Tetracore®

Tetracore® Inc.

RedLine Alert™ Test

An immunochromatographic test kit for the presumptive identification of *Bacillus anthracis* colonies from culture plates

For *In Vitro* Diagnostic Use

16 February 2010

RedLine Alert™ Test

NAME AND INTENDED USE

The Tetracore® ***RedLine Alert***™ Test is an immunochromatographic test intended for the rapid, ***in vitro*** qualitative presumptive identification of ***Bacillus anthracis*** from non-hemolytic ***Bacillus*** colonies cultured on sheep blood agar plates. The test is intended for use in clinical, public health, and hospital laboratories in conjunction with other markers and testing for the presumptive identification of ***Bacillus anthracis***.

Warning: The ***RedLine Alert***™ Test has not been evaluated for use with spore preparations, suspicious powders, or samples other than colonies from culture growth.

SUMMARY AND EXPLANATION

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, non-hemolytic, spore-forming, rod-shaped bacterium. ***B. anthracis*** vegetative cells have two surface structures, a capsule and an S-layer in addition to the cytoplasmic membrane and peptidoglycan layers found in other bacteria. The ***RedLine Alert***™ Test identifies those colonies that produce a protein that is part of the S-layer.

Anthrax is primarily a zoonotic disease of herbivores; however, humans can naturally acquire this disease directly from contact with infected herbivores, or indirectly via their products, such as hair, wool, and hides (USAMRIID, 2001). Spores are the usual infective form. Anthrax presents clinically as three distinct syndromes, depending on the route of infection: cutaneous, gastrointestinal, and inhalational disease. ***B. anthracis*** may be recovered by culture from blood, sterile fluids, and swabs (oropharyngeal ulcers, rectal, vesicular fluid, eschar material, stools, or biopsy specimens depending on the type of infection).

Current CDC guidelines[1] for culturing and the presumptive identification of ***B. anthracis*** include culture on standard 5% sheep blood agar (SBA) and observation of the following characteristics:

- **Colony morphology:** After 15-24 hours incubation on sheep blood agar plates at 35-37°C, well-isolated colonies are 2-5mm in diameter. The flat or slightly convex colonies are irregularly round, with edges that are slightly undulate and have a ground-glass appearance. Colonies typically have a tenacious consistency, i.e., teasing with an inoculating loop causes colony to 'stand up' like beaten egg white.
- **Hemolysis:** Colonies of ***B. anthracis*** are non-hemolytic.
- **Motility:** ***B. anthracis*** is non-motile.
- **Sporulation, microscopic observation:** Spores appear after 18-24 hours incubation at 35-37°C in a non-CO_2 atmosphere. Oval, central to sub-terminal spores that do not appreciably swell may be observed by Gram stain, wet mount, or malachite green stain.

Currently, the CDC recommends that cultures with the above-mentioned characteristics undergo confirmatory testing by gamma-phage lysis and direct fluorescence assay (DFA) of polysaccharide cell wall and capsule (De et al., 2002).

(Footnotes) [1]http://www.bt.cdc.gov/agent/anthrax/levelaprotocol/anthraxlabprotocol.pdf
http://www.bt.cdc.gov/documentsapp/Anthrax/ApprovedLRNTests.asp

PRINCIPLES OF THE PROCEDURE

The ***RedLine Alert***™ Test is for testing bacterial colonies that have been evaluated by other microbiological tests recommended by the CDC including Gram stain, hemolysis, motility, and sporulation. The ***RedLine Alert***™ Test is a rapid, ***in vitro*** qualitative test that detects the presence of a cell surface protein found in ***Bacillus anthracis*** vegetative cells. The presence or absence of this protein can be used to differentiate ***B. anthracis*** from other non-hemolytic ***Bacillus*** colonies. The test is a lateral flow immunoassay based on capillary (wicking) action and has a monoclonal antibody specific for the cell surface protein being detected. Polyclonal antibodies (from rabbits immunized with ***B. anthracis***) are immobilized on the membrane to form the "test line." A control line on the membrane is coated with an anti-murine antibody.

The assay is initiated by adding the prepared colony suspension to the Sample Port. The suspension wicks to the conjugate pad and re-hydrates the monoclonal antibody labeled with colored microparticles (conjugate). The rehydrated conjugate migrates via capillary action along the membrane. If test organisms have the cell surface protein, the labeled monoclonal binds with that target antigen to form an antigen-conjugate complex that will be captured by the immobilized rabbit antibody at the test line. Concentration of the conjugate (visualizing solution) in the test line zone produces a clearly visible, reddish colored line, indicating a positive result.

If the cell surface antigen is not present in the sample, the conjugate does not form a complex with the antigen and continues to migrate to the control line where it is captured at the control line. Since there is an excess of conjugated antibody, both positive and negative specimens will produce a reaction at the control line. A visible colored line at the control line zone verifies that capillary flow has occurred, and that the reagents are functional.

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PRODUCT DESCRIPTION

Materials Provided

RedLine Alert™ Test Kit

Product Catalogue Numbers:
TC-5123-010 (10 cassettes and 4 positive controls/kit)
TC-5123-020 (20 cassettes and 8 positive controls/kit)
TC-5123-200 (200 cassettes and 80 positive controls/kit)

Component	Part Number	Contents
RedLine Alert™ Test Cassette	S-5123-001	A membrane coated with rabbit polyclonal anti-***B. anthracis*** IgG antibody, conjugated murine monoclonal antibody, and capture solution housed in a plastic cassette
Colony Isolation Buffer	S-5123-002	Tris EDTA buffer with 0.1% sodium azide, 10mL vial
Positive Control	S-5123-003	Antigen derived from ***B. anthracis*** (Sterne) with 0.1% sodium azide

The ***RedLine Alert***™ Test Cassette and Colony Isolation Buffer are ready to use and do not require any preparation. The Positive Control is supplied as a liquid and is used as supplied. No dilutions are necessary.

Materials and Equipment Required, but Not Provided

- Micropipettor with tips (capable of delivering 150µL and 200µL)
- Vortex (optional)
- Sheep Blood Agar Plates, 5% sheep blood
- Sterile inoculating loops (10µL or other)
- Disposable specimen vials with caps (1.5mL)

STORAGE AND STABILITY

Store the test kit and kit reagents at room temperature (15-30˚C or 59-86˚F). The Tetracore® ***RedLine Alert***™ Test is stable until the assigned expiration date indicated on the outer package label and on each individual component. Do not use past the assigned expiration date.

PRECAUTIONS

Biosafety Level 2 practices, containment equipment, and facilities are recommended for activities using clinical materials and diagnostic quantities of infectious cultures of ***B. anthracis.*** [Reference: Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition; U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health, May 1999. Available from http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4/bmbl4toc.htm.]

For ***in vitro*** diagnostic use only.

Store the test cassette in the sealed pouch until ready to use. Do not use the test cassette if the pouch is not intact. Exposure to humidity can affect assay performance.

Sodium azide (NaN_3) is used as a preservative in the Colony Isolation Buffer and in the Positive Control. Sodium azide may react with lead and copper in plumbing to form explosive compounds. When disposing, flush drains with water to minimize buildup of metal azide compounds.

Buffer suspensions containing test bacteria must be decontaminated before disposing.

Use only fresh colonies (12-24 hour incubation). The ***RedLine Alert***™ Test has been evaluated on colonies incubated for up to 48 hours; results from the ***RedLine Alert***™ Test were valid at all time points between 12 and 48 hours. However, the use of colonies older than 24 hours may result in variable Gram stain and hemolysis. The reliability of results from colonies older than 48 hours has not been evaluated by the ***RedLine Alert***™ Test.

Incubation times or temperatures other than those specified may give unreliable results.

If the control line fails to appear, consider the test invalid. Results from an invalid test should not be used.

Do not use the test kit or components beyond the specified expiration date.

Avoid contamination of buffer. Do not use if buffer appears to have cloudiness, particulates, or precipitates.

Do not touch the Sample Port of the test cassette.

All reagents in this kit can be substituted across kit lot numbers, provided the expiration date is not exceeded.

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PREPARATION OF SAMPLE

Use colonies from sheep blood agar plates that have been incubated at 37˚C +/- 1˚C for 12-24 hours (ambient air or 5% CO_2). Do not use colonies cultured for longer than 24 hours[2].

Observe the colony for the lack of ß-hemolysis (***B. anthracis*** colonies are non-hemolytic) and perform other testing as appropriate (e.g., Gram stain, catalase, motility, etc.).

Dispense 200µL of Colony Isolation Buffer into a 1.5mL plastic vial.

Select an isolated 1-2mm diameter colony[3]. Transfer the colony using a sterile inoculating loop (diameter of colony ~1/3 to 2/3 of 10µL inoculating loop) to the vial containing the 200µL of Colony Isolation Buffer. To facilitate transfer, twirl the loop in buffer for 5 seconds to dislodge colony.

Place the cap on the tube and mix using a vortex for approximately 5 seconds or, alternatively, pipet the mixture up and down approximately 10 times to emulsify the colony.

Allow the buffer suspension to sit for approximately 2 minutes and then re-mix or re-vortex for approximately 5 seconds.

The buffer suspension is now ready to be added to the ***RedLine Alert***™ Test Sample Port on the test cassette.

(Footnotes)
[2] The ***Redline Alert***™ Test Kit has been evaluated on colonies incubated for up to 48 hours; results from the ***Redline Alert***™ Test were valid at all time points between 12 and 48 hours. However, the use of older colonies (i.e., cultured for more than 24 hours) may result in variable Gram stain and hemolysis patterns.

[3] Colonies of 3-5mm can be used; however, only half of the colony should be used for the test. For colonies larger than 5mm, use a portion of the colony corresponding to a 1-2mm diameter colony.

TEST PROCEDURE

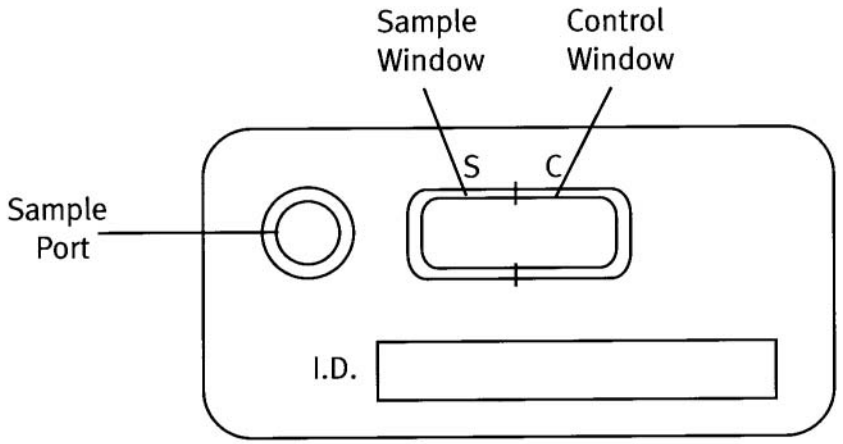
Step 1: Remove the test cassette from the protective pouch and place it on a flat surface.

Step 2: Label the test cassette with the sample code or identification number.

Step 3: Dispense 0.15mL (150µL) of sample into the Sample Port on the ***RedLine Alert***™ Test cassette.

Step 4: Wait for clearly visible red colored lines to appear. Read the results at 15 minutes.

NOTE: Positive results may be observed in as short as 30 seconds depending on the concentration of ***B. anthracis*** antigen in the sample. Read negative results at 15 minutes. **Colored lines that appear after 20 minutes are not valid and results should not be used.**



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POSITIVE CONTROL

Individual Positive Control tubes, consisting of protein derived from ***B. anthracis*** (Sterne), have been included in the kit as an external control.

Procedure for use:

(1) Add 150µL of Positive Control to the Sample Port on the ***RedLine Alert***™ Test cassette designated for Positive Control.

(2) Wait for clearly visible colored lines to appear. Read the results at 15 minutes.

QUALITY CONTROL

Internal controls: The ***RedLine Alert***™ Test includes internal controls for each test cassette. The presence of a "Control Line" indicates a valid reaction and demonstrates appropriate reagent flow in the test cassette. The background should remain near-colorless, indicating the reagents provided in the kit will not interfere with the test results.

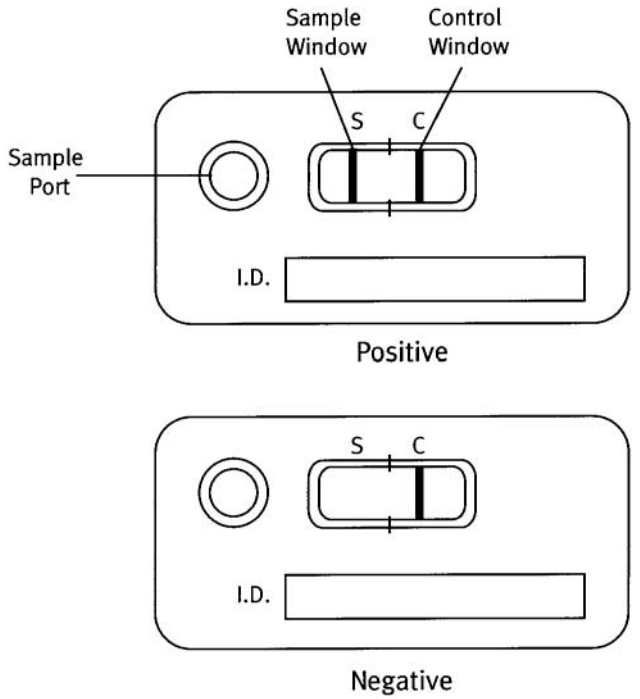
External controls: A Positive Control has been included in the test kit. For a negative control, another non-hemolytic ***Bacillus*** species, such as ***B. megaterium***, or a non-***Bacillus*** species, such as ***S. aureus***, may be used. Additional positive controls may be run, such as freshly cultured colonies of ***B. anthracis*** Sterne, prepared as described in "Sample Collection and Storage" section above.

External controls should be run with each new kit lot, or more frequently, according to established laboratory procedures.

The use of external positive and negative controls ensures the test kit is performing properly and ensures valid results. Controls should be processed in the same manner as test samples.

If controls do not behave as expected, results must be considered invalid. Results from invalid tests should not be used.

INTERPRETATION OF RESULTS



Negative Results: The test is negative if a single colored line appears only in the C (control) window.

Positive Results: The test is positive if two colored lines appear. One colored test line will appear in the S (sample) window and one in the C (control) window. Any colored line in the S (sample) window should be considered positive. Colored S (sample) lines may be lighter or darker than the C (control) line.

Invalid Results: The test is invalid if no colored line appears in the C (control) window even if a colored line appears in the S (sample) window.

If the test is positive and the bacterial colony is non-hemolytic, ***B. anthracis*** cannot be ruled out. Submit the sample to the State Public Health Laboratory or State Public Health Department for confirmation. The state public health lab director should be consulted if ***B. anthracis*** is suspected, (even when the ***RedLine Alert***™ Test is negative or hemolysis is questionable). Refer to state and local regulations for reporting requirements and to current CDC guidelines [http://www.bt.cdc.gov/agent/anthrax/levelaprotocol/anthraxlabprotocol.pdf; www.bt.cdc.gov/documentsapp/Anthrax/ApprovedLRNTests.asp].

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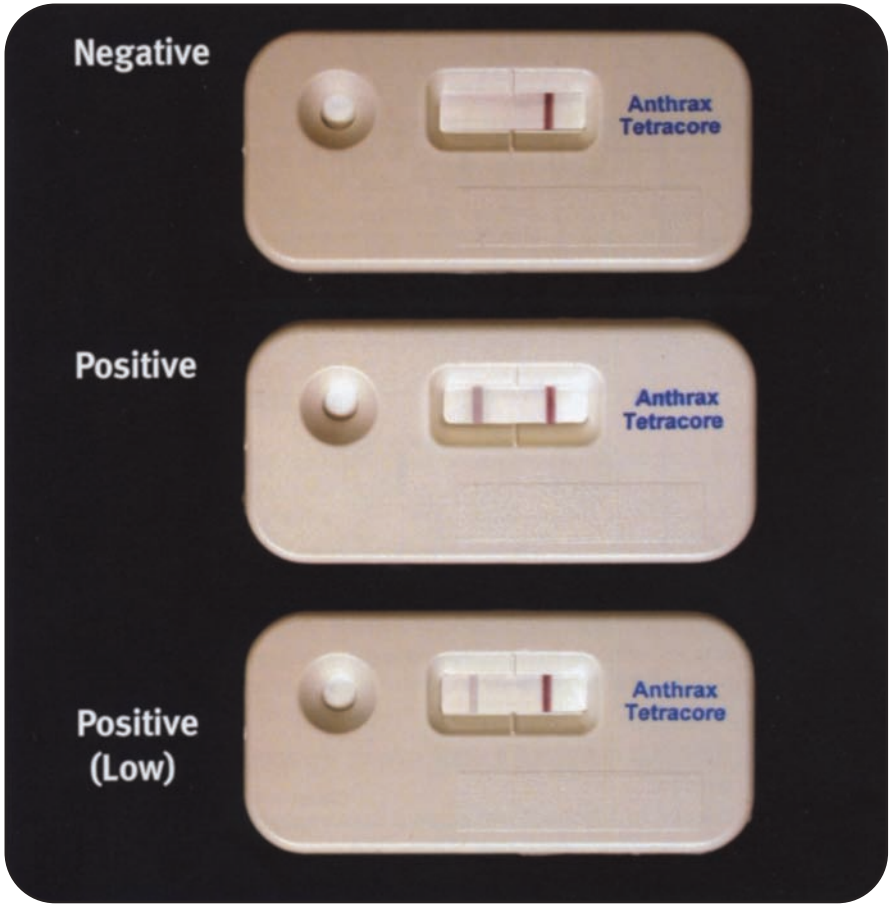
Reading Guide for Interpretation of Negative and Positive Results



The above photographs contain cassettes as they appear after a test has been run. The cassette in the top photo shows a negative result, i.e., a colored line appears only in the "C" (control) window. The cassettes in the lower two photos show positive results, i.e., a colored line appears in both the "S" (sample) window and the "C" (control) window*. The cassette in the middle photo shows a representative positive result for a 1mm colony of ***B. anthracis.*** The cassette in the lower photo shows a low (or weak) positive result.

***[**Note: The colored line in the "S" window may be lighter than the "C" window; a line in the "S" window should be scored as positive as long as it is reddish in color and is clearly visible, even if it is lighter than the line in the "C" window.]

PRESUMPTIVE IDENTIFICATION OF *B. ANTHRACIS*

A presumptive identification of ***B. anthracis*** is made when the following criteria are met:

(1) Colony consists of Gram-positive, rod-shaped ***Bacillus***; colony has typical ***B. anthracis*** morphology (i.e., ground-glass appearance, irregularly round with edges that slightly undulate, tenacious consistency like "beaten egg white" when teased with inoculating loop).

(2) Colony cultured on sheep blood agar plate is negative for ß-hemolysis, i.e. bacteria are non-hemolytic.

(3) Colony tests positive in ***RedLine Alert***™ Test [i.e., a clearly visible colored line appears in both the "S" (sample) area and the "C" (control) area on the cassette].

(4) Additional CDC-recommended tests show bacteria to be non-motile and spore-forming.

LIMITATION OF THE PROCEDURE

1. Use the ***RedLine Alert***™ Test on bacterial colonies from sheep blood agar (SBA) only. Performance with samples from other sources has not been evaluated. Growth of bacterial colonies on media other than SBA will not allow for determination of hemolysis; both positive reactivity on the ***RedLine Alert***™ Test ***and*** observation of non-hemolysis of colonies grown on SBA are needed to reliably use ***RedLine Alert***™ results.

2. False negative results may occur when levels of specific antigen are below the detectable concentration level (sensitivity) of the test. Using the recommended colony size should produce a sufficiently dense suspension and assure sufficient antigen is used. Suspensions less than a 1 McFarland may yield barely visible lines.

3. The test cassette cannot be reused. Once the test cassette is used, discard the test cassette as biohazardous waste.

4. Testing of hemolytic ***Bacillus*** species is not recommended due to positive results on ***RedLine Alert***™ Test cassettes with some ***B. cereus*** and ***B. thuringiensis*** isolates.

5. Unknown cross-reactions may cause a false positive result.

6. This test can be used for a presumptive identification of ***B. anthracis.*** Isolates that produce a negative result but are suspected of being ***B. anthracis*** by other criteria should be subjected to further testing.

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PERFORMANCE CHARACTERISTICS

(a) *B. anthracis*

One hundred forty-five (145) ***Bacillus anthracis*** isolates from geographically diverse locations world-wide including isolates from 28 countries were tested with the ***RedLine Alert***™ Test. The isolates encompass the major diversity groups of Sterne-Ames, Southern Africa, Kruger, Western North America, and Vollum strains and cover the genetic diversity of ***B. anthracis*** genotypes, as determined by multiple-locus variable-number tandem repeat analysis (Keim et al., 2000). One hundred forty three (143)[4] were positive in the ***RedLine Alert***™ Test.

These 145 ***B. anthracis*** isolates were also tested for sensitivity to gamma phage lysis. One hundred forty one (141) were positive by gamma phage analysis. The two false negative results with the ***RedLine Alert***™ Test and the four false negative results with gamma phage lysis did not coincide, thus emphasizing that utilization of more than one detection method can aid in the identification of ***B. anthracis.***

	Gamma phage +	Gamma phage -	
RedLine Alert™ +	139	4	143
RedLine Alert™ -	2	0	2
	141	4	145

Redline Alert % Correct = 98.6% (143/145) [Confidence Levels: 95.1-99.8%]
Gamma phage % Correct = 97.2% (141/145) [Confidence Levels: 93.1-99.2%]

(b) Non-*anthracis Bacillus* species

Forty-nine (49) non-hemolytic ***Bacillus spp.*** (other than ***B. anthracis***), in addition to fifty-two (52) hemolytic ***Bacillus spp.,*** were tested with the ***RedLine Alert***™ Test. The species (and number of each) are listed below[5]:

Non-*anthracis Bacillus* spp.

Non-hemolytic (n=49)
B. mycoides (n=5)
B. sphaericus (n=3)
B. subtilis (n=11)
B. amyloliquifaciens
B. atrophaeus
B. circulans (n=2)
B. coagulans
B. fusiformis
B. globigii
B. licheniformis (n=9)
B. medusa Delaporte
B. megaterium (n=6)
B. polymyxa
B. pumilis (n=2)
B. subtilis var. niger (n=2)
Brevibacillus parabrevis
Paenibacillus macerans

Hemolytic (n=52)
B. mycoides (n=3)
B. sphaericus (n=1)
B. subtilis (n=4)
*B. cereus (n=23)**
*B. thuringiensis (n=20)**
Paenibacillus alvei

(Footnotes)
[4] One of the two isolates that tested negative had atypical colony morphology.
[5] Certain strains of three of these species, i.e., ***B. mycoides, B. sphaericus,*** and ***B. subtilis***, have been reported as either hemolytic or non-hemolytic.

The ***RedLine Alert***™ Test gave the correct negative test result with all forty-nine (49) non-hemolytic, non-anthracis ***Bacillus*** cultures.

* Six out of the 23 ß-hemolytic ***B. cereus*** strains and six out of the 20 ß-hemolytic ***B. thuringiensis*** strains gave a positive test result in the ***RedLine Alert***™ Test. All other ß-hemolytic species or strains in this group gave a negative test result.

Reactivity of ß-hemolytic *Bacillus* species: Genetically, ***Bacillus cereus*** and ***Bacillus thuringiensis*** are the most closely related species to ***Bacillus anthracis*** (Radnedge et al. 2003); all three species are members of the group 1 bacilli. While ***B. anthracis*** is phylogenetically monomorphic, ***B. cereus*** and ***B. thuringiensis*** are genetically more diverse, with some strains particularly close genetically to ***B. anthracis*** (Radnedge et al. 2003). One morphological difference between these closely related organisms is the ability to cause ß-hemolysis when cultured on sheep blood agar (SBA) plates: (i) ***B. anthracis*** is non-hemolytic; (ii) ***B. cereus*** and ***B. thuringiensis*** are ß-hemolytic. It is this difference in hemolysis that accounts for one of the major determinants in the CDC algorithm for ***B. anthracis*** identification, i.e., that any ***Bacillus*** species which exhibits ß-hemolysis when cultured on SBA plates is ruled out as ***B. anthracis.*** Despite the fact that ***B. cereus*** and ***B. thuringiensis*** organisms would have been ruled out early in the CDC algorithm due to ß-hemolysis and, therefore, not candidates for further testing, several strains of ***B. cereus*** and ***B. thuringiensis*** were evaluated in the ***RedLine Alert***™ Test. The majority of ***B. cereus*** and ***B. thuringiensis*** strains tested negative in the ***RedLine Alert***™ Test. However, some strains of ***B. cereus*** (6/23) and ***B. thuringiensis*** (6/20), amongst those genetically closest to ***B. anthracis,*** can cross-react in the ***RedLine Alert***™ Test. No cross-reaction was seen with ß-hemolytic strains of ***B. mycoides***, ***B. sphaericus, B. subtilis***, and ***Paenibacillus alvei***.

(c) Non-*Bacillus* laboratory pathogens

Twenty-six (26) non-Bacillus laboratory pathogens were tested. All twenty-six (26) non-Bacillus laboratory pathogens gave a negative result in the ***RedLine Alert***™ Test.

The organisms tested included:

Non-*Bacillus* Laboratory Pathogens (n=26)

Campylobacter jejuni
Candida albicans
Clostridium histolyticum
Clostridium tertium (n=2)
Clostridium perfringens
Corynebacterium pseudodiptheriticum
Escherichia coli
Erysipelothrix rhusiopathiae
Klebsiella pneumoniae
Lactobacillus acidophilus
Listeria monocytogenes
Mycobacterium fortuitum subsp. fortuitum
Nocardia pseudobrasiliensis
Pseudomonas aeruginosa
Rhodococcus equi
Staphylococcus aureus (n=3)
Staphylococcus epidermidis
Streptococcus dysgalactiae subsp. Equisimilis (n=2)
Streptococcus equi
Streptococcus pneumoniae
Streptococcus pyogenes
Yersinia pseudotuberculosis

RedLine Alert™ Test

REPRODUCIBILITY

Study 1

A reproducibility study of the ***RedLine Alert***™ Test was conducted at three (3) sites using a panel of blinded specimens. The blinded specimen panel contained three (3) ***B. anthracis*** specimens and three (3) non-anthrax related ***Bacillus*** species. Each of the six (6) specimens was tested in triplicate in two (2) runs on each of three (3) days (n=108). One hundred eight (108) of the 108 specimens tested produced the expected result 100% of the time.

Study 2

An interlaboratory study was conducted at four (4) clinical sites using a panel of thirty (30) blinded culture specimens. The blinded panel consisted of thirty (30) ***Bacillus*** spp. There were sixteen (16) non-anthracis, non-hemolytic ***Bacillus,*** four (4) non-hemolytic ***B. anthracis*** Sterne used as positive controls, and ten (10) hemolytic ***Bacillus*** organisms. Of the 10 hemolytic ***Bacillus*** organisms in the panel, two (2) were known to give equivocal results. Each site tested each specimen in duplicate for a total of two-hundred forty (240) determinations. There was agreement in the one-hundred sixty (160) out of one-hundred sixty (160) tests completed on non-hemolytic ***Bacillus*** for a reproducibility of 100% (160/160 non-anthracis, non-hemolytic ***Bacillus*** and non-hemolytic ***B. anthracis*** Sterne specimens). For the 10 hemolytic ***Bacillus*** organisms, there was agreement with eighty (80) out of eighty (80) determinations, but the twenty (20) determinations of the known equivocal samples, as expected, gave both negative and positive results among the four clinical sites.

REFERENCES

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Radnedge L, Agron PG, Hill KK, Jackson PJ, Ticknor LO, Keim P, Anderson GL. 2003. Appl Environ Microbiol 69:2755.

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