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Detection of *Bacillus anthracis* in animal tissues using InBios active anthrax detect rapid test lateral flow immunoassay

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Abstract

The Active Anthrax Detect (AAD) Rapid Test lateral flow immunoassay is a point-of-care assay that was under investigational use for detecting *Bacillus anthracis* capsular polypeptide (polyglutamic acid) in human blood, serum and plasma. Small sample volumes, rapid results and no refrigeration required allow for easy use in either the field or laboratory. Although the test was developed for use in suspect cases of human inhalation anthrax, its features also make it a potentially powerful tool for testing suspect animal cases. We tested animal tissue samples that were confirmed or ruled out for *B. anthracis*. The AAD Rapid Tests were also deployed in the field, testing animal carcasses during an anthrax outbreak in hippopotami *(Hippopotamus amphibius)* and Cape buffalo *(Syncerus caffer)* in Namibia. Evaluation of all samples showed a specificity of 82% and sensitivity of 98%. However, when the assay was used on specimens from only fresh carcasses (dead for <24 h), the specificity increased to 96%. The AAD Rapid Test is a rapid and simple screening assay, but confirmatory testing needs to be done, especially when the age of the sample (days animal has been deceased) is unknown.

Keywords

animal tissues; anthrax; *Bacillus anthracis*; capsular antigen; lateral flow immunoassay; polyglutamic acid

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Conflict of Interest

No conflict of interest declared.

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Introduction

Bacillus anthracis, the causative agent of anthrax, is enzootic in many locations worldwide. Often, humans are exposed and may become infected through contact with animal products or by consuming meat from animals that have died of anthrax. By identifying infected animal carcasses, control and prevention measures may be implemented sooner, resulting in fewer human infections. Currently, suspected anthrax animal carcasses are either not tested or tested using classic microbiological methods (microscopy, culturing or PCR) that often require more than a day turn-around time and may pose a health risk for those working with the samples. Also, in remote areas, laboratories may not be near outbreak sites, making sample transportation necessary, further delaying results. A simple and fast diagnostic test that can be used in the field or laboratory would reduce the time to obtain results, allowing for earlier implementation of control and treatment measures. It would also be useful in low-resource settings where access to biosafety equipment, such as a certified biological safety cabinet, may be limited. Previously described rapid lateral flow tests for detection of *B. anthracis* are limited, and required higher-risk collection procedures such as the use of needles for obtaining whole blood samples from cattle or have not been tested in animals and showed low sensitivities using bacterial culture (Muller *et al.* 2015; Zasada *et al.* 2015). The Active Anthrax Detect (AAD) Rapid Test is a lateral flow test that was under investigational use for detecting *B. anthracis* capsular antigen (polyglutamic acid (PGA)) in human sera, plasma and blood (Gates-Hollingsworth *et al.* 2015, unpublished data). It is easy to perform, requires no refrigeration of cassettes or reagents, uses a small sample volume (10–20 μl) with minimal sample processing and is read by eye in 15 min. Here, we investigate the use of the AAD Rapid Test lateral flow immunoassay for testing animal tissues for *B. anthracis*.

Results and discussion

Animal tissue samples tested included experimental spiked and nonspiked tissues and filtrates from tissues, as well as known positive and negative clinical animal outbreak samples. The AAD Rapid Test correctly identified 140 true-negatives and 41 true positives, and misidentified 1 positive (false-negative) and 30 negatives (false-positives). The spiked and nonspiked canine, coyote and store-bought cow and bison meat or tissues (n = 12) all gave expected results (positive and negative, respectively). Fifty of the 51 confirmed-negative US cow ear tips were negative, and 13 of the 14 confirmed-positive nonhuman primate (NHP) tissue filtrates were positive by the AAD Rapid Test (one cow ear was a false-positive and one NHP lung filtrate was a false-negative). Eighty of the 108 confirmed-negative white-tailed deer samples were negative by the AAD Rapid Test. The rest of the deer samples (n = 28) gave false-positive results (ruled out by the Laboratory Response Network (LRN) real-time PCR), generally increasing in frequency with the time of decomposition, often peaking on day 3 (Table 1). We hypothesized that this may be due to other PGA-producing bacteria present during decomposition. PGA expression and gene homologues to the *B. anthracis cap* genes have been reported in some other species of bacteria (Hoffmaster *et al.* 2004; Beesley *et al.* 2010). However, these species either do not cause disease or cause a clinical illness that is not diagnostically confused with anthrax in

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animals, with the exception of some of the rare *Bacillus cereus* strains, such as *B. cereus* biovar anthracis (Klee *et al.* 2006). These deer data also suggested that ear tissue was an optimal specimen over rectal or nasal swabs, as there were no false-positive ear tissues on days 0 and 1. This was likely due to the fact that the microbiota of ear tissue may not be as diverse or is different from that of nasal and rectal sites.

Specificity was calculated as the number of true-negatives divided by the sum of true-negatives and false-positives, multiplied by 100; sensitivity was calculated as the number of true-positives divided by the sum of true-positives and false-negatives, multiplied by 100. The specificity of the AAD Rapid Test was 82% ((140/170) × 100), and the sensitivity was 98% ((41/42) × 100). However, the specificity increased to 96% ((85/89) × 100) if only specimens from deer carcasses that were dead <24 h were included.

For the Namibia animal outbreak specimens, confirmation of *B. anthracis* was based on any specimen type from an animal being positive by previously established confirmatory methods (culture, LRN real-time PCR or IHC). For specimens from confirmed-positive animals, the AAD Rapid Test showed better sensitivity (100%) than the gold standard of culture (28%), the LRN real-time PCR (69%) or IHC (88%) (Table 2). Given these results, this assay showed high utility for presumptive identification of *B. anthracis* on specimens from animal carcasses, especially those individuals that have been dead <24 h and showed signs consistent with anthrax infection. Additional testing, such as microscopy, culture, PCR or other methods, is still recommended for confirmation.

Materials and methods

Samples

Spiked ($n = 6$) and nonspiked ($n = 6$) tissues included domestic dog and coyote *(Canis latrans)* liver and kidney, and store-bought bison and domestic cow meat. Additional negative samples tested included 51 ear tips from US beef cattle provided by the US Department of Agriculture (USDA), and 108 ear tissue and nasal and rectal swabs from nine deceased Georgia (US) white-tailed deer *(Odocoileus virginianus)* carcasses, allowed to decompose naturally in a field in Athens, GA over a 5-day period. Time points for specimen collection were 0, 1, 3 and days postmortem. NHP *(Macaca fascicularis)* tissue filtrates ($n = 14$) received from the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health (NCEH), were prepared at Battelle Biomedical Research Center and include brain, kidney, liver, lymph node, spleen, lung and heart from two NHPs experimentally infected with anthrax. Outbreak samples received by the CDC's Zoonoses and Select Agent Laboratory (ZSAL) for diagnostic testing included tissues or swabs from bovines, hippopotami and an impala ($n = 27$) (Table 3). Use of the white-tailed deer samples was approved under the Southeastern Cooperative Wildlife Disease Study's IACUC (AUP A2018 02–010) at the University of Georgia. Use of the NHP samples was approved under Battelle Biomedical Research Center's IACUC Protocol 3283.

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Processing

Tissues from the two NHPs were collected at time of death, and repeatedly rinsed in a EDTA-free protease-inhibitor cocktail (SigmaFAST Protease Inhibitor Cocktail Tablets, EDTA-free Cat. No. S8830 (Sigma-Aldrich, St. Louis, MO) or EDTA-free protease inhibitor cocktail with similar inhibitor panel), then homogenized by grinding in a cell lysis solution and centrifuged. The supernatant was syringe-filter sterilized and used for testing. Swabs tested in ZSAL were processed using the swab extraction tube system (SETS) (Roche Applied Science, Indianapolis, IN) as previously described (Dauphin *et al.* 2012). An aliquot of the resulting liquid was used for testing. For tissues, *c.* 4 mm cubes were washed with 600 μl sterile phosphate-buffered saline (PBS) by pipetting and used for testing. Spiked samples were washed by pipetting with 600 μl of PBS containing $2–3 \times 10^5$ CFU per ml encapsulated *B. anthracis* culture. Samples were pulse-vortexed on high speed for 10 s. In the field, swabs were treated the same as tissues (not using SETS), and samples were either vortexed as described above or shaken vigorously by hand for 30 s.

All samples were tested for B. *anthracis* using culture, the Laboratory Response Network's (LRN) real-time PCR, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) lethal factor detection, and/or capsule and cell wall detection using immunohistochemistry (IHC) (Hoffmaster *et al.* 2002; Guarner *et al.* 2003; Boyer *et al.* 2007, 2011; Gallegos-Candela *et al.* 2018). For animals in which multiple specimens were tested, the animal was still considered positive even if only one of the samples was confirmed-positive by these methods. For culture, 100 μl aliquots of the processed tissues and swabs were inoculated onto trypticase soy agar plates containing 5% sheep blood agar (SBA) or in heart infusion broth (HIB) and incubated overnight at 37°C. All samples that were initially overgrown and appeared negative for *B. anthracis* culture were then heat-shocked at 65°C for 30 min to select for spore-formers, and subsequently inoculated onto SBA and incubated as above. DNA for real-time PCR was extracted from swabs using the Qiagen QIAamp DNA Blood Mini kit. For tissues, overnight digestion and extraction was performed as described in the tissue protocol of the Qiagen QIAamp DNA Mini and Blood Mini kit handbook. The LRN real-time PCR, MALDI-TOF MS and IHC were all performed as previously described (Hoffmaster *et al.* 2002; Guarner *et al.* 2003; Boyer *et al.* 2007, 2011; Gallegos-Candela *et al.* 2018).

AAD rapid test

The samples were tested following kit instructions. Briefly, for cartridges or strips, 10 or 20 μl, respectively, of sample supernatant or filtrate was added to the sample pad of the lateral flow, followed by two to three drops of Chase Buffer Type A solution. Positive and negative controls were supplied with the kits. An additional positive control of encapsulated *B. anthracis* ($2–3 \times 10^5$ CFU per ml) was used in the laboratory. Controls were performed once per day of testing. Results were read by eye after 15 min. Each strip or cartridge included test and control lines. For a positive result, both lines must be visible. For a negative result, only the control line was visible. The result was invalid if the control line was not visible, regardless of the test line (Fig. 1).

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Significance and Impact of the Study

In countries where anthrax is endemic, many human outbreaks are often caused by epizootics. Earlier detection of infected animals may allow for identification of exposed people, early implementation of prevention and control methods, and ultimately lessen the number of people and animals affected. Detection of *Bacillus anthracis* in animal tissues using a simple, rapid and field-deployable method would allow for faster outbreak response. We evaluated a simple sample collection and processing method for use with the Active Anthrax Detect Rapid Test lateral flow immunoassay to screen dead animals for anthrax.

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Figure 1.
The Anthrax Active Detect Lateral Flow Immunoassay cartridges, showing positive (left) and negative (right) reactions. ‘T’ is the Test line and ‘C’ is the Control line.

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Table 1

Number of Active Anthrax Detect (AAD) Rapid Test lateral flow false-positive specimens in nine confirmed-negative* white-tailed deer

	Number of days of decomposition			
	0	**1**	**3**	**5**
Ear Tissue†	0	0	2	7
Nasal Swab†	1	2	4	1
Rectal Swab†	1	2	6	2
Total	2	4	12	10

*Animals were confirmed-negative by the Laboratory Response Net work's real-time PCR.

†Number of specimens tested was nine per day for each specimen type (one from each animal) or 27 specimens total per day.

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Table 2

Sensitivity* of methods used for detection of *Bacillus anthracis* in various types of positive Namibian animals†

	Ear tissue	Eyelid tissue	Eye blood swab	Nasal swab	Rectal swab	Total
Culture	0/4 (0%)	2/4 (50%)	1/1 (100%)	2/5 (40%)	0/3 (0%)	5/18 (28%)
LRN Real-time PCR	3/4 (75%)	4/4 (100%)	1/1 (100%)	3/4 (75%)	0/3 (0%)	11/16 (69%)
IHC	4/5 (80%)	3/3 (100%)	NA	NA	NA	7/8 (88%)
AAD	6/6 (100%)	4/4 (100%)	1/1 (100%)	6/6 (100%)	3/3 (100%)	20/20 (100%)

LRN, Laboratory Response Network; PCR, polymerase chain reaction; IHC, immunohistochemistry; AAD, Anthrax Active Detect.

* (No. of positives/total no. of specimens tested from positive animals) × 100.

† Animals were considered positive if any sample from an animal was confirmed by culture, the Laboratory Response Network's real-time PCR or immunohistochemistry.

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Table 3

Detection of *Bacillus anthracis* in animal outbreak specimens using the Active Anthrax Detect (AAD) Rapid Test lateral flow immunoassay

ID	Origin	Anima	Specimen	Anthrax confirmed?*	Field or lab AAD rapid test result
001	BNP, Namibia	Buffalo	Ear	Yes	+
001	BNP, Namibia	Buffalo	Nasal swab	Yes	+
005	BNP, Namibia	Buffalo	Ear	Yes	+
007	BNP, Namibia	Hippo	Ear	Yes	+
007	BNP, Namibia	Hippo	Eyelid	Yes	+
007	BNP, Namibia	Hippo	Nasal swab	Yes	+
007	BNP, Namibia	Hippo	Rectal swab	Yes	+
008	BNP, Namibia	Hippo	Eyelid	Yes	+
008	BNP, Namibia	Hippo	Ear	Yes	+
008	BNP, Namibia	Hippo	Nasal swab	Yes	+
009	BNP, Namibia	Hippo	Eye blood swab	Yes	+
009	BNP, Namibia	Hippo	Nasal swab	Yes	+
009	BNP, Namibia	Hippo	Eyelid	Yes	+
009	BNP, Namibia	Hippo	Ear	Yes	+
009	BNP, Namibia	Hippo	Rectal swab	Yes	+
012	BNP, Namibia	Hippo	Nasal swab	Yes	+
012	BNP, Namibia	Hippo	Ear	Yes	+
012	BNP, Namibia	Hippo	Eyelid	Yes	+
012	BNP, Namibia	Hippo	Rectal swab	Yes	+
2000032045	Texas, US	Cow	Ear	Yes	+
3000015281	Montana, US	Bison	Spleen	Yes	+
2002734829	Zhemgang District, Bhutan	Bovine	Ear	Yes	+
013	BNP, Namibia	Impala	Ear	No	+
002	BNP, Namibia	Buffalo	Ear	No	–
013	BNP, Namibia	Impala	Rectal swab	No	–
013	BNP, Namibia	Impala	Nasal swab	No	–
013	BNP, Namibia	Impala	Eyelid	No	–

BNP, Bwabwata National Park; US, United States; AAD, Anthrax Active Detect.

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* Animals were confirmed-positive by culture, the Laboratory Response Network's real-time PCR and/or immunohistochemistry.