 84, 85, 87, 89	29/29	29/29	29/29
Other isolates	16	1950–1993 Africa, Asia, Europe, N. America	13, 14, 21, 24, 47, 62, 69, 73, 77, 79, 82	16/16	16/16	16/16
Outbreak isolates	317	2001 U.S. outbreak	62	317/317	317/317	317/317
pXO1 cured	5	1956–1974 North America		5/5	0/5	5/5
pXO2 cured	1	Africa		0/1	1/1	1/1

aMLVA, multiple-locus variable-number tandem repeat analysis as described by Keim et al. (15).
bBa1, Ba2, and Ba3 primer/probe sets as described in Materials and Methods.

including the *Bacillus* spp. isolate, Ba813_11, resulting in 100% specificity (95% CI 94% to 100%). Results were also negative for 88 clinical and environmental isolates, which were determined by standard microbiologic methods not to be *B. anthracis* (specificity 100%, 95% CI 96% to 100%).

The limit of detection on the LightCycler, Smart Cycler, and ABI Prism 7700 instruments, as determined by using DNA extracted from vegetative cells of the Sterne and Pasteur reference strains, was 1 pg DNA (approximately 167 cells based on a 5.5 Mbp genome size). Five to 10 spores could be detected on the ABI Prism 7700 instrument for the Ames (2000031656), Pakistan-sheep (20000 31648), French-bovine (2000031651), and Sterne (2000031075) strains of *B. anthracis*.

The recent bioterrorism-associated anthrax outbreak demonstrated the need for sensitive, specific, and rapid methods for diagnosis and confirmation of anthrax, both for identification of suspect *B. anthracis* isolates and direct detection of *B. anthracis* DNA in clinical specimens. When tested on >500 strains, representing *B. anthracis* and five other *Bacillus* species, the LRN PCR exhibited 100% sensitivity and specificity.

To date, designing PCR assays for identification of *B. anthracis* has primarily focused on genes located on the plasmids (1–4). Patra et al. used a PCR that targeted two chromosomal loci, *vrrA* and Ba813, and found numerous environmental *Bacillus* isolates other than *B. anthracis* that were positive for both Ba813 and *vrrA* (11). While assays focusing on plasmid targets allow for a high level of specificity, a specific chromosomal target for detection of avirulent and plasmid-cured *B. anthracis* strains is needed. Thus, the LRN PCR includes a chromosomal target in addition to targets on each of the two virulence plasmids, pXO1 and pXO2.

Closely related *B. cereus* and *B. thuringiensis*, notorious for generating false-positive results using assays designed to be specific for *B. anthracis* (11,13), were consistently negative in this real-time PCR assay. *B. anthracis, B. cereus*, and *B. thuringiensis* are so closely related that their distinction as separate species is frequently questioned based on DNA-DNA hybridization studies, multiple-locus enzyme electrophoresis, and 16S rRNA sequence similarity (19–21). We have selected non–*B. anthracis* isolates that were primarily of clinical as opposed to environmental origin. *B. cereus* and *B. thuringiensis* clinical isolates are even more closely related to *B. anthracis* than their environmental counterparts (19,22), and they are more likely to cause false-positive results. We also tested the *Bacillus* spp. isolate that caused the one false-positive result in the Qi et al. report (13). Despite all of these challenges, all three targets of this real-time PCR assay have demonstrated 100% specificity and sensitivity in identification of *B. anthracis* when tested against our panel of *Bacillus* spp. strains and in identification of 317 outbreak-associated *B. anthracis* isolates. This LRN PCR is currently the only real-time PCR assay that detects both plasmid and chromosomal targets with 100% specificity and sensitivity. In addition, real-time PCR assays using fluorescent probes provide great sensitivity; this assay was able to detect 1 pg of purified DNA from vegetative cells (equivalent to 167 cells) or directly detect 5–10 spores.

The high level of sensitivity and specificity of the LRN PCR assay can be attributed to several factors. An extensive panel of DNA samples (non-*Bacillus* gram-positive bacterial species, gram-negative bacterial species, and human, vertebrate, and invertebrate DNA) were tested (data not shown). Having more than a single target decreases the rate of both false-negative and false-positive results, as they are not dependent on a single locus. The use of multiple targets also decreases the risk of false-positive results from contamination because each target is amplified as a separate PCR reaction. Finally, 5′ nuclease

assays makes use of a fluorescent oligonucleotide probe, in addition to the forward and reverse primers, that allows for a lower limit of detection compared to conventional PCR, eliminates the need for post-PCR processing, and increases specificity (23,24).

The LRN PCR was shown to be important for use on environmental and clinical specimens during the 2001 bioterrorism-associated anthrax outbreak. A supplement covering the use of this assay on these specimens can be seen online (available from: URL: http://www.cdc.gov/ncid/EID/vol8no10/02-0393_sup.htm). The LRN PCR assay is widely available at over 200 laboratories in several countries and all 50 states of the United States through the Laboratory Response Network. The system is designed to be accessed through the State Department of Health.

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Industry-Related Outbreak of Human Anthrax, Massachusetts, 1868

To the Editor: In Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States, Jernigan et al. noted that in the mid-1800s inhalational anthrax related to the textile industry became known as woolsorters' disease (in England) and ragpickers' disease (in Germany and Austria) because of the frequency of infection in mill workers exposed to imported animal fibers contaminated with *Bacillus anthracis* spores (1). During the 1800s, as in Europe, industry-related human cases of anthrax also occurred in the United States.

In 1868, Silas Stone, a physician, reported that "an unusual number of cases of a rather rare affection have come under my observation within the past 14 months" (2). Stone described eight patients with "malignant pustules" who worked in or were associated with an animal hair factory in Massachusetts. The patients' cutaneous lesions were described as dark red, dark purple, purplish-black, and black; six of the patients had "slough" lesions. Stone treated his patients with tincture of iodine, iron, and quinine. Since antibiotics were not available, six of the eight patients had severe clinical disease, and two died. Stone's patients demonstrated the full spectrum of anthrax, including gastrointestinal, mediastinal, and meningeal involvement. Four patients had gastrointestinal symptoms, including epigastric distress and pain, nausea, and vomiting. Three patients had mediastinal involvement, manifested by chest distress and pain, dyspnea, and tachypnea. In the two fatal cases, meningitis appeared to have been the immediate cause of death; both of these patients were described as delirious.

Among Stone's eight patients, most remarkable was case 5, which was strikingly similar to case 8 of Jernigan et al.; the signs and symptoms of both patients included chills, headache, fatigue, vomiting, chest pain, tachypnea, tachycardia, and cutaneous lesions. Stone's description of the 7-day clinical course of patient 5, a laborer at the hair factory, is as follows: "Called November 17. Had been sick since the Thursday previous (November 14). Was taken with chills, pain in head and back, and suffered loss of strength. When first seen, was in bed . . . had not slept well the previous night. Pain and distress in epigastrium and back. Pulse 120 . . . breathing hurried. Discovered a dark purple spot surrounded by yellow vesicles . . . pressure on slough produced no pain. November 18: Slough doubled in size. November 19: Vomited . . . severe chill. November 20: Sleep restless . . . slough one inch by half an inch, much raised above surrounding skin, with a red areola about an inch in width. November 21: a.m.: Delirious part of night . . . slept but little . . . pain in chest. 3 p.m.: Distress at epigastrium great . . . delirium more violent. 8 p.m.: Distress and delirium greater . . . pulse failing . . . sinking rapidly . . . died soon after visit."

Stone perceptively noted that each of his patients was directly or indirectly exposed to hair or dirt from the animal hair factory, and that in the surrounding population not so exposed, no cases were seen. Stone realized that he was dealing with an industry-related disease and hypothesized that the cause was "a specific poison, and not simply putrescent animal matter." Nine years after Stone's 1868 report, Robert Koch in Germany reported isolation and cultivation of *B. anthracis*, the formation of its spores, the production of anthrax disease with pure cultures, and the recovery of *B. anthracis* from experimental infection (3).

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Evaluating Real-Time Polymerase Chain Reaction Assay for Rapid Identification of *Bacillus anthracis*

Appendix

During the recent outbreak of bioterrorism-associated anthrax in the United States, 11 patients were diagnosed with inhalational anthrax and 7 with cutaneous anthrax (Table 1 and 2) (*1*–*6*). During the extensive epidemiologic investigation, >125,000 clinical and environmental specimens were collected and analyzed for *Bacillus anthracis*, the causative agent of anthrax. We used the Laboratory Response Network (LRN) polymerase chain reaction (PCR) assay (real-time PCR assay) during the anthrax outbreak to detect *B. anthracis* DNA in environmental samples and clinical specimens. This assay provided 100% sensitivity and specificity when evaluated and validated on our panel of diverse bacterial isolates. On clinical specimens, this assay was one of three used to confirm anthrax cases when isolation of *B. anthracis* failed after antimicrobial drug treatment was initiated. In these culture-negative cases, laboratory confirmation was based on at least two supportive laboratory tests including this PCR, immunohistochemical stain (IHC), or anti-protective antigen (PA) titer (immunoglobulin [Ig]G enzyme-linked immunosorbent assay [ELISA]). PCR assays have not been previously used in an outbreak setting to detect *B. anthracis* directly in clinical specimens in a real-time manner. We evaluated the use of this assay on the clinical specimens and environmental samples received during the outbreak.

Real-time PCR performance was evaluated by using clinical specimens collected from the nine confirmed cases of inhalational anthrax and seven confirmed cases of cutaneous anthrax identified during the bioterrorism-associated anthrax outbreak from October to December 2001. An effort was made to obtain the exact time of collection for each clinical specimen; however, when these data were not available, estimates were made based on other evidence from the medical record. A confirmed case of anthrax was defined as a clinically compatible case of

cutaneous or inhalational illness that was either 1) laboratory confirmed by isolation of *B. anthracis* from an affected tissue or site or 2) accompanied with other laboratory evidence of *B. anthracis* infection based on at least two supportive laboratory tests, including (a) evidence of *B. anthracis* DNA by PCR from specimens collected from an affected tissue or site, (b) demonstration of *B. anthracis* in a clinical specimen by IHC, or (c) fourfold rise in anti-PA IgG. Further testing will be necessary for full evaluation of the utility of these methods on clinical samples. However, as more specimens became available, the LRN PCR was used as part of the laboratory confirmation of anthrax in this outbreak setting. Although real-time PCR results were part of initial confirmation of the diagnosis in 2 patients, all 18 patients were subsequently found to have sufficient laboratory evidence (i.e., culture, serologic testing, or IHC) to confirm case status without considering real-time PCR assay results (Table 1 and 2). In addition, 14 of 18 patients had sufficient laboratory evidence (i.e., real-time PCR, serologic testing, and IHC) to confirm case status without considering culture results (Table 1 and 2).

During the course of the outbreak investigation, clinical specimens were available from 74 patients who had initial symptoms similar to those of anthrax, but in whom the diagnosis was excluded after further evaluation. The exclusion of the diagnosis in these patients was based on the following: 1) the subsequent clinical course was not consistent with anthrax, 2) no laboratory evidence of *B. anthracis* infection was found, and 3) patient had sufficient negative laboratory evidence to establish that the confirmed-case definition could not be met (i.e., negative culture results or negative results on at least two other supportive laboratory tests).

The clinical performance of real-time PCR on clinical specimens was evaluated by using two approaches. In the first approach, traditional culture methods were used as the standard for evaluating real-time PCR detection of *B. anthracis* DNA in clinical specimens. In the second approach, the confirmed-case definition was used as the standard for comparing real-time PCR and traditional culture methods as diagnostic tests for anthrax.

A total of 279 clinical specimens were tested in parallel fashion by both traditional culture methods and by real-time PCR (Table 3). Two aliquots were prepared from each specimen. From one aliquot DNA was extracted with a MagNa Pure LC instrument (Roche Diagnostics GmbH, Mannheim, Germany) by employing a DNA isolation kit I with the “High Performance” protocol. In addition, select specimens were extracted in duplicate with a Qiagen

DNA Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions. A second aliquot was used to inoculate bacteriological media for isolation of *B. anthracis* (*7*).

Specimens from patients meeting the definition for confirmed anthrax and from those in whom the diagnosis was excluded were tested by LRN PCR assay and traditional culture using the methods described above. For specimens that were unavailable for testing at Centers for Disease Control and Prevention (CDC), culture results reported by the clinical laboratories of the patient's treating facility were used for case confirmation.

The performance of the LRN PCR assay was compared to that of traditional culture methods by testing environmental specimens collected from throughout the United States during the course of the outbreak by both methods. *B. anthracis* spores were eluted from swab specimens and other environmental samples in 2.5% pluronic F-68 (Sigma, St. Louis, MO) and then collected by centrifugation through an Ultrafree-CL, 0.45 uM, PVDF membrane filter (Millipore, Bedford, MA). Spores were eluted from the filters with 2.5% pluronic F-68, used to inoculate bacteriologic media, and added directly to real-time PCR assays without further purification or DNA extraction.

Two hundred seventy-nine clinical specimens were tested by both culture and real-time PCR: 92 were from 9 patients with inhalational anthrax, 33 from 7 patients with cutaneous anthrax, 12 from 4 patients with suspect cutaneous anthrax, and the remaining 142 from 74 patients in whom anthrax was excluded (Table 3). Of the 92 specimens from the inhalational anthrax cases, 5 (all blood specimens) were positive by both methods. Of the remaining 87, all were culture negative, but 29 (33%) were positive by the PCR assay. These included serum, sputum, pleural fluid, and tissue specimens (Table 3). Of the 33 specimens from the cutaneous anthrax cases, none were culture positive, but positive PCR results were obtained on a single blood specimen and two skin biopsy specimens. None of the 142 specimens from 74 patients without anthrax had positive results on culture or PCR.

A total of 382 clinical specimens from 94 patients were tested by real-time PCR, culture, or both. Real-time PCR was performed on specimens from 14 patients with anthrax in whom the diagnosis could have been confirmed using non-PCR methods, including 9 inhalational anthrax patients and 5 cutaneous patients (2 were confirmed by culture, 3 by IHC and serology, and 2 by

IHC and PCR). PCR was also performed on specimens from 74 patients in whom anthrax was excluded.

Culture was performed on specimens from 13 anthrax patients in whom the diagnosis could be confirmed using non-culture methods, including 8 patients with inhalational anthrax and 5 patients with cutaneous anthrax. Culture was also performed on specimens from 74 patients in whom anthrax was excluded.

One hundred forty-two specimens tested in the patients with inhalational anthrax included blood (n=74), serum (n=36), sputum (n=2), tissue (n=7), pleural fluid (n=19), and other (n=4). One hundred eighty-six specimens tested in the patients without anthrax included blood (n=74), swabs (n=15), serum (n=41), sputum (n=4), tissue (n=38), and other (n=14).

Inhalational Cases

Of the 11 patients with inhalational anthrax, 8 had blood cultures performed before the initiation of antimicrobial drug therapy, and cultures were positive in all eight at the hospital where patients were initially treated. At CDC, *B. anthracis* was also isolated from blood cultures of patient 5 (two blood cultures collected immediately before the start of the antimicrobial drug therapy), patient 6 (one blood culture collected on the same day antimicrobial drug therapy started),and patient 11 (two blood cultures collected the day before antimicrobial drug therapy). In contrast, 44 blood specimens were cultured from five patients (patients 2, 8, 9, 10, 11) (Table 1 and 2) after administration of antimicrobial drug therapy, and all were negative, including those from four patients (patients 2, 8, 10, 11) who had blood cultures obtained within 48 h of administration of antimicrobial drugs. Of the four patients (patients 2, 3, 5, 6) who had PCR performed on blood collected before the administration of antimicrobial drug therapy, all four had a positive PCR result (Table 4). In contrast, six patients had PCR testing of blood specimens collected after administration of antimicrobial drug therapy, and four (patients 2, 8, 10, 11) had a positive PCR result. A single patient (patient 2) had blood cultures collected ≥5 days after antimicrobial agent administration; a total of 26 blood specimens were collected past day 5, and 8 were PCR positive, ranging from day 7 to day 10 (Figure).

Pleural fluid was available for testing from five patients (patients 1, 2, 8, 10, 11) with inhalational anthrax (Table 4). Of the pleural fluid specimens collected ≤5 days after the administration of antimicrobial drug therapy, none grew *B. anthracis* in culture, whereas all five

had a positive PCR result. Pleural fluid specimens collected >5 days after antimicrobial agent administration were available only from a single patient (patient 2): all four of these specimens were PCR positive (Figure).

Seven postmortem tissue specimens were collected from three patients. Samples from a lymph node and lung tissue from one patient (patient 10) and a lymph node sample from another patient (patient 11) were PCR positive. All others were negative (Table 4).

Two sputum samples were tested. A sputum sample from patient 2 was received 5 days after the administration of antimicrobial drugs, and it was PCR negative. The second sputum was obtained on day 2 after the administration of antimicrobial drugs and was PCR positive (patient 11).

Cutaneous Cases

Of the seven patients with cutaneous anthrax, two had blood cultures performed before administration of antimicrobial drug therapy at the medical facility where patients were treated, and one patient had a positive result (Table 2, patient 5). All seven patients had blood cultures performed after initiation of antimicrobial drug therapy, and none had a positive result. All seven patients had PCR testing of blood specimens collected after administration of antimicrobial drug therapy, and of these, one was positive, from a patient with an extensive lesion and systemic complications of cutaneous anthrax. The blood sample was obtained 3 days after onset of the lesion.

At the local facility, two patients had wound swabs obtained from ulcerative skin lesions before antimicrobial drugs were initiated; of these, one had evidence of gram-positive rods on Gram stain with *B. anthracis* isolated on culture (patient 7).

Nine tissue samples were obtained from seven confirmed cases of cutaneous anthrax, including five patients with both fixed and frozen tissue and two patients with only fixed tissue. Eight samples were obtained after the administration of antimicrobial drugs. Culture was negative on all eight tissue samples; PCR was positive on one fixed tissue sample (patient 2), obtained 14 days after onset date, and on a fresh frozen tissue (patient 6) received 6 days after antimicrobial drugs were administered. In addition, one frozen tissue sample was received from a single patient before antimicrobial drug therapy; both culture and PCR were negative.

Four additional cutaneous cases were defined as suspect because only one supportive laboratory test was positive; for three of the cases, serologic testing was positive, and for the fourth, IHC of an arm biopsy specimen was positive. A total of 12 specimens (7 blood specimens, 3 sera, 2 swabs) collected from these four patients were tested by PCR and culture; all were PCR and culture negative.

Patients without Anthrax

One hundred eighty-six clinical specimens were collected from 74 patients who were subsequently determined not to have either inhalational or cutaneous anthrax; 142 specimens were culture and PCR negative (PCR specificity of 100%, 95% confidence interval 99% to 100%), and the remaining 44 tested by PCR only were also negative.

Real-Time PCR in Environmental Specimens

One hundred forty environmental specimens were analyzed by both culture and real-time PCR. A wide variety of samples were tested, including dust, paper towels, a syringe, vent filters, HVAC filters, vacuum cleaner debris, a cellulose sponge, and clothing; however, most samples were surface swabs (n=82). Of the 140 environmental specimens tested by both PCR and culture, 35 were positive by both methods, 7 were positive by culture only, and 4 were positive by PCR only.

Discussion

Isolation of *B. anthracis* from primarily sterile sites in culture has long been considered the standard of diagnosis for anthrax. However, this method is associated with a diagnostic delay of 12–24 h, and sensitivity is greatly diminished in the setting of prior antimicrobial administration (*1*). The LRN PCR was invaluable in diagnosing anthrax in patients when culturing *B. anthracis* failed and has rapidly become an integral part of the laboratory confirmation of anthrax. This real-time PCR also appears to be less affected by prior administration of antimicrobial drugs than culture, a property with important clinical ramifications. PCR positive results were obtained directly on clinical specimens, especially pleural fluids, in one case up to 11 days after the initiation of antimicrobial treatment.

When the LRN PCR and culture were simultaneously performed on clinical specimens, PCR was positive in every specimen from which *B. anthracis* was isolated. PCR was also positive in an additional 29 (33%) specimens that were culture negative. All PCR-positive specimens were collected from patients in whom the diagnosis of anthrax was confirmed by other methods, suggesting that LRN PCR has a higher positive predictive value than culture. The LRN PCR also appears to have high clinical specificity; no positive tests on clinical specimens were collected from patients in whom the diagnosis of anthrax was considered, but ultimately ruled out based on clinical course and additional diagnostic tests.

By using the confirmed case definition as the standard for diagnosis, the clinical performance characteristics of culture and LRN PCR during the 2001 outbreak can be directly compared to one another. Blood cultures appear to have a sensitivity of 100% (8 of 8 patients) if collected before the administration of antimicrobial drug therapy in patients with inhalational anthrax, but the sensitivity falls to zero if the blood is collected after administration of antimicrobial drugs. Similarly, PCR assay of blood has a sensitivity of 100% (6 of 6 patients) if the blood is collected before antimicrobial drug therapy. In contrast to blood culture, PCR assay can detect *B. anthracis* in the blood after administration of antimicrobial drug therapy. However, the sensitivity seems to decrease within 24 h after initiation of antimicrobial drugs; three of four inhalational anthrax patients who had PCR assay performed on blood collected within 24 h of antimicrobial administration had a positive result, while one of five patients who had PCR performed on blood collected $\geq$24 h after the start of antimicrobial drug therapy had a positive result.

The LRN PCR assay was particularly useful for testing pleural fluid specimens. No patient (n=5) in whom pleural fluid specimens were received at CDC had a culture positive result; however, all tests were performed after the administration of antimicrobial drug therapy. In contrast, all five patients who had the LRN PCR performed on pleural fluid specimens had a positive result including all three from whom pleural fluid was collected $\geq$24 hours after the administration of antimicrobial therapy. The sensitivity of the real-time LRN PCR on pleural fluid specimens appears to be less affected by the administration of antimicrobial drugs than does the LRN PCR of blood.

Laboratory confirmation for the seven cutaneous cases primarily relied on IHC and serology as only two clinical samples (one blood and one tissue sample) from two patients grew *B. anthracis* at the medical facility where the patients were examined and treated. However, the LRN PCR was subsequently attempted on 11 blood samples and 8 tissue samples from six cutaneous cases. Only one blood sample and two tissue samples from three patients were PCR positive (Table 3). CDC received all specimens from patients with cutaneous anthrax after the initiation of antimicrobial drug therapy. This success rate is similar to results of the LRN PCR on fluids (with the exception of pleural fluids) and tissue taken after the initiation of antimicrobial drug therapy on patients with inhalational anthrax.

Overall, *B. anthracis* was isolated from 8 (73%) of 11 patients with confirmed inhalational anthrax while the LRN PCR was positive for 8 (89%) of 9 patients tested. One case in which only two blood cultures were tested yielded negative results for both culture and PCR. Of the seven patients with confirmed cutaneous anthrax, *B. anthracis* was isolated from two patients (29%), and the LRN PCR was positive for three (43%).

One advantage of the LRN PCR assay is its rapidity; as a rule, results can be obtained within 1 h from the time samples have been prepared for testing. This rapid result is in striking contrast to the results for all other methods used for laboratory confirmation of anthrax. For example, standard culture methods require at least 24 h, while IHC results can be obtained within 8 h. On the other end of the spectrum is serology that requires paired sera collected at least 10 days apart, making this approach the least helpful in situations where therapeutic and public health decisions need to be instigated rapidly.

Evaluation of the LRN PCR and its performance on clinical specimens was not conducted as a true prospective study as we were, to a degree, limited by the number and type of specimens available, as well as by the emergent response needed to establish the microbiologic diagnosis. However, the number and variety of clinical samples were substantial enough to allow statistically significant comparisons with the current standard, culture. Also, the fact that laboratory confirmation was obtained by either culture or a combination of other supportive laboratory methods allowed for case-based evaluation of the LRN PCR's sensitivity and specificity. A major advantage of the LRN PCR was its lack of any false-positive results (100% specificity) when used on cultures and directly on clinical specimens. Of the 110 patients

clinically suspected to have anthrax, 74 had clinical samples collected and tested by at least three diagnostic approaches (culture, PCR, IHC, or serology) that would allow for a case to be defined as confirmed (culture positive or two supportive tests positive) or suspect (one supportive case positive). Samples from all of these patients were negative in all tests applied, including this LRN PCR. Given the extent and cost of public health and other actions taken after the laboratory confirmation of each anthrax cases in this epidemic, a false-positive PCR could have resulted in unnecessary waste of resources.

In addition to its invaluable use on clinical specimens, the LRN PCR also allowed for the rapid analysis of hundreds of diverse environmental samples throughout the outbreak investigation. If present in these specimens, *B. anthracis* was in the form of spores. Because *B. anthracis* spores contain DNA on their surfaces as a result of the sporulation process, environmental specimens can be analyzed in the PCR assay without having to do the DNA extraction, which eliminates the need for complicated and usually inefficient spore lysis methods. Culture methods and LRN PCR results were in agreement 92% (129/140) of the time. For the remaining 11 specimens, 4 were PCR positive and culture negative, and 7 were PCR negative and culture positive. The occasional discrepancies between culture and PCR could be due to inefficient removal of PCR inhibitors, detection of nonviable spores by PCR, and sampling error and volume effects when very few spores were present (5 μL for PCR vs. 100–200 μL for culture).

The LRN PCR assay evaluated and validated in this study detects a *B. anthracis*–specific chromosomal target as well as targets on both plasmids that are required for full virulence. This assay has served as an important aid in epidemiologic investigations of the recent bioterrorism-associated anthrax outbreak and was rapidly established as a valuable component of laboratory confirmation of anthrax cases. Highly specific results are obtained within a few hours of specimen arrival, making rapid and appropriate actions possible. At the same time, unnecessary panic and administration of antimicrobial drugs and vaccines were prevented when *B. anthracis* was rapidly excluded from differential diagnosis.

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Table 1. Laboratory methods used for confirmation of 11 inhalational anthrax cases[a,b]

Patient no.[c]	Laboratory confirmation	Other laboratory tests positive for *Bacillus anthracis*
1	CSF culture	IHC of multiple (postmortem) tissues, blood culture
2	PCR of pleural fluid; IHC of pleural fluid; serology	Transbronchial biopsy IHC, pleural biopsy IHC
3	Blood culture	PCR of blood; serology
4	Blood culture	Serology
5	Blood culture	IHC of mediastinal lymph nodes; PCR of blood
6	Blood culture	IHC of mediastinal lymph nodes; PCR of blood
7	Blood culture	Serology
8	PCR of pleural fluid; IHC of pleural fluid	Serology
9	IHC of pleural fluid and bronchial biopsy; serology	
10	Blood and pleural fluid culture	IHC of multiple organs; PCR of multiple organs
11	Blood culture	PCR of multiple organs; IHC of multiple organs

[a]All initial isolation of *Bacillus anthracis* from clinical specimens took place at the local health facility where the patients were treated.
[b] CSF, cerebrospinal fluid; IHC, immunohistochemical stain; PCR, polymerase chain reaction.
[c]Patients 1–10 described in Jernigan et al. (1) and patient 11 in Barakat et al. (5).

Table 2. Laboratory methods used for confirmation of seven cutaneous anthrax cases[a,b]

Patient no.	Laboratory confirmation
1	Chest biopsy IHC, serology
2	Arm biopsy IHC and PCR, serology
3	Arm biopsy IHC, serum PCR
4	Face biopsy IHC, serology
5	Blood culture
6	Forehead biopsy IHC and PCR
7	Face biopsy culture

[a]All initial isolation of *Bacillus anthracis* from clinical specimens took place at the local health facility where the patients were treated.
[b]IHC, immunohistochemical stain; PCR, polymerase chain reaction.

Table 3. Results of real-time PCR and culture testing performed on 382 clinical specimens[a,b]

Cases	PCR only		PCR and culture				Total
Nine inhalational cases	+	-	PCR + C -	PCR + C +	PCR - C -	PCR - C +	
Blood specimens	5	20	9	5	35	0	74
Swab specimens	0	0	0	0	0	0	0
Serum specimens	2	15	3	0	16	0	36
Sputum specimens	0	1	1	0	0	0	2
Tissue specimens	0	1	3	0	3	0	7
Pleural fluid specimens	5	0	11	0	3	0	19
Other specimens	1	0	2	0	1	0	4
Totals	50		92				142
Seven cutaneous cases							
Blood specimens	0	0	1	0	10	0	11
Swab specimens	0	0	0	0	3	0	3
Serum specimens	0	2	0	0	11	0	13
Sputum specimens	0	0	0	0	0	0	0
Tissue specimens	0	2	2	0	4	0	8
Other specimens	0	0	0	0	2	0	2
Totals	4		33				37
Four suspect cases							
Blood specimens	0	2	0	0	7	0	9
Swab specimens	0	0	0	0	2	0	2
Serum specimens	0	1	0	0	3	0	4
Sputum specimens	0	0	0	0	0	0	0
Tissue specimens	0	2	0	0	0	0	2
Other specimens	0	0	0	0	0	0	0
Totals	5		12				17
Other							
Blood specimens	0	16	0	0	58	0	74
Swab specimens	0	1	0	0	14	0	15
Serum specimens	0	11	0	0	30	0	41
Sputum specimens	0	0	0	0	4	0	4
Tissue specimens	0	14	0	0	24	0	38
Other specimens	0	2	0	0	12	0	14
Totals	44		142				186

[a]Sixteen patients with laboratory-confirmed anthrax, four suspect cases of anthrax, and 74 patients on whom anthrax has been ruled out.
[b]PCR, polymerase chain reaction; C, culture.

Table 4. Results of real-time PCR and culture testing performed on 142 clinical specimens collected from nine patients with inhalational anthrax[a]

Patient no.[b]	Specimen type	Antimicrobial drug therapy: Post therapy	Antimicrobial drug therapy: Interval (days)	Culture	Real-time PCR
1	Pleural fluid[c]	Yes	3	Negative	Positive
	Serum[c]	Yes	3	Negative	Negative
	Serum[C]	Yes	3	Not done	Negative
	Pleural fluid[c]	Yes	4	Not done	Positive
	Pleural fluid[c]	Yes	4	Negative	Positive
	Blood[c]	Yes	4	Not done	Negative
	Right lung tissue (frozen)[c]	Yes	4	Not done	Negative
	Heart blood[c]	Yes	4	Not done	Negative
	Pericardial fluid[c]	Yes	4	Not done	Positive
2[c]	Blood (5)			Not done	Positive
	Blood (18)			Not done	Negative
	Blood (5)			Negative	Positive
	Blood (21)			Negative	Negative
	Serum (2)			Not done	Positive
	Serum (14)			Not done	Negative
	Serum (2)			Negative	Positive
	Serum (13)			Negative	Negative
	Pleural fluid (3)			Not done	Positive
	Pleural fluid (5)			Negative	Positive
	Pleural fluid (1)			Negative	Negative
	Body fluid (1)			Negative	Negative
	Respiratory wash (1)			Negative	Positive
	Sputum (1)			Not done	Negative
3	Blood culture	No	-1	Negative	Positive
5	Blood culture	No	-1	Positive	Positive
	Blood culture	No	-1	Positive	Positive
6	Blood culture	No	0	Positive	Positive
8	Blood	Yes	.5	Negative	Positive
	Blood	Yes	.5	Negative	Positive
	Serum	Yes	.5	Negative	Positive
	Serum	Yes	2	Negative	Negative
	Pleural fluid	Yes	2	Negative	Positive
	Pleural fluid	Yes	2	Negative	Positive
	Blood	Yes	37	Negative	Negative
9	Blood	Yes	2	Negative	Negative
	Blood	Yes	2	Negative	Negative
10	Pleural fluid	Yes	1	Negative	Positive
	Pleural fluid	Yes	1	Negative	Positive
	Blood	Yes	1	Negative	Positive
	Thioglycolate broth[c]	Yes	3	Negative	Negative
	CSF[c]	Yes	3	Negative	Positive
	Lung tissue (frozen)[c]	Yes	3	Negative	Positive
	Lymph node tissue (frozen)[c]	Yes	3	Negative	Positive
11	Blood culture	No	-1	Positive	Positive
	Blood culture	No	-1	Positive	Positive
	Sputum	Yes	2	Negative	Positive
	Body fluid/pleural fluid	Yes	2	Not done	Positive
	Blood	Yes	2	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Liver tissue (frozen)[c]	Yes	4	Negative	Negative
	Lymph node tissue (frozen)[c]	Yes	4	Negative	Positive
	Blood[c]	Yes	4	Negative	Negative
	Pleural fluid[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Lung tissue (frozen)[c]	Yes	4	Negative	Negative
	Spleen tissue (frozen)[c]	Yes	4	Negative	Negative
	Blood[c]	Yes	4	Negative	Negative
	Pleural fluid[c]	Yes	4	Negative	Negative

[a] PCR, polymerase chain reaction

Patient no.[b]	Specimen type	Antimicrobial drug therapy: Post therapy	Interval (days)	Culture	Real-time PCR

[b]Patients 1–10 described in Jernigan et al. (1) and patient 11 in Barakat et al. (5).
[c]Samples collected postmortem.
[d]Due to the large number of samples from patient #2, samples were summarized by type and result in Figure.

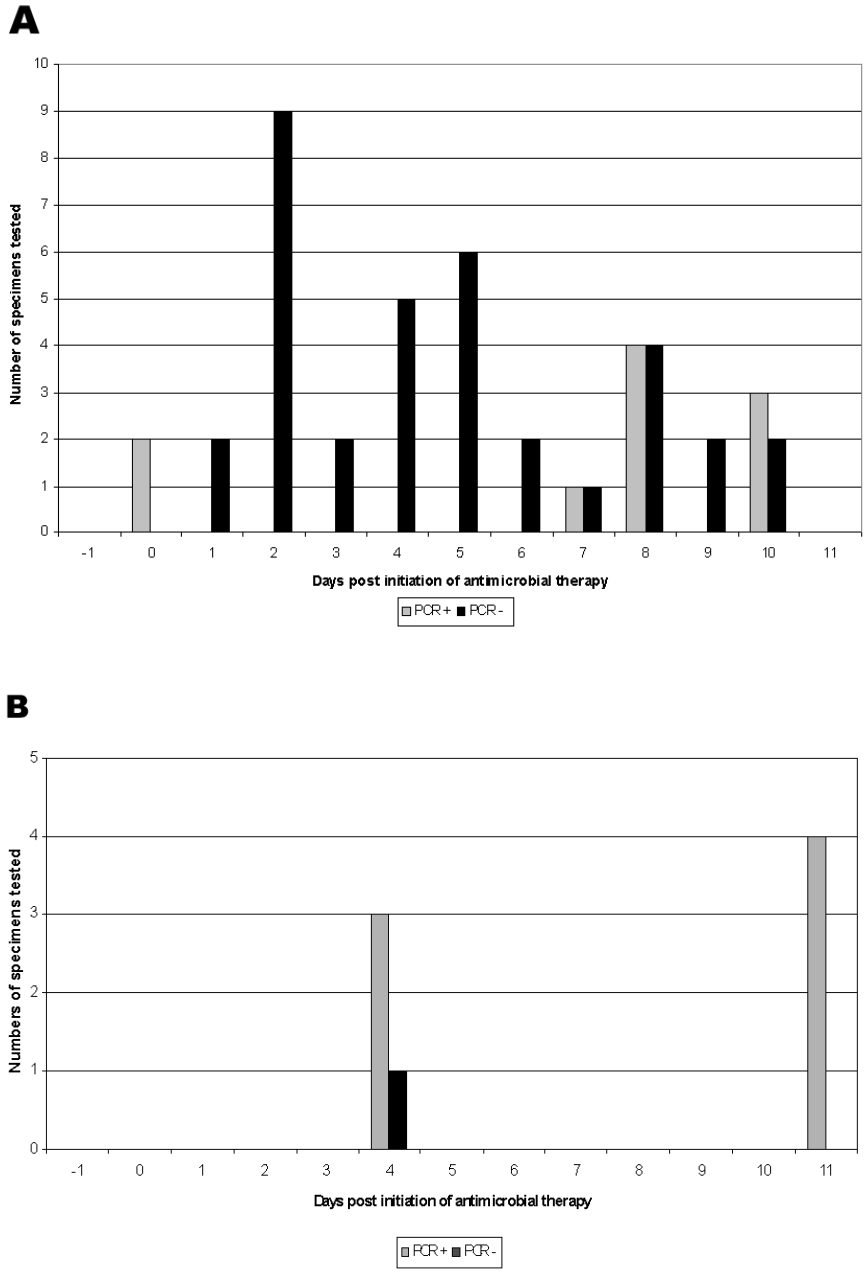

Figure. Results of polymerase chain reaction testing of clinical specimens (for which dates of collection were available) from a patient with inhalational anthrax (patient 2), are illustrated by date of collection relative to the initiation of antimicrobial drug therapy. *Bacillus anthracis* was not recovered from any of these specimens on which culture was attempted (data not shown). A. Blood, n=45; B. Pleural fluid, n=8.