

An FDA Cleared In vitro Diagnostic Real-Time PCR System for Detection of Bacillus anthracis

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ABSTRACT

Background: Recent history has highlighted the need for rapid, reliable methods for identifying *Bacillus anthracis*. The Joint Biological Agent Identification and Diagnostic System (JBAIDS) Anthrax Detection Kit is a real-time polymerase chain reaction (PCR) based test that has been cleared by the Food and Drug Administration (FDA) for presumptive identification of *B. anthracis*. **Methods:** The system is composed of the JBAIDS Instrument and two freeze-dried assays for detecting gene sequences on the pXO1 (Target 1) and pXO2 (Target 2) plasmids. The system can be used to test whole blood, colonies, and positive blood cultures. Each JBAIDS run requires a positive and negative control. An inhibition control (IC) is also included for each patient sample. Analytic studies were conducted to establish the limit of detection (LOD), analytic sensitivity and specificity, and reproducibility of the system. A multicenter clinical trial was also performed. **Results:** LOD for whole blood samples is 1000 colony forming units (CFU)/mL (67/68; 98% tested positive with both assays). Analytic sensitivity is 100% with all virulent *B. anthracis* isolates (34/34) testing positive with both assays. Analytic specificity was 100% (42/42) for unrelated organisms and 94% (47/50) for phylogenetically related organisms. The three discrepant results occurred with *B. cereus* isolates associated with anthrax-like illnesses. Reproducibility for whole blood samples was examined by testing a 10-sample panel (2 negatives, 3 weak positives, 3 moderate positives and 2 strong positives) at 4 different sites on 3 different days, yielding 100% (120/120) concordance with the expected results. In the clinical trial, all 150 whole blood samples collected from patients having symptoms consistent with anthrax tested negative for *B. anthracis* by both standard blood culture and the JBAIDS system. Clinical sensitivity could not be established due to the absence of anthrax infections. **Conclusions:** The JBAIDS Anthrax Detection System provides reliable test results in a few hours and is a useful adjunct to standard identification methods.

INTRODUCTION

The JBAIDS Anthrax Detection System is the first PCR-based test to be cleared by the FDA for the presumptive identification of *B. anthracis* in clinical samples. The system is composed of the JBAIDS Instrument with laptop computer, JBAIDS software, and two different freeze-dried reagent assays (in one kit) for the detection of sequences on the pXO1 plasmid (Target 1) and the pXO2 plasmid (Target 2). The JBAIDS Instrument is a ruggedized real-time thermocycler that has been designed for use by the Department of Defense for pathogen detection in both environmental and clinical samples. The JBAIDS software uses a diagnostic wizard to provide a separate and clearly defined workflow for *in vitro* diagnostic (IVD) assays. To reduce the possibility of false positive results caused by amplicon contamination, all samples are initially tested using the *B. anthracis* Target 1 assay. The Target 2 assay is reserved for confirmation of samples with a positive Target 1 result.

METHODS

The system can be used to test positive blood cultures, organisms cultured on blood agar, and human whole blood collected from individuals suspected of having anthrax. The testing process starts by isolating DNA from patient samples or cultures using the Idaho Technology IT 1-2-3™ FLOW Sample Purification Kit for whole blood and the Idaho Technology IT 1-2-3™ SWIPE Sample Purification Kit for blood culture and direct culture samples. Both sample purification kits use mechanical lysis (bead beating) followed by purification of nucleic acids using a silica-based spin filter. Each purified sample is used to reconstitute an Unknown and Inhibition Control (IC) vial. Positive (PC) and Negative (NC) Controls are included with every JBAIDS run. Results for a typical negative patient sample are shown in Figure 1. Failure of the PC or NC results in "Invalid" test results for all samples in the JBAIDS run. Failure of the IC to demonstrate robust amplification results in an "Inhibited" result for the associated sample.

In support of our 510(k) application for FDA clearance, the following performance characteristics of the JBAIDS Anthrax Detection System were determined in analytic testing and in a multicenter clinical trial. Results of the key analytic studies are presented here.

- Whole Blood Limit of Detection (LOD)
- Validation of the Direct Culture and Blood Culture techniques
- Assay Sensitivity, or the ability to detect diverse strains or isolates of *B. anthracis*
- Assay Specificity, or the ability to correctly exclude non-target organisms, including phylogenetically related organisms and organisms that are likely to be found in clinical samples
- System reproducibility
- Carry-over or cross-contamination
- Evaluation of potentially interfering substances
- Sample Transport and Storage Conditions, both before and after sample purification

RESULTS

Whole Blood LOD

Sixty-seven (67) out of 68 whole blood samples, spiked at 1000 CFU/mL using live vegetative *B. anthracis*, were positive for both assays resulting in an analytic sensitivity of 98% (95% CI 93 – 100). The LOD was not determined for *B. anthracis* spores as spores would not be present in clinical samples. To estimate the number of target copies per PCR reaction, a standard curve was constructed by plotting the crossing point (Cp) values obtained when testing known concentrations of cloned Target 1 or Target 2 amplicon. The Cp values obtained in the organism LOD study were plotted on the standard curve resulting in an estimated target copy LOD of 48 copies/reaction for Target 1 and 12 copies/reaction for Target 2 (see Table 1).

Direct Culture and Blood Culture Validation Studies

LOD assessments were not performed for direct culture or blood culture due to the high concentration of organisms found in this sample types. The methods were validated by testing replicates of *B. anthracis* grown on blood agar plates or in blood culture media. For blood culture samples, live *B. anthracis* was spiked into blood culture media (containing human whole blood) and the samples were tested when the blood culture instrument indicated bacterial growth. The concentration of *B. anthracis* in a positive blood culture was determined by performing viable plate counts for 10 blood cultures that had been positive for less than one hour. The average CFU/mL of blood culture media was 1.5 x 10⁷ with a range of 1.0 x 10⁷ to 3.2 x 10⁷. All direct culture and blood culture samples tested positive for both assays. The Cp values and estimated target copies per PCR reactions are shown in Table 1.

Table 1 – JBAIDS Cp Values and Estimated Target Copy Number per PCR Reaction for Different Sample Types

Sample Type	Target 1			Target 2		
	Average Cp (SD)	Range of Cp values	Estimated average target copy per PCR reaction	Average Cp (SD)	Range of Cp values	Estimated average target copy per PCR reaction
Whole blood specimens spiked with live <i>B. anthracis</i> at assay LOD levels (1000 CFU/mL)	34.2 (1.0) n= 136	31.8 - 38.5	48	34.9 (1.0) n= 136	32.5 – 37.7	12
<i>B. anthracis</i> positive blood culture samples (Average 1.5 x 10 ⁷ CFU/mL of blood culture media)	16.7 (0.9) n= 154	15.1 - 19.1	10 ⁶	17.0 (0.8) n= 154	16.0 – 19.4	10 ⁶
<i>B. anthracis</i> direct culture samples	20.3 (4.2) n=206	15.2 - 31.0	10 ⁶	21.4 (4.2) n=150	16.2 – 31.0	10 ⁵

N= the number of individual PCR reactions evaluated.
* These values are estimated based on extrapolation of a standard curve ending at 1000 copies/reaction.

Assay Sensitivity and Specificity

Sensitivity testing was performed using three panels containing 34 different strains of virulent *B. anthracis* representing the known genetic and geographic diversity of *B. anthracis*. As shown in Table 2, all virulent strains of *B. anthracis* were positive with both the Target 1 and Target 2 assays, however *B. anthracis* Vollum 1b was only detected when tested at a high concentration (approximately 200 times the assay's LOD). For specificity, the three panels contained 50 phylogenetically related organisms as well as 42 organisms that are likely to be encountered in clinical samples (e.g., organisms associated with pneumonia and sepsis). All (42/42) unrelated organisms tested negative for both the Target 1 and Target 2 assays and 94% (47/50) of the phylogenetically related organisms were also negative. The three unexpected findings were *B. cereus* 03BB102, which tested positive for both *B. anthracis* Target 1 and Target 2, and *B. cereus* 03BB87 and G9241, both of which tested positive with the Target 1 assay. These results were not completely unanticipated as all three of these *B. cereus* organisms have been associated with anthrax-like illnesses and death.^{1,2} Follow-up testing yielded results that were in agreement with the JBAIDS results indicating the presence of Target 1 and Target 2 sequences in these strains.

Table 2 – Testing Panel Composition and Number of Correct JBAIDS Results

Evaluation	Organism Type	DNA Panel (# Correct/# tested)	Colony Panel (# Correct/# tested)	Blood Culture Panel (# Correct/# tested)	Total # of Unique Stains Tested (# Correct/# tested)
Assay sensitivity	Virulent <i>B. anthracis</i>	11 /11	23/23	11/11	34/34 – 100%
Assay specificity	Phylogenetically related organisms (nearest neighbors)	25/25	22/25	2/2	47/50 – 94%
	Clinically relevant unrelated organisms	30/30	11/11	10/10	42/42 – 100%

Table 3 – Between-Site Reproducibility Results for Whole Blood and Blood Culture *B. anthracis* Target 1 and Target 2 Assays

Matrix	Sample ID	Target 1					Target 2				
		Site 1	Site 2	Site 3	Site IT	All	Site 1	Site 2	Site 3	Site IT	All
Whole Blood	Matrix blank	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Matrix blank	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Low	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Low	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Low	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Medium	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Medium	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	Medium	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	High	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	High	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
Total Agreement for Target 1					120/120	Total Agreement for Target 2					120/120
Blood Culture	Matrix blank	2/3	3/3	3/3	3/3	11/12	3/3	3/3	3/3	3/3	12/12
	Low	3/3	3/3	3/3	3/3	12/12	3/3	3/3	3/3	3/3	12/12
	High	2/3	3/3	3/3	3/3	11/12	3/3	3/3	3/3	3/3	12/12
	Total Agreement for Target 1					34/36	Total Agreement for Target 2				

Table 4 – Between-Site Reproducibility Results for Direct Culture *B. anthracis* Target 1 and 2 Assay

Organism Tested	Target 1			Target 2		
	Site 1	Site 2	Between Site	Site 1	Site 2	Between Site
<i>B. anthracis</i> GT 3	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 10	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 23	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 29	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 30	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 35	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 41	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 51	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 62	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 77	3/3	3/3	6/6	3/3	3/3	6/6
<i>B. anthracis</i> GT 85	3/3	3/3	6/6	3/3	3/3	6/6
<i>H. influenzae</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>S. aureus</i>	3/3	2/3	5/6	3/3	2/3	5/6
<i>S. pneumoniae</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>K. pneumoniae</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>E. coli</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>A. calcoaceticus</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>P. vulgaris</i>	3/3	3/3	6/6	3/3	3/3	6/6
<i>E. aerogenes</i>	3/3	3/3	6/6	3/3	3/3	6/6
Group A <i>Streptococcus</i>	3/3	3/3	6/6	3/3	3/3	6/6
Total	100% (60/60)	98.3% (59/60)	99.2% (119/120)	100% (60/60)	98.3% (59/60)	99.2% (119/120)

Reproducibility

A multicenter reproducibility study was performed to determine the between-site and overall reproducibility. Sample panels were prepared by spiking whole blood or simulated blood culture matrix with irradiated *B. anthracis* vegetative cells at four concentrations. Samples were tested at three external sites and at our facility on three different days. As shown in Table 3, all samples yielded the expected results with the exception of two discrepant results that occurred in a single JBAIDS run. These unexpected results were most likely caused by a sample identification error as the correct results were obtained when the same samples were retested on the same day by the same operator.

Reproducibility was also assessed for colonies by testing a panel of 20 microorganisms at two external sites on three different days (see Table 4). The single incorrect result, positive result for both Target 1 and 2 when testing *S. aureus*, was likely caused by cross-contamination of the sample with *B. anthracis* organism or DNA during sample purification. Retesting of the same organism on subsequent days gave the expected negative result.

Cross-Contamination and Carry-Over

To evaluate potential carry-over, 7 negative samples were processed and tested in alternating order with 7 strongly positive samples (spiked with *B. anthracis* at approximately 200 times the assay LOD). The samples were tested with both the Target 1 and Target 2 assays. For the whole blood panel, there was no evidence of cross-contamination (all 14 negative capillaries were Negative for both the Target 1 and Target 2 assays). For the blood culture panel, 1 of the 14 Target 1 negative capillaries was positive but all Target 2 capillaries were negative. Retesting of the same sample on the same day gave a Negative test result, suggesting that the cross-contamination occurred during PCR reaction setup. The overall carry-over rate for the blood culture samples in this evaluation is 3.5% (1/28).

Clinical Performance

A multicenter clinical study was conducted at three U.S. military medical installations in Texas, Washington D.C., and Egypt. All 150 subjects were hospitalized, exhibited the clinical definition of Systemic Inflammatory Response Syndrome (SIRS) and had a blood culture ordered as part of their medical care. The study subjects ranged in age from 18 – 90 years (mean 48.5), and 56% were male. The clinical specificity of the JBAIDS Anthrax Detection System was assessed by comparing the JBAIDS results (positive/negative) to the blood culture results. As shown in Table 5, all 150 samples included in the study gave the expected negative results for both the Target 1 and Target 2 assays, and none of the blood cultures taken from the 150 patients were positive for *B. anthracis*. The clinical specificity of the JBAIDS Anthrax Detection System was 100% (95% CI 98 – 100%). Clinical sensitivity could not be determined due to the absence of clinical samples containing *B. anthracis*.

Table 5 – Results of Clinical Evaluation of the JBAIDS Anthrax Detection System

Site	Subjects Tested (n)	Subjects with Positive Blood Cultures	Organism Identified in Blood Cultures		Negative JBAIDS Results (n)		Subjects with Blood Culture Negative for <i>B. anthracis</i> (n)
			Coagulase negative staph (n=8) Escherichia coli (n=4) Staphylococcus epidermidis (n=2) Gemella Morbillorum (n=1)	Staphylococcus aureus (n=6) Enterococcus faecalis (n=3) Propionibacterium acnes (n=1) Candida albicans (n=1)	Target 1	Target 2	
1	39	15 (38.5%)			39	39	39
2	70	9 (12.9%)	Coagulase negative staph (n=3) Escherichia coli (n=1) Klebsiella pneumoniae (n=1)	Staphylococcus aureus (n=3) E. cloacae (n=1)	70	70	70
3	41	10 (24.4%)	Coagulase negative staph (n=6) Staph aureus (n=2) Candida parapsilosis (n=1)	a-hemolytic streptococcus (n=4) Klebsiella pneumoniae (n=2)	41	41	41
Total	150	34 (26.7%)			150	150	150

DISCUSSION

Performance testing of the JBAIDS Anthrax Detection System has shown the test system to be highly sensitive and specific for the detection of virulent forms of *B. anthracis*. All virulent isolates of *B. anthracis*, as well as a few rare cases of virulent *B. cereus*, were identified while non-target organisms were correctly excluded. The test system also proved to provide reproducible test results, with the few discordant test results being associated with errors in sample processing. The clinical specificity of the JBAIDS Anthrax Detection System is at least 98% (lower bound of 95% confidence interval). High analytic and clinical specificity is important to this test system as the majority of samples tested will likely be negative for *B. anthracis*.

Due to the rarity of clinical anthrax infections, it was not possible to determine the ability of the test system to detect positive patient samples. The test system can reliably detect as little as 1000 CFU/mL of human whole blood. While the level of organisms present in the early stages of anthrax infection is not known, the level of organisms in diagnosed cases is often extraordinarily high (>10⁸ CFU/mL)³, making it likely that the test system would be clinically useful.

As with all PCR-based assays, false positives due to cross-contamination are a concern. In these studies, a low rate of cross-contamination was observed, with the most likely source being the DNA contained in the PC/IC reagent vials. During the clinical trial, all samples tested Negative for both targets, however cross-contamination during set-up of the freeze-dried reagents resulted in 0.7% (1/150) of Target 1 and 2.7% (4/150) of Target 2 tests giving Uncertain results. In all cases, retesting of these samples (as described in the product literature) provided the correct negative test result. Cross-contamination during sample purification can also occur, especially with samples containing large concentrations of *B. anthracis* (such as in the case of positive cultures). Careful adherence to good laboratory technique will help to reduce the incidence of false positives. In addition, the incidence of false positives caused by amplicon contamination is greatly reduced by reserving the Target 2 assay only for confirming samples testing positive with the Target 1 assay.

CONCLUSIONS

The clinical course of inhalational anthrax is dramatic. Patients often present with non-specific influenza-like symptoms but then rapidly decline, developing severe respiratory distress and septic shock. Rapid diagnosis is necessary for patient survivability. Definitive diagnosis of inhalation anthrax is made by isolation of *Bacillus anthracis* from the blood or sputum of a patient with a compatible clinical syndrome. These gold standard tests may take from 24 to 48 hours, and confirmation may require sending the samples to a high-level public health laboratory. In a 2001⁴ report, the Center for Disease Control recognized detection of *B. anthracis* DNA using PCR assays as supportive laboratory testing in the identification of clinical anthrax infections. The JBAIDS Anthrax Detection System is a real-time PCR test system that can provide reliable test results in just a few hours. This testing system should be a useful adjunct to standard microbiologic techniques for the rapid identification of *B. anthracis*.

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