Health Security
Volume 17, Number 4, 2019 © Mary Ann Liebert, Inc.
DOI: 10.1089/hs.2019.0038

Rapid Presumptive Identification of *Bacillus anthracis* Isolates Using the Tetracore RedLine Alert™ Test

Segaran P. Pillai, Kristin W. Prentice, Jason G. Ramage, Lindsay DePalma, Jawad Sarwar, Nishanth Parameswaran, Melissa Bell, Andrea Plummer, Alan Santos, Ajay Singh, Christine A. Pillai, Nagarajan Thirunavvukarasu, Gowri Manickam, Julie R. Avila, Shashi K. Sharma, Alex Hoffmaster, Kevin Anderson, Stephen A. Morse, Kodumudi Venkat Venkateswaran, and David R. Hodge

A comprehensive laboratory evaluation of the Tetracore RedLine Alert test, a lateral flow immunoassay (LFA) for the rapid presumptive identification of *Bacillus anthracis,* was conducted at 2 different test sites. The study evaluated the sensitivity of this assay using 16 diverse strains of *B. anthracis* grown on sheep blood agar (SBA) plates. In addition, 83 clinically relevant microorganisms were tested to assess the specificity of the RedLine Alert test. The results indicated that the RedLine Alert test for the presumptive identification of *B. anthracis* is highly robust, specific, and sensitive. RedLine Alert is a rapid test that has applicability for use in a clinical setting for ruling-in or ruling-out nonhemolytic colonies of *Bacillus* spp. grown on SBA medium as presumptive isolates of *B. anthracis*.

Keywords: Anthrax, Lateral flow assay, Rapid diagnostics, *Bacillus anthracis*

Anthrax is an acute infection caused by the gram-positive, rod-shaped, spore-forming facultative anaerobic bacterium *Bacillus anthracis*.[1-5] Cells of *B. anthracis* are nonmotile, nonhemolytic, encapsulated, and arranged in chains. The vegetative form of this bacterium is not easily transmitted to humans, but the spores, which are the infectious form, are resistant to harsh environmental conditions, including ultraviolet light, ionizing radiation, heat, and various chemicals.[4,6-8] Cutaneous anthrax infections make up the vast majority of reported human cases

Segaran P. Pillai, PhD, is Director, Office of Laboratory Science and Safety, FDA Office of the Commissioner, Department of Health and Human Services, Silver Spring, MD. Kristin W. Prentice, MS, is an Associate, and Lindsay DePalma, MS, is a Staff Life Scientist; both at Booz Allen Hamilton, Rockville, MD. Jason G. Ramage, MS, MBA, PMP, is Assistant Vice Chancellor for Research and Innovation and Director of Research Compliance, University of Arkansas, Fayetteville, AR. Jawad Sarwar, MS, is a Senior Research Scientist, and Nishanth Parameswaran is a Research Scientist; both at Omni Array Biotechnology, Rockville, MD. Melissa Bell, MS, is a Microbiologist, and Alex Hoffmaster, PhD, is Chief, Bacterial Special Pathogens Branch; both in the National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA. Andrea Plummer and Alan Santos are Microbiologists, and Kodumudi Venkat Venkateswaran, PhD, is Chief Scientist; all at Tetracore, Inc., Rockville, MD. Ajay Singh, PhD, is a Research Scientist, Laulima Government Solutions, Contractor Support to USAMRICD Neurobiological Toxicology Branch, Analytical Toxicology Division, Aberdeen Proving Ground, MD. Christine A. Pillai, Nagarajan Thirunavvukarasu, PhD, and Gowri Manickam, PhD, are ORISE Fellow Research Scientists, and Shashi K. Sharma, PhD, is a Research Microbiologist; all with the FDA Center for Food Safety and Applied Nutrition, Molecular Methods Development Branch, Division of Microbiology, Office of Regulatory Science, College Park, MD. Julie R. Avila, MS, is a Scientific Associate, Biosciences and Biotechnology Division, Lawrence Livermore National Laboratory, Livermore, CA. Kevin Anderson, PhD, and David R. Hodge, PhD, are Program Managers, Science and Technology Directorate, US Department of Homeland Security, Washington, DC. Stephen A. Morse, MSPH, PhD, is a Senior Advisor, CDC Division of Select Agents and Toxins, and is currently with IHRC, Inc., Atlanta, GA.

worldwide, accounting for more than 95% of human anthrax cases; if left untreated, up to 20% of cases can be fatal.[3,9,10] Oropharyngeal or gastrointestinal anthrax is the second most common form of the disease, characterized by lesions in the oral cavity or stomach/intestinal tract due to consumption of contaminated meat or by swallowing aerosolized spores.[11] Lesions cause massive swelling and blockage of the airway, stomach or intestinal perforation, or hemorrhage, and, if left untreated, 25% to 60% of cases are fatal.[10,11] Inhalation anthrax, which is the most dangerous form of *B. anthracis* infection, with untreated fatality rates close to 100%, can be acquired through occupational exposure (eg, mill workers) or from an intentional release of spores, as happened in the anthrax attacks in 2001.[3,9,12-16] In recent years, a fourth form, injection/septicemic anthrax, has emerged among intravenous drug users in western Europe.[17] The most recent outbreak of injectional anthrax was believed to be the result of contaminated heroin that originated in Pakistan or Iran.[18]

B. anthracis ranks high on the list of potential agents of bioterrorism, as mortality rates can be significant when illness results from the inhalation of aerosolized spores.[19-21] Experts consistently rank anthrax spores as a potential bioweapon when taking into consideration characteristics such as stability and ease of dissemination.[20,21] Bioterrorism events and planning scenarios have emphasized the need for rapid and accurate detection and diagnostics to protect public health. Various tests have been developed to detect and/or identify *B. anthracis* in clinical specimens. Many of these tests have a high sensitivity and/or high specificity, such as real-time PCR, antigen detection tests, gamma phage susceptibility, and direct fluorescent antibody tests.[22,23] However, these tests have limitations or high complexity testing.[24,25] Because of these limitations, the time to obtain results may be delayed, which can hinder timely and critical treatment decisions.

Lateral flow immunochromatographic assays (LFAs) were commercially introduced for pregnancy testing in 1988.[26] Simple to use and requiring minimal training,[27] these LFAs are ideal for use by first responders and law enforcement officers to test suspicious materials in field settings. LFAs such as the BioThreat Alert® assays have previously been evaluated for the detection of several biothreat agents including orthopoxviruses,[28] ricin,[29] abrin,[30] *B. anthracis,*[31] and *Yersinia pestis*.[32]

The purpose of this study was to conduct a comprehensive test and evaluation of the FDA cleared RedLine Alert LFA (Tetracore, Inc., Rockville, MD) (510K Approval No. K030370) for presumptive identification of *B. anthracis* from nonhemolytic bacillus colonies grown on sheep blood agar (SBA) plates. The RedLine Alert LFA uses a combination of a polyclonal and a monoclonal antibody directed against extractable antigen 1 (EA1). The presence of EA1 in the sample forms a complex with the colloidal gold-labeled monoclonal antibody that migrates along the membrane. An immobilized rabbit capture antibody binds the colloidal gold-labeled antibody-antigen complex to form a colored line in the results window. An internal control line is also present in the results window to ensure that the test has been performed correctly.[33,34] The evaluation included the likelihood of false-negative results (assay is negative but the analyte is present at a concentration below the limit of detection, or LOD), false-positive results (assay is positive but the target analyte is not present in the sample), and robustness and reproducibility of an assay that can be incorporated into the Level A protocol for *B. anthracis*.[25] This will enable local hospital and commercial clinical laboratories to obtain a rapid presumptive diagnosis of anthrax infections, including those resulting from an intentional biological attack, so that appropriate notification and clinical intervention can be initiated in a timely manner to save lives.

This study was designed and executed through an interagency collaboration with participation from subject matter experts from the Department of Homeland Security (DHS) Science and Technology Directorate (S&T) Chemical and Biological Defense Division (CBD) and First Responders Group (FRG); Health and Human Services (HHS) Office of the Assistant Secretary for Preparedness and Response/Biomedical Advanced Research and Development Authority (ASPR/BARDA); HHS Centers for Disease Control and Prevention (CDC); HHS Food and Drug Administration (FDA), Office of Laboratory Science and Safety; FDA Center for Food Safety and Applied Nutrition (CSFAN); Department of Justice (DOJ) Federal Bureau of Investigation (FBI); the US Department of Agriculture (USDA); and others.

Materials and Methods

The Level A protocol for the sentinel level clinical laboratory for the presumptive identification of *B. anthracis* is to inoculate the specimen onto an SBA plate (and chocolate agar, and MacConkey or eosin methylene blue agar, depending on the type of specimen). After incubation at 35°C to 37°C in 5% to 10% CO_2 for 18 to 24 hours (growth of *B. anthracis* may be observed as early as 8 hours), γ-hemolytic (no hemolysis) nonpigmented colonies with a ground glass appearance on the blood agar plate are gram stained. Those consisting of large, gram-positive rods are tested for the presence of catalase and motility. Cultures of γ-hemolytic, nonmotile, catalase positive, and gram-positive rods are considered as presumptive *B. anthracis* and sent to a CDC Laboratory Response Network (LRN) reference laboratory for confirmatory testing and further characterization.[25] *B. anthracis* can be ruled out if the colonies are α- or β-hemolytic or if the organism is catalase negative and/or motile.[25] RedLine Alert test strips (catalog number TC-5123-001) were obtained from Tetracore, Inc., in Rockville, MD. In Phase 1, the sensitivity of the assay was determined using the virulent *B. anthracis* Ames strain and an inclusivity panel of 15 additional strains. The virulent *B. anthracis* strains were grown, samples prepared, and 4 replicates tested at the Zoonoses and Select Agent Laboratory, Bacterial Special Pathogens Branch, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta, GA. In Phase 2, assay specificity was determined using the avirulent Sterne strain of *B. anthracis* (positive control) and a panel composed of 81 clinically relevant strains. Most of the testing was done at Omni Array Biotechnology, Rockville, MD, by 4 different operators from DHS S&T and FDA CFSAN according to manufacturers' recommendations. Select agent organisms in the clinical panel were grown, samples prepared, and 4 replicates tested at Tetracore, Inc., Rockville, MD.

Phase 1: Inclusivity Panel

All inclusivity strains of *B. anthracis* ($N=16$) representing clades A1.a, A1.b, A3, A3.a, A3.b, A4, B1, B2, and C were typed using Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) and characterized by plasmid profile analysis and 16S typing at CDC (Table 1).

Prior to testing, the inclusivity panel strains were inoculated onto SBA plates and incubated at 37°C under aerobic conditions until visible colonies (2-4 mm in diameter) were observed. A colony was removed using a sterile loop and resuspended in 200 µL of colony isolation buffer (CIB) in a vial. To facilitate transfer, the loop was twirled in the buffer for 5 seconds to dislodge the colony. The tube was vortexed for 5 seconds to resuspend and to evenly distribute the cells in the buffer and then allowed to sit at room temperature for about 2 minutes. Next, the vials were gently vortexed, and 150 µL of the test sample was transferred to the sample well of the RedLine Alert test strip. Four replicates of each strain were tested, and visual readings were noted between 15 and 30 minutes after the addition of the sample.

Phase 2: Clinical Panel

After consultation with subject matter experts, a clinical panel consisting of 81 human pathogens was assembled (Table 2). The panel of bacteria consisted of agents known to cause dermal, pulmonary, gastrointestinal, and septicemic infections.

This panel was used to evaluate the specificity of the RedLine Alert LFA assay. Strains were grown either on selective medium or SBA by Lawrence Livermore National Laboratory, Livermore, CA, or Tetracore, Inc., Rockville, MD, and shipped to Omni Array, where they were subcultured onto SBA plates and incubated for 16 to 24 hours at 37°C to ensure purity. For long-term storage, individual bacterial seeds were prepared using MICROBANK™ (Pro-Lab, Ontario, Canada), based on manufacturer's guidelines and stored at −80°C. Two days prior to testing, the frozen stocks were retrieved, inoculated onto SBA plates, and incubated overnight at 37°C. The following day, cultures were examined for purity, and a single isolated colony was

Table 1. Inclusivity Strains of *B. anthracis*

S. No.	*Strain ID*	*Clade*	*Genotype*	*pX01*	*pX02*
1	K8960; A0369; 2011756210	A1.a	GT7	Yes	Yes
2	K1256; A0193; 2000031657	A1.a	GT10	Yes	Yes
3	K9002; A0149; Turkey #32; 2000031650	A1.b	GT23	Yes	Yes
4	K7948; A0264; 2000031659	A1.b	GT28	Yes	Yes
5	K2802; A0248; 2000031652 Ohio ACB	A3	GT68	Yes	Yes
6	K4516; A0376; 2000031654 1015	A3.a	GT51	Yes	Yes
7	AO467; 2002013028	A3.a	GT91	Yes	Yes
8	K7816; Sterne	A3.b	GT59	Yes	No
9	K1694; A0462; Ames; 2000031656	A3.b	GT62	Yes	Yes
10	K7222; A0379; 2000031653 SK-102	A4	GT69	Yes	Yes
11	K4596; A0488; Vollum 1B; 2000031666	A4	GT77	Yes	Yes
12	K1129; A0337; 2008724774	A4	GT74	Yes	Yes
13	K8101; A0382; 2008724769 1035	B1	GT82	Yes	Yes
14	K2762; A0465; 2000031651 RA3	B2	GT80	Yes	Yes
15	Clade C Wild type; 2002013094	C	GT133	Yes	Yes
16	K5135; A0463; 2000031648 PAK-1	[a]	[a]	Yes	Yes

[a]Strain K5135 received as GT29, but the allele size for vrrC1 does not match (should be 520 but is an unusual size of 548).

Table 2. List of Clinically Relevant Background Organisms (Route of infection – Red color cell)

#	Clinical Panel Microorganism	Hemolysis	Respiratory	Gastro-intestinal	Sepsis	Skin / Wound
1	*Acinetobacter baumanii* ATCC 19606, 2208	α	Red	Red	Red	Red
2	*Acinetobacter calcoaceticus* ATCC 14987	α	Red	Green	Green	Green
3	*Aeromonas hydrophila* ATCC 7966	α	Green	Red	Red	Red
4	*Bacillus fusiformis* DSN no:493	α	Green	Green	Green	Red
5	*Citrobacter braakii* ATCC 10053	α	Green	Green	Red	Green
6	*Citrobacter koseri*	α	Green	Green	Green	Red
7	*Citrobacter youngae*	α	Green	Green	Green	Red
8	*Enterobacter aerogenes*	α	Green	Green	Green	Red
9	*Escherichia coli* 0157:H7 ATCC 43895:CDC EDL 933	α	Green	Red	Red	Green
10	*Escherichia coli* ETEC ATCC 35401	α	Green	Red	Green	Green
11	*Escherichia coli* STEC ATCC MP-9, Serogroup O103:H11 ATCC BAA-2215	α	Green	Red	Green	Green
12	*Escherichia coli* STEC ATCC MP-9, Serogroup O111 ATCC BAA-2440	α	Green	Red	Green	Green
13	*Escherichia coli* STEC ATCC MP-9, Serogroup O121:H19 ATCC BAA-2219	α	Green	Red	Green	Green
14	*Escherichia coli* STEC ATCC MP-9, Serogroup O145 ATCC BAA-2192	α	Green	Red	Green	Green
15	*Escherichia coli* STEC ATCC MP-9, Serogroup O26:H11 ATCC BAA-2196	α	Green	Red	Green	Green
16	*Escherichia coli* STEC ATCC MP-9, Serogroup O45:H2 ATCC BAA-2193	α	Green	Red	Green	Green
17	*Klebsiella oxytoca*	α	Green	Green	Green	Red
18	*Klebsiella pneumoniae* ATCC 10031; FDA PCI 602; CDC 401-68	α	Red	Green	Red	Red
19	*Morganella morganii* ATCC 49993	α	Green	Green	Red	Green
20	*Plesiomonas shigelloides* ATCC 14029	α	Green	Red	Green	Red
21	*Proteus mirabilis* ATCC 29906; CDC PR14	α	Red	Green	Red	Red
22	*Providencia rattgeri*	α	Green	Green	Green	Red
23	*Providencia stuartii* ATCC 25825	α	Green	Green	Red	Green
24	*Pseudomonas aeruginosa* ATCC 15442	α	Red	Red	Red	Red
25	*Pseudomonas stutzeri*	α	Green	Green	Green	Red
26	*Salmonella dublin* ATCC 15480	α	Green	Green	Red	Green
27	*Salmonella enterica* subsp. enterica (serotype Enteritidis) ATCC 4931	α	Green	Red	Red	Green
28	*Serratia marcescens* ATCC 13880	α	Red	Green	Red	Green
29	*Stenotrophomonas maltophilia* ATCC 13637; NCIMB 9203	α	Red	Green	Red	Red
30	*Streptococcus pneumonia* ATCCC 6301; BCRC 10794; CNCTC 5810	α	Red	Green	Red	Green
31	*Streptococcus viridans* ATCC BAA-1455	α	Green	Green	Red	Green
32	*Vibrio cholera ATCC* 14104	α	Green	Red	Green	Green
33	*Vibrio vulnificus* ATCC 29307	α	Green	Green	Red	Red
34	*Bacillus cereus* 172560W; UK-04	β	Green	Red	Green	Green
35	*Bacillus cereus* ATCC 4342; BACI083; NRS 731	β	Green	Red	Green	Green
36	*Bacillus cereus* E33L/ZK	β	Green	Red	Green	Green
37	*Bacillus cereu* s FRI-13; D17	β	Green	Red	Green	Green
38	*Bacillus cereus* FRI-41; 3A; BACI228	β	Green	Red	Green	Green
39	*Enterobacter cloacae* ATCC 10699	β	Red	Green	Red	Green
40	*Streptococcus pyogenes* ATCC 8133; group a, type 23	β	Red	Green	Red	Red
41	*Vibrio mimicus*	β	Green	Red	Green	Green

(*continued*)

Table 2. (Continued)

#	Clinical Panel Microorganism	Hemolysis	Respiratory	Gastro-intestinal	Sepsis	Skin / Wound
42	*Achromobacter* spp.	γ				
43	*Acinetobacter lwoffii*	γ				
44	*Argobacter radiobacter*	γ				
45	*Bartonella bacilliformis* ATCC 35685C5	γ				
46	*Bordetella bronchiseptica* ATCC 19395	γ				
47	*Bordetella parapertussis* ATCC 15311	γ				
48	*Brevibacterium linens* ATCC 9172	γ				
49	*Brucella abortus* 544 ATCC 23444 (2008724321)	γ				
50	*Brucella melitensis* 16M ATCC 23456 (2011756247)	γ				
51	*Brucella suis* 1330 ATCC 23444 (2008724321)	γ				
52	*Burkholderia mallei* ATCC 23344	γ				
53	*Burkholderia pseudomallei* ATCC 11668	γ				
54	*Citrobacter freundii*	γ				
55	*Corynebacterium diphtheria* ATCC 13812	γ				
56	*Corynebacterium jeikeium* ATCC 43734	γ				
57	*Enterobacter durans*	γ				
58	*Enterobacter raffinosus*	γ				
59	*Enterococcus faecalis* ATCC 10100	γ				
60	*Enterococcus faecium* ATCC 349	γ				
61	*Erysipelothrix rhusiopathiae* ATCC 35427	γ				
62	*Escherichia coli* ATCC 35150	γ				
63	*Francisella tularensis* LVS	γ				
64	*Klebsiella rhinoscleromatis* ATCC 6908	γ				
65	*Lactobacillus* spp	γ				
66	*Listeria monocytogenes* ATCC 7302; BCRC 15329	γ				
67	*Moraxella catarrhalis* ATCC 8176	γ				
68	*Nocardia asteroides* ATCC 9504	γ				
69	*Pantoea agglomerans*	γ				
70	*Pasteurella multocida* ATCC 6533; ATCC 9658	γ				
71	*Salmonella cholerasius* ATCC 13312	γ				
72	*Salmonella typhimurium* ATCC 14028	γ				
73	*Salmonella virchow* ATCC 51955	γ				
74	*Shigella sonnei* ATCC 9290	γ				
75	*Staphylococcus aureus* ATCC 700699; CIP 106414; Mu 50, MRSA	γ				
76	*Staphylococcus epidermidis* ATCC 14990	γ				
77	*Streptococcus agalactiae* ATCC 624	γ				
78	*Streptococcus dysgalactiae* ATCC 9926	γ				
79	*Yersinia enterocolitica* ATCC 23715	γ				
80	*Yersinia pestis* CO99-3015	γ				
81	*Yersinia pseudotuberculosis* ATCC 13979	γ				

Table 3. Informational Panel Organisms (Route of infection – Red color cell)

#	Informational Panel Microorganism	Hemolysis	Respiratory	Gastro-intestinal	Sepsis	Skin / Wound
1	***Bacillus cereus biovar Anthracis* CA**	γ				
2	***Bacillus cereus biovar Anthracis* CI**	γ				

selected and streaked onto a new SBA plate. On the day of testing, the medium surrounding the colonies was examined. Cultures showing a darkening or discoloration of the medium surrounding the colonies demonstrated alpha-hemolysis. Cultures showing clear halos around and under the colonies exhibited beta-hemolysis. An isolated colony measuring 2 to 4 mm in diameter was removed with a sterile inoculating loop and transferred to a vial containing 200 μL colony isolation buffer. Vials were vortexed for 5 seconds to suspend and evenly distribute the cells in the buffer and allowed to stand at room temperature for at least 2 minutes prior to testing. The vials were gently vortexed, and 150 μL of cell suspension was added to the sample well of each strip. Strips were read visually 15 to 30 minutes following sample addition. Strips on which the control line did not appear were discarded. Each organism was tested by 4 different operators and visual observations recorded.

Phase 3: Informational Panel

Bacillus cereus biovar *anthracis* strains CA and CI were obtained from the Center for Biological Threats and Special Pathogens, Robert Koch Institute, Berlin Germany.[35] Strains were grown and suspensions prepared for testing as described above for the inclusivity panel (Table 3).

Biosafety Considerations

The virulent *B. anthracis* and *B. cereus* biovar *anthracis* strains used in this study were handled with appropriate biosafety conditions at the CDC according to institutional biosafety guidelines. Virulent *B. anthracis* strains were handled in a BSL-3 laboratory. All other organisms, including low-risk bacterial strains, were handled, processed, and tested under safety protocols in accordance with *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*.[36] To minimize risk of aerosols, cultures were handled using BSL-2 practices that also required personal protective equipment and procedures such as gowning, use of gloves, protective eyewear, and working in a certified Class II biosafety cabinet (BSC). All work areas before and after the testing were cleansed with 10% bleach, while disposal of stock cultures or biomedical waste was done in accordance with institutional guidelines.

Statistical Analysis

The performance of the lateral flow assay was assessed by calculating its sensitivity, specificity, and accuracy based on the results from all the testing done in this study. Sensitivity is defined as the proportion of true positives that are correctly identified by the test and is calculated as:

$$Sensitivity = 100 \times \frac{True\ Positive}{False\ Negative + True\ Positive}$$

Specificity is defined as the proportion of true negatives that are correctly identified by the test and is calculated as:

$$Specificity = 100 \times \frac{True\ Negative}{False\ Positive + True\ Negative}$$

Accuracy is defined as the proportion of true positives and true negatives correctly identified by the test, the overall probability that the test correctly classifies the presence of the analyte (*Bacillus anthracis* in this instance), and is calculated as:

$$Accuracy = 100 \times \frac{True\ Positive + True\ Negative}{True\ Positive + False\ Negative + True\ Negative + False\ Positive}$$

Sensitivity, specificity, and accuracy from the visual results of the lateral flow assay were calculated using MedCalc Statistical Software version 18.11.3 (MedCalc Software bvba, Ostend, Belgium; http://www. medcalc.org; 2019).

Results

RedLine Alert test results of all 3 phases of testing—Phase 1, inclusivity panel; Phase 2, clinical panel; and Phase 3, informational panel—microorganisms are shown in Table 4. Number of samples tested as positive controls and negative controls in each phase are also given in the table.

Phase 1: Inclusivity Panel Testing

Four replicates of 16 geographically diverse *B. anthracis* strains that comprised the inclusivity panel were tested. A

Table 4. RedLine Alert test results of 3 phase testing comprising *B. anthracis* Phase 1 inclusivity panel, Phase 2 clinical panel, and Phase 3 informational panel microorganisms

Description	*No. of Samples Tested*	*No. of Positive Controls Tested*	*No. of Negative Controls Tested*	*Total*
Phase 1: Inclusivity panel testing	64	16	48	128
Phase 2: Clinical panel testing	324	20	44	388
Phase 3: Informational panel testing	8	4	4	16
Total	396	40	96	532

combined total of 64 tests were performed in this phase. *B. anthracis* Sterne strain was used as a positive control, and the manufacturer-supplied CIB was used as a negative control. Forty-eight negative controls and 16 positive controls were also tested in Phase 1 in addition to the inclusivity panel organism testing. All samples containing *B. anthracis* produced positive visual results; no reactivity was observed for any of the negative controls.

Phase 2: Clinical Panel Testing

The clinical panel used in the current study consisted of 40 (49.4%) γ-hemolytic organisms, 8 (9.9%) β-hemolytic microorganisms, and 33 (40.7%) α-hemolytic organisms (Table 2). As in Phase 1 testing, a combined total of 324 tests were performed in Phase 2 along with 44 negative controls and 20 positive controls.

Testing of the clinical panel by 4 operators yielded negative results for 80 of 81 (98.76%) organisms shown in Table 2 by visual inspection. The only positive result occurred with a β-hemolytic *Bacillus cereus* strain. When evaluating the assay using data from all organisms regardless of hemolytic activity, the sensitivity of the RedLine Alert assay was 100% and its specificity was 98.76% (Table 5). The sensitivity and specificity of the assay using results from only the nonhemolytic organisms in the clinical panel (40 out of 81) were 100% and 100%, respectively (Table 6). The data show that the assay is robust and does not cross-react with other clinically relevant organisms tested based on the testing algorithm. Nevertheless, the manufacturer's product disclosure notes that the RedLine Alert test may cross-react with *B. cereus* and *B. thuringiensis;* however, these organisms are β-hemolytic, so the predefined algorithm would exclude them for testing.

Phase 3: Informational Panel Testing

B. cereus biovar *anthracis* is an emerging pathogen that has caused fatal anthrax-like infections in nonhuman primates such as chimpanzees and gorillas in West Africa.[35] Human infections with this organism have not been reported but cannot be ruled out due to the paucity of clinical laboratories in areas where this organism has been found. These organisms possess some phenotypic properties of *B. anthracis* (γ-hemolytic, colony morphology), which may make them difficult to identify in a clinical laboratory. However, *B. cereus* biovar *anthracis* is motile, which would rule out *B. anthracis* prior to testing with the RedLine Alert assay. In a study limited by the availability of strains, *B. cereus* biovar *anthracis* strains CA and CI were tested 4 times each; 4 replicates of negative controls and 4 replicates of positive controls were also tested during the informational panel testing. Strain CA gave negative results, but strain CI gave positive results in the RedLine Alert test. Results of the informational panel testing were not included in the sensitivity, specificity, and accuracy calculations shown in Table 5. While these results are interesting, additional strains need to be tested before we can state with certainty that this organism can occasionally produce false-positive test results if the manufacturer's algorithm is not followed.

Analytical Sensitivity, Specificity, and Accuracy

Calculation of the analytical sensitivity, specificity, and accuracy of the RedLine Alert test is shown in Table 5 using the results from testing organisms in the inclusivity panel and the clinical panel; Table 6 uses the results from testing of clinically relevant nonhemolytic organisms (40) from the clinical panel and the inclusivity panel. Results of positive

Table 5. 2 x 2 Contingency table and statistical analysis assessing the accuracy of RedLine Alert Assay based on the data from Phase 1 testing and all clinical panel microorganisms in Phase 2

Result	*Anthrax Positive*	*Anthrax Negative*	*Total*
Red Line Alert Test positive	64	0	64
Red Line Alert Test negative	4	320	324
Total	68	320	388

Parameter	*Percentage*	*Confidence Interval*
Sensitivity	100.00%	94.40% to 100.00%
Specificity	98.77%	96.87% to 99.66%
Accuracy	98.97%	97.38% to 99.72%
Area under the curve	0.99	(0.99 to 1.00)
Negative predictive value	100.00%	—

Table 6. 2 x 2 Contingency table and statistical analysis assessing the accuracy of RedLine Alert assay based on the data from Phase 1 testing and Phase 2 testing of only nonhemolytic strains

Result	*Anthrax Positive*	*Anthrax Negative*	*Total*
Red Line Alert Test positive	64	0	64
Red Line Alert Test negative	0	160	160
Total	64	160	224
Parameter	*Percentage*	*Confidence Interval*	
Sensitivity	100.00%	94.40% to 100.000%	
Specificity	100.00%	97.66% to 100.000%	
Accuracy	100.00%	98.34% to 100.00%	
Area under the curve	1.00	(0.98 to 1.00)	
Negative predictive value	100.00%	—	

controls and negative controls tested in each phase were not included in the statistical analysis and calculations.

Of the 388 RedLine Alert tests performed in this study, 64 tests were positive (ie, "*B. anthracis* present in sample"), while 320 were negative (ie, "*B. anthracis* absent from sample"), and 1 negative sample *"Bacillus cereus"* yielded a false-positive result which equates to 4 tests. Based on these data, the assay sensitivity was calculated to be 100% and specificity at 98.76%, with an area under the curve value of 0.99. Since the test is FDA cleared for use in a clinical setting for testing unidentified γ-hemolytic bacterial colonies for presence of *B. anthracis*, analysis using data from only the γ-hemolytic organisms produced a sensitivity and specificity of 100% and 100%, respectively, with an area under the curve value of 1.

Discussion

B. anthracis is high on the list of potential bioterrorism agents because of its ease of dissemination, stability and hardiness of the spore in the environment, high morbidity

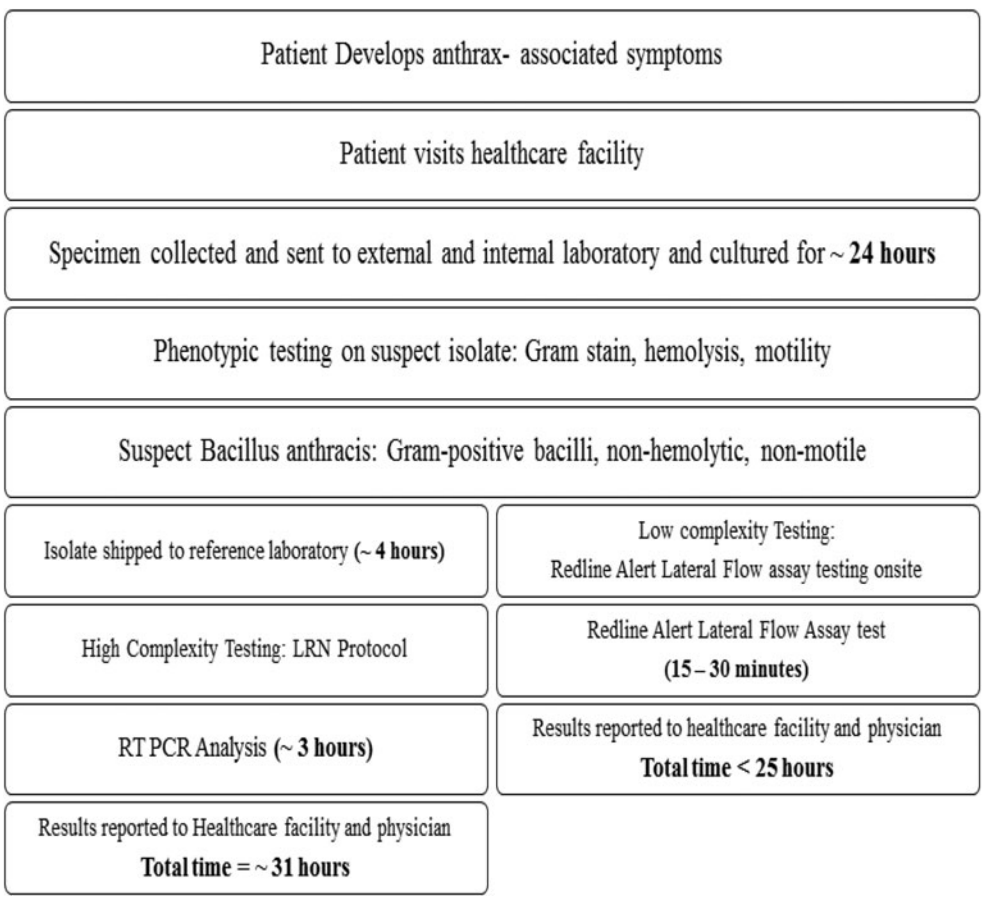

Figure 1. Concept of operation for use of *RedLine Alert*™ test

and mortality rate, and past development as a biowarfare agent. One key to saving lives is rapid diagnosis and clinical intervention with appropriate antibiotics. The current Level A protocol used in clinical laboratories in the United States involves the identification of gram-positive, rod-shaped bacteria, which grow as γ-hemolytic (nonhemolytic) colonies on SBA plates, produce catalase, and are nonmotile.[25] However, there are some drawbacks to relying solely on these methods. Laboratory personnel require additional training and certification to accurately identify these uncommon agents, using the appropriate biosafety practices, and to package and transfer the agents to a nearby LRN laboratory for secondary analysis and confirmation.[24] The ability to rapidly identify *B. anthracis* in a clinical laboratory will greatly benefit the workflow and support timely therapeutic decisions.

The results of this extensive evaluation led us to conclude that the FDA-cleared RedLine Alert is a highly robust, sensitive, and specific test for the presumptive identification of *B. anthracis* from γ-hemolytic (nonhemolytic) colonies on SBA. It is important to emphasize that a motility test should be performed to differentiate *B. anthracis* (nonmotile) from *B. cereus* biovar *anthracis* (motile), as some strains of the latter organism may give a positive result with the RedLine Alert test.

The diagnostic methods used for the presumptive identification of *B. anthracis* by Level A LRN laboratories include culture on 5% SBA plates to determine colony morphology and gram stain characteristics, hemolysis (*B. anthracis* is γ-hemolytic), motility (*B. anthracis* is nonmotile), and microscopic observation for spores.[37] The RedLine Alert test can reduce the time required for a presumptive identification of *B. anthracis* in the absence of a confirmed outbreak or biological attack. During an outbreak or biological attack, the assay can be used to confirm the presence of *B. anthracis* in order that appropriate antibiotic treatment can be started as quickly as possible.

This test may also support timely diagnosis in resource-limited endemic regions where there is a high prevalence of anthrax. For use in a low-prevalence region such as the United States, this test can serve as an important diagnostic tool in the event of an outbreak or biological attack. The 2001 anthrax incident in the United States prompted an extensive review of how clinical and public health laboratories should respond to bioterrorism. The LRN, which is capable of detecting, confirming, and reporting potential bioterrorism agents, was established prior to 2001.[24] The LRN consists of 3 tiers of laboratories: (1) sentinel laboratories (Level A), which use Level A protocols developed by the American Society for Microbiology (ASM)—these laboratories generally function as a first line of defense for detecting possible outbreaks or infections caused by biothreat agents and alerting state and federal agencies;[25] (2) reference laboratories, which are generally public health laboratories that perform confirmatory tests to produce high-confidence test results for threat analysis and intervention by public health and public safety authorities; and (3) national laboratories (eg, CDC) for definitive characterization.[24] Figure 1 indicates that up to 31 hours may elapse between the time a symptomatic patient arrives at a clinic, an initial diagnosis is made, and presumptive and confirmatory testing are completed. Therefore, it is vital that a simple, rapid method be incorporated to rule in the presence of *B. anthracis* in a clinical sample as quickly as possible so that proper treatment can be started. Equally important, the 100% Negative Predictive Value (NPV, Table 6) of the RedLine Alert assay suggests that it can be used to rapidly rule out γ-hemolytic colonies of gram-positive bacilli as potential *B. anthracis.*

The RedLine Alert kit is a very simple, easy-to-use, objective test for the presumptive identification of *B. anthracis*. This test can reduce the diagnostic time by several hours. Additionally, the RedLine Alert lateral flow assay has been approved by the FDA since 2003 for identifying *B. anthracis* in specimens from symptomatic patients cultured on SBA. However, it is critical that non–select agent registered laboratories (such as most hospital and commercial labs) promptly notify the CDC Select Agent Program and forward all samples and specimens to the closest LRN laboratory for further characterization to avoid violating the select agent regulation prohibiting possession of a select agent by an unregistered laboratory. Once testing has been completed and cultures/specimens forwarded to an LRN laboratory, remaining samples associated with the patient should be destroyed by the unregistered laboratory using appropriate methods within the time period stated in the select agent regulations.

Acknowledgments

This work was funded by US Department of Homeland Security Science and Technology Directorate Contract #HSHQDC-12-C-00071. The article reflects the views of the authors and should not be construed to represent the views or policies of DHS or HHS.

References

1. Bouzianas DG. Medical countermeasures to protect humans from anthrax bioterrorism. *Trends Microbiol* 2009;17(11):522-528.
2. Brossier F, Mock M. Toxins of *Bacillus anthracis. Toxicon* 2001;39(11):1747-1755.
3. Jamie WE. Anthrax: diagnosis, treatment, prevention. *Prim Care Update Ob Gyns* 2002;9:117-121.
4. Koehler TM. *Bacillus anthracis* physiology and genetics. *Mol Aspects Med* 2009;30(6):386-396.
5. Helgason E, Okstad OA, Caugant DA, et al. *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl Environ Microbiol* 2000;66(6):2627-2630.
6. Pilo P, Frey J. *Bacillus anthracis:* molecular taxonomy, population genetics, phylogeny and patho-evolution. *Infect Genet Evol* 2011;11(6):1218-1224.

7. Dragon DC, Rennie RP. The ecology of anthrax spores: tough but not invincible. *Can Vet J* 1995;36(5):295-301.
8. Fraise AP, Maillard JY, Sattar S, eds. *Russell, Hugo and Ayliffe's Principles and Practice of Disinfection, Preservation and Sterilization.* 5th ed. Wiley-Blackwell; 2012.
9. Balali-Mood M, Moshiri M, Etemad L. Medical aspects of bio-terrorism. *Toxicon* 2013;69:131-142.
10. Cavallo JD, Ramisse F, Girardet M, Vaissaire J, Mock M, Hernandez E. Antibiotic susceptibilities of 96 isolates of *Bacillus anthracis* isolated in France between 1994 and 2000. *Antimicrob Agents Chemother* 2002;46(7):2307-2309.
11. Cote CK, Welkos SL, Bozue J. Key aspects of the molecular and cellular basis of inhalational anthrax. *Microbes Infect* 2011;13(14-15):1146-1155.
12. Beatty ME, Ashford DA, Griffin PM, Tauxe RV, Sobel J. Gastrointestinal anthrax: review of the literature. *Arch Intern Med* 2003;163(20):2527-2531.
13. Centers for Disease Control and Prevention (CDC). Update: investigation of bioterrorism-related anthrax, 2001. *MMWR Morb Mortal Wkly Rep* 2001;50(45):1008-1010.
14. Friedlander AM, Welkos SL, Pitt ML, et al. Postexposure prophylaxis against experimental inhalation anthrax. *J Infect Dis* 1993;167(5):1239-1243.
15. Hanna PC, Ireland JA. Understanding *Bacillus anthracis* pathogenesis. *Trends Microbiol* 1999;7(5):180-182.
16. Holty JE, Bravata DM, Liu H, Olshen RA, McDonald KM, Owens DK. Systematic review: a century of inhalational anthrax cases from 1900 to 2005. *Ann Intern Med* 2006; 144(4):270-280.
17. Hanczaruk M, Reischl U, Holzmann T, et al. Injectional anthrax in heroin users, Europe, 2000-2012. *Emerg Infect Dis* 2014;20(2):322-323.
18. Price EP, Seymour ML, Sarovich DS, et al. Molecular epidemiologic investigation of an anthrax outbreak among heroin users, Europe. *Emerg Infect Dis* 2012;18(8):1307-1313.
19. Rao SS, Mohan KV, Atreya CD. Detection technologies for *Bacillus anthracis:* prospects and challenges. *J Microbiol Methods* 2010;82(1):1-10.
20. Inglesby TV, O'Toole T, Henderson DA, et al. Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA* 2002;287(17):2236-2252.
21. Morse SA, Kellogg RB, Perry S, et al. Detecting biothreat agents: the Laboratory Response Network. *ASM News* 2003; 69(9):433-437.
22. Tatti KM, Greer P, White E, et al. Morphologic, immunologic, and molecular methods to detect *Bacillus anthracis* in formalin-fixed tissues. *Appl Immunohistochem Mol Morphol* 2006;14(2):234-243.
23. Hoffmaster AR, Fitzgerald CC, Ribot E, Mayer LW, Popovic T. Molecular subtyping of *Bacillus anthracis* and the 2001 bioterrorism-associated anthrax outbreak, United States. *Emerg Infect Dis* 2002;8(10):1111-1116.
24. Wagar E. Bioterrorism and the role of the clinical microbiology laboratory. *Clin Microbiol Rev* 2016;29(1):175-189.
25. Buchan BW, Mahlen SD, Relich RF. *Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases.* Washington, DC: American Society for Microbiology; 2018. https://www.asm.org/ASM/media/Policy-and-Advocacy/Biosafety_Sentinel_Guideline_October_2018_FINAL.pdf. Accessed June 12, 2019.
26. Gubala V, Harris LF, Ricco AJ, Tan MX, Williams DE. Point of care diagnostics: status and future. *Anal Chem* 2012; 84(2):487-515.
27. Andreotti PE, Ludwig GV, Peruski AH, Tuite JJ, Morse SS, Peruski LF Jr. Immunoassay of infectious agents. *Biotechniques* 2003;35(4):850-859.
28. Townsend MB, MacNeil A, Reynolds MG, et al. Evaluation of the Tetracore Orthopox BioThreat® antigen detection assay using laboratory grown orthopoxviruses and rash illness clinical specimens. *J Virol Methods* 2013;187(1):37-42.
29. Hodge DR, Prentice KW, Ramage JG, et al. Comprehensive laboratory evaluation of a highly specific lateral flow assay for the presumptive identification of ricin in suspicious white powders and environmental samples. *Biosecur Bioterror* 2013; 11(4):237-250.
30. Ramage JG, Prentice KW, Morse SA, et al. Comprehensive laboratory evaluation of a specific lateral flow assay for the presumptive identification of abrin in suspicious white powders and environmental samples. *Biosecur Bioterror* 2014; 12(1):49-62.
31. Ramage JG, Prentice KW, DePalma L, et al. Comprehensive laboratory evaluation of a highly specific lateral flow assay for the presumptive identification of *Bacillus anthracis* spores in suspicious white powders and environmental samples. *Health Secur* 2016;14(5):351-365.
32. Tomaso H, Thullier P, Seibold E, et al. Comparison of hand-held test kits, immunofluorescence microscopy, enzyme-linked immunosorbent assay, and flow cytometric analysis for rapid presumptive identification of *Yersinia pestis. J Clin Microbiol* 2007;45(10):3404-3407.
33. Mangold BL, Aldrich JL. Spore Specific Antibodies. Tetracore, United States patent 7,772,374 B2. 2010.
34. Cohen MN, Robison RA, Venkateswaran N, et al. Evaluation of a lateral flow assay kit for the detection of *Bacillus anthracis* and its application in laboratory diagnosis of anthrax. Bacillus ACT Annual meeting; 2015; New Delhi, India.
35. Klee SR, Ozel M, Appel B, et al. Characterization of *Bacillus anthracis*-like bacteria isolated from wild great apes from Cote d'Ivoire and Cameroon. *J Bacteriol* 2006;188(15):5333-5344.
36. Chosewood LC, Wilson DE, eds. *Biosafety in Microbiological and Biomedical Laboratories.* 5th ed. Washington, DC: US Department of Health and Human Services; 2009. https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF. Accessed June 12, 2019.
37. Centers for Disease Control and Prevention. Anthrax. Reviewed January 31, 2017. http://www.cdc.gov/anthrax. Accessed June 12, 2019.

Manuscript received March 21, 2019;
revision returned May 17, 2019;
accepted for publication May 17, 2019.

Address correspondence to:
Segaran P. Pillai, PhD
Director, Office of Laboratory Science and Safety
FDA Office of the Commissioner
Department of Health and Human Services
Silver Spring, MD

Email: Segaran.Pillai@FDA.HHS.GOV