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Implications of Limits of Detection of Various Methods for *Bacillus anthracis* in Computing Risks to Human Health▽†

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Used for decades for biological warfare, *Bacillus anthracis* (category A agent) has proven to be highly stable and lethal. Quantitative risk assessment modeling requires descriptive statistics of the limit of detection to assist in defining the exposure. Furthermore, the sensitivities of various detection methods in environmental matrices are vital information for first responders. A literature review of peer-reviewed journal articles related to methods for detection of *B. anthracis* was undertaken. Articles focused on the development or evaluation of various detection approaches, such as PCR, real-time PCR, immunoassay, etc. Real-time PCR and PCR were the most sensitive methods for the detection of *B. anthracis*, with median instrument limits of detection of 430 and 440 cells/ml, respectively. There were very few peer-reviewed articles on the detection methods for *B. anthracis* in the environment. The most sensitive limits of detection for the environmental samples were 0.1 CFU/g for soil using PCR-enzyme-linked immunosorbent assay (ELISA), 17 CFU/liter for air using an ELISA-biochip system, 1 CFU/liter for water using cultivation, and 1 CFU/cm^2 for stainless steel fomites using cultivation. An exponential dose-response model for the inhalation of *B. anthracis* estimates of risk at concentrations equal to the environmental limit of detection determined the probability of death if untreated to be as high as 0.520. Though more data on the environmental limit of detection would improve the assumptions made for the risk assessment, this study's quantification of the risk posed by current limitations in the knowledge of detection methods should be considered when employing those methods in environmental monitoring and cleanup strategies.

According to the Centers for Disease Control (CDC), a category A agent is an organism that poses a risk to national security because it can be easily disseminated or transmitted from person to person, results in high mortality rates, has the potential for major public health impact, might cause public panic and social disruption, and requires special action for public health preparedness (http://emergency.cdc.gov/agent/agentlist-category.asp). Quantitative information on category A agents in environmental matrices (soil, air, fomite, and water) is very limited (62). However, from the literature, it has been concluded that *Bacillus anthracis* is the most environmentally stable category A agent overall (62).

After the release of *B. anthracis* through mail envelopes in 2001, assessment of the decontamination process revealed an important question: could the detection methods effectively determine if the environment is clean? An evaluation of the effectiveness of sampling methods at a U.S. postal facility in Washington, DC, that was contaminated with *B. anthracis* spores concluded that neither of the sampling methods used (HEPA vacuum or wipes) were sensitive enough to ensure that spores had been removed completely. In addition, the event exposed the necessity of quantifying recovery and extraction efficiency during sample collection and processing to improve the method limit of detection (61, 67).

In this literature review, the limit of detection of methods for *B. anthracis* is characterized as either an instrument limit of detection or an environmental limit of detection. An instrument limit of detection is generally evaluated with pure cultures. An environmental limit of detection is evaluated with cultures/cells spiked into an environmental matrix (soil, air, fomites, water), which then undergoes various recovery and concentration procedures (i.e., filtration and extraction or direct extraction) before detection (see Fig. S1 in the supplemental material).

Compared to an instrument limit of detection, the establishment of an environmental limit of detection poses more challenges, including dilute target concentrations, environmental impurities, background inhibitors, organisms in a viable but not cultivable state, and overall processing efficiency. There are many steps for processing environmental samples prior to detection. At each process step, there can be a loss of the initial target organism, and thus, each step has a recovery efficiency, which could be interpreted as a set number, distribution, or range (see Fig. S1 in the supplemental material). Since recovery efficiency directly affects the limit of detection, improving recovery efficiency would result in a more sensitive detection method.

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In determining if an environmental site is "clean," another component that should be evaluated is the quantification and characterization of the potential health risk. Quantitative microbial risk assessment (QMRA) is a method used to assess the likelihood of infection based on specific exposures to hazardous pathogenic organisms. QMRA risk modeling has been used with water and food and could be useful for management decisions during a disease outbreak or a bioterrorism attack (35). Environmental monitoring is used to inform the exposure assessment and the efficiency of disinfection. The limit of detection is a critical criterion for any method, which dictates the application and usefulness of demonstrating a "zero" during environmental monitoring. The limit of detection of a chosen analytical method is also an input variable for the QMRA model; a statistical distribution quantifying the variability in limit of detection is preferred for realistic modeling.

The objectives of this study were to review, in the literature, the instrument limit of detection and the environmental limit of detection for methods to detect *B. anthracis* and to compare the estimated risk at the instrument limit of detection and the environmental limit of detection. Though the number of articles on *B. anthracis* was extensive, there was a paucity of articles that specifically included environmental limits of detection. This information is essential for a QMRA of *B. anthracis* in the establishment of future environmental monitoring strategies and cleanup goals.

MATERIALS AND METHODS

Journal articles were searched on the ISI Web of Science database for *B. anthracis* and the following keywords: method, sensitivity, limit of detection, detection limit, limit, water, air, soil, fomite, surface, specificity, PCR sensor, environmental, rapid, assay, diagnostic, immunoassay, antibody, real time, real-time PCR, microfluidic, polymerase, quantitative, bioaerosol, aerosol, microarrays, biosensor, electrochemiluminescence (ECL), Raman spectrometry, and mass spectrometry. Approximately 1,700 references (and abstracts, when available) were retrieved and were saved in an EndNote file. Though the search defaults were set for the years 1900 through 2007, the oldest article used to evaluate the limit of detection was published in 1994. Abstracts were manually screened for information on the detection of *B. anthracis*. Some studies used a surrogate for *B. anthracis* to determine the limit of detection. It was assumed that *B. anthracis* would behave as the surrogate and was included in this review. If the abstract pertained to a detection method, then the full article was downloaded, saved in another database, and reviewed for quantitative data describing the limit of detection. The remaining references and abstracts that were not used in this literature review either did not indicate information about detection methods or were not retrievable. At the end, 71 articles were retrieved and analyzed to obtain the instrument limit of detection or the environmental limit of detection.

Instrument limit of detection. The instrument limit of detection was extracted from the articles describing a method that detected *B. anthracis* in a pure culture without spiking *B. anthracis* into an environmental matrix (soil, air, fomite, or water). Raw data that were extracted were recorded in numbers of units of cells, spores, DNA, CFU, protective antigens, and genomic copies in volumes that ranged from liters to microliters. Articles that used units of protective antigens were not used in this literature review due to the unknown conversion factor from antigens to cells. All data were converted into standard units of cells per milliliter of reaction solution, and the data by method were graphed and compared.

Environmental limit of detection. In studies reporting the environmental limit of detection, *B. anthracis* spores were spiked into the matrix, extracted, and detected using various detection methods. The articles that reported the environmental limit of detection of *B. anthracis* were categorized according to the matrix in which *B. anthracis* was detected (soil, air, fomite, or water). Additional parameters extracted from the articles varied with the matrix (see Fig. S2 in the supplemental material). These included the following parameters. (i) For soil, they include the amount of soil, sample concentration, extraction volume, volume of extracted sample added to the reaction, and total volume. In addition, the type of pretreatment or extraction method and the soil type or location were noted (Table 1). (ii) For air, they include the sample volume, airflow rate, duration, sample concentration, extraction volume, volume of extracted sample added to the reaction, and total volume. (iii) For fomites, they include the surface area, sample concentration, surface seeding method, extraction volume, and total volume. In some cases, recovery efficiency and extraction efficiency were available and noted. In addition, the type of fomite, sampling method, extraction method, and culturing method were noted (Table 2). (iv) For water, they included the sample volume, sample concentration, extraction volume, volume of extracted sample added to the reaction, and total volume. In addition, the condition of the water was noted.

Quantifying limits of risk estimates. The risk of mortality by inhalation of *B. anthracis* spores was estimated for concentrations corresponding to the instrument limit of detection and the environmental limit of detection in the air. For each limit of detection, a distribution of risks was calculated by the Monte Carlo method using 100,000 replicates in Crystal Ball 7.3.1 (2007; Oracle). The number of replicates was chosen at the point where the 90% confidence interval was stable over a range from 1/10 to 10 times the number of replicates used.

A recent evaluation of dose-response data for *B. anthracis* spores through the inhalation exposure route found that the dose-response relationship could be modeled by the exponential equation (4)

$$P(d) = 1 - e^{-kd}$$

where $P(d)$ is the probability of death (P) (when untreated) at dose d, and k is the probability that one organism will survive to initiate the response (4). In this study, a k value generated from a pooled guinea pig and rhesus monkey data set was used. A distribution of 10,000 best-fit k values generated using bootstrap replicates of that data set was provided by Timothy Bartrand of Drexel University and fit to a gamma distribution. The dose was calculated as

$$d = C_{\text{air}} \cdot R \cdot t$$

where C_{air} is the number of spores per cubic meter of air (instrument limit of detection or environmental limit of detection), R is the breathing rate (m^3/h), and t is the duration of exposure (h). When C_{air} was evaluated as a range of limits of detection, it was modeled as a lognormal distribution; otherwise, it was evaluated as a point estimate. The breathing rate, R, was modeled as a Pareto distribution fit to the short-term breathing rates of adults (18 years of age and up) of both sexes from rest to moderate activity (71). The exposure time, t, was modeled as a uniform distribution from 1 min to 8 h.

Five risk scenarios were evaluated with this model using different values for C_{air}. For each risk scenario, either the instrument limit of detection or environmental limit of detection, a sensitivity analysis was generated using Crystal Ball 7.3.1 (2007; Oracle). The median real-time PCR instrument limit of detection and the range of real-time PCR instrument limit of detection were two scenarios used to explore the effect of instrument limit of detection on risk. For the instrument limit of detection scenarios, it was assume that all *B. anthracis* spores in a cubic meter of air could be collected without any loss and concentrated into 1 ml of solution for analysis. Log-transformed real-time PCR and PCR instrument limits of detection were checked for normality with a Lilliefors test and compared using analysis of variance. Then, the range of PCR instrument limits of detection was combined with the range of real-time PCR instrument limits of detection to increase the data in the distribution.

There were three environmental limit of detection scenarios; C_{air} was set to the environmental limits of detection reported for *B. anthracis* detected in the air. There were only two articles on the environmental limit of detection in the air. Due to the lack of data on the environmental limit of detection, the two limits of detection were referred to as the lower and upper environmental limits of detection. These two risk scenarios were evaluated as point estimates. The last risk scenario assumed that the environmental limit of detection for the air fit the same distributions as the lognormal instrument limit of detection, ranging from 17,000 to 50,000 CFU/m^3 (this may not be the true range).

RESULTS AND DISCUSSION

Instrument limit of detection. Out of 56 articles on the instrument limit of detection, 17 articles were on real-time PCR (6, 7, 11, 23, 25, 27, 39, 41, 45, 49, 51, 53, 56, 58, 60, 70, 72), 6 were on PCR (13, 31, 48, 57, 76), 10 were on biosensors (1, 3, 21, 22, 33, 36, 40, 52, 73, 74), 5 were on microarray/PCR (5, 19, 50, 64, 75), 6 were on immunoassay (29, 30, 32, 46, 65,

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TABLE 1. Parameters for the environmental limit of detection in soil

Detection method[a]	Amt of soil	Sample concn	Pretreatment/extraction method (company)	Time (h)	Difficulty level	Extraction vol (μl)	Vol added to reaction (μl)	Total vol (μl)	Limit of detection (CFU/g soil)	Soil type/location	Reference
PCR-ELISA	100 g	1–100 CFU/100 g	Easy DNA kit (Invitrogen)	2.5	2	100	60	60	0.1	Nonsuspicious sites	10
	100 g	1–100 CFU/100 g	Easy DNA kit (Invitrogen)	2.5	2	100	60	60	1.0	Contaminated sites with organic compounds and tanning agents	10
Nested PCR + 2× cultivation in TSB	1 g	0, 1, 10, 10^2, 10^3 CFU/g	FastDNA SPIN kit	36.0	4			25	1.0	Garden soil with 3% peat	24
Nested PCR + cultivation in TSB	1 g	0, 1, 10, 10^2, 10^3 CFU/g	FastDNA SPIN kit	18	3			25	1.0×10^2	Garden soil with 3% peat	24
Nested PCR	1 g	0, 1, 10, 10^2, 10^3 CFU/g	FastDNA SPIN kit	2	2			25	1.0×10^3	Garden soil with 3% peat	24
	100 mg	10^6 CFU/100 mg	Three freeze-thaw cycles/glass beads and glass milk	3.5	5	30	5	25	1.0×10^5	Litter, meadow, cultivated, swamp, and lawn	63
PCR	1 g	2.5×10^3–2.5×10^7 CFU/g	Hot detergent/bead mill homogenization	1	3	100	10	100	2.5×10^3	Anthony fine sandy loam from New Mexico agriculture fields	44
Immunofluorescence	1 g	10^3–10^7 CFU/g	Aqueous polymer two-phase system	0.75	2	100	20	40	5.6×10^3	Sand	2
	1 g	10^3–10^7 CFU/g	Aqueous polymer two-phase system	0.75	2	100	20	40	1.4×10^4	Garden	2
Real-time PCR	0.1 g	10^3–10^7CFU/g	Heat treatment with 1.22 g/ml sucrose-0.5% Triton X-100	0.75	3	1,000	5	25	1.0×10^4	National Institute of Health—Korea	60
Multiplex PCR	0.1 g	10^3–10^7 CFU/g	Heat treatment with 1.22 g/ml sucrose-0.5% Triton X-100	0.75	3	1,000	1	25	1.0×10^5	National Institute of Health—Korea	60
	0.1 g	10^3–10^7 CFU/g	Heat treatment with sterilized water and 10% Triton X-100-PBS	1.5	3	1,000	1	25	1.0×10^8	National Institute of Health—Korea	60
IM-ECL	1 mg	0–10^6 CFU/assay	IM separation performed twice and resuspended in PBS	1.5	3				1.0×10^5	Moist, dark brown to black soil and dry, light yellowish sandy soil from diverse military and agriculture fields	18
	1 mg	0–10^6 CFU/assay	IM separation performed twice and resuspended in PBS	1.5	3				1.0×10^6	Moist, dark brown to black soil and dry, light yellowish sandy soil from diverse military and agriculture fields	18
	1 mg	0–10^6 CFU/assay	IM separation performed twice and resuspended in PBS	1.5	3				1.0×10^7	Moist, dark brown to black soil and dry, light yellowish sandy soil from diverse military and agriculture fields	18
Biosensor assay	1 mg/ml powder in PBS	3.2×10^3–3.2×10^5 CFU/ml	Washing with 1 ml PBST	0.25	1	30	5	25	3.2×10^8	Talc-based powder, cornstarch, confectioners' sugar, baking soda, and *Bacillus thuringiensis*-based pesticide	69

[a] TSB, Trypticase soy broth.

TABLE 2. Parameters for the environmental limit of detection on fomites

Study authors (reference)	Surface area	Sample concn	Surface seeding	Sampling method	Extraction method
Hodges et al. (38)	10 cm^2	0.2–3,000 CFU/cm^2	Inoculated with 0.5 ml spore solution	Macrofoam swab	Vortex in 5 ml PBST for 2 min at 10-s intervals
Rose et al. (59)	25 cm^2	2×10^4 CFU/cm^2	Inoculated with 0.5 ml spore solution	Cotton swab, macrofoam swab	Vortex in 5 ml PBST for 2 min at 10-s intervals
Brown et al. (15)	25 cm^2	100–10,000 CFU/cm^2	Dry aerosol deposition	Polyester-rayon blend gauze wipe	Sonication and heat treatment
Brown et al. (16)	25 cm^2	100–10,000 CFU/cm^2	Dry aerosol deposition	Rayon swab	Sonication and heat treatment
Brown et al. (14)	100 cm^2	100–10,000 CFU/cm^2	Dry aerosol deposition	Vacuum filter sock	Sonication and heat treatment
Buttner et al. (20)	1 m^2	10^5 CFU/m^2	Inoculated with spore solution	BiSKit—wet/dry	Foam compression

[a] The limit of detection was not recorded in the article and was calculated to be at least 20 CFU/cm^2.

68), 3 were on ECL (17, 34, 77), 2 were on enzyme-linked immunosorbent assay (ELISA) (12, 26), 3 were on Raman spectroscopy (37, 55, 78), and 4 were on mass spectrometry (8, 9, 28, 43) (Fig. 1). Limits of detection ranged from 10 cells/ml (for real-time PCR) to 10^8 cells/ml (for mass spectrometry). Considering the median instrument limit of detection, real-time PCR and PCR were the most sensitive methods, with median instrument limits of detection of 430 and 440 cells/ml, respectively. It should be noted that there was one instrument limit of detection (4.29×10^6 cells/ml) that was not added to the distribution for real-time PCR because it was a multiplex assay, and the other instrument limits of detection in the distribution were from a singleplex assay (42). The least-sensitive methods were Raman spectroscopy and mass spectrometry, with median instrument limits of detection of approximately 1.0×10^7 and 8.0×10^7 cells/ml, respectively.

The number of journal articles on real-time PCR and biosensors allowed limits of detection to be fit to a statistical distribution. When fewer articles were published, as was true for the other eight methods, assigning distributions was not possible. ECL, ELISA, Raman spectroscopy, and mass spectrometry (having less than four articles) were the methods with the least-sensitive instrument limits of detection. With limited information on these methods, the median instrument limit of detection may not properly represent these detection methods' capabilities for detecting *B. anthracis*. For example, the instrument limit of detection for ECL had only three published articles, with limits of detection ranging from 10^2 cells/ml to 10^6 cells/ml. For some emerging techniques, such as immunomagnetic ECL (IM-ECL) and aptamer-magnetic bead-ECL, limits of detection differed by 4 orders of magnitude. While the instrument limit of detection gives insight into the instruments' capabilities, when evaluating cleanup goals and assessing risk, the environmental limits of detection are needed to understand the challenges and capabilities for addressing the contamination.

Environmental limit of detection. Out of 15 articles on the environmental limit of detection, 8 articles were on detection in soil (2, 10, 18, 24, 44, 60, 63, 69), 2 were on detection in the air (47, 66), 6 were on detection on fomites (14, 15, 16, 20, 38, 59), and 1 was on detection in water (54). The results for the environmental limit of detection could not be reported as distributions due to the limited number of articles for each matrix. The two most predominant methods used for the environmental limit of detection were cultivation and PCR-based methods.

Soil. The environmental limit of detection of *B. anthracis* spiked into soil ranged from 0.1 (reported as 10 CFU/100 g of soil) to 3.2×10^8 CFU/g of soil, with a median limit of detection of 1.2×10^4 CFU/g of soil (Table 1). The median environmental limit of detection for soil should be used with caution, since there is a 9-orders-of-magnitude range due to the many approaches used to evaluate the environmental limit of detection. The approximate time for the extraction method (Table 1) was the time for one sample to be processed based on the information reported. If it was not an automated extraction procedure, then with the increase in samples, there would be an increase in extraction process time. The difficulty level for the extraction process (1 to 5, easy to difficult) was based on the number of steps in the procedure, the preparation time, and the approximated time for the extraction (Table 1). The biosensor assay, the easiest extraction method, resulted in the poorest limit of detection (3.2×10^8 CFU/g of soil). The detection methods with the most-sensitive limits of detection (PCR-ELISA, nested PCR, and PCR) had extraction methods with difficulty levels ranging from 2 to 5 (Table 1).

The environmental limit of detection depended highly on the pretreatment/extraction process; for instance, in 2003, Ryu et al. (60) used multiplex PCR and reported a difference of 3 orders of magnitude between heat treatment with 1.22 g/ml sucrose–0.5% Triton X-100 and heat treatment with sterilized water and 10% Triton X-100–phosphate-buffered saline (PBS) (Table 1). Similar results were found by Bruno and Yu in 1996 (18) when using IM-ECL as the detection method.

Differences in the environmental limit of detection were also based on the location or the type of soil. In 1999, Beyer et al. (10) reported that the PCR-ELISA method was more sensitive when using soils from nonsuspicious locations compared to

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TABLE 2—*Continued*

Extraction vol	Total vol	Recovery efficiency (%)	Extraction efficiency (%)	Culture medium	Limit of detection
5 ml		31.7–49.1	93.4	Sheep blood agar	12 CFU/cm^2
5 ml	100 μl	41.7 for cotton swab, 43.6 for macrofoam swab	93.9 for cotton swab, 93.4 for macrofoam swab	Trypticase soy agar with 5% sheep blood	20 CFU/cm^2[a]
30 ml	1 ml	35 for stainless steel, 29 for painted wallboard	93	Brain heart infusion agar	90 CFU/cm^2 for stainless steel, 105 CFU/cm^2 for painted wallboard
10 ml	1 ml	41 for stainless steel, 41 for painted wallboard	76	Brain heart infusion agar	1 CFU/cm^2 for stainless steel and painted wallboard
30 ml	1 ml	29 for stainless steel, 25 for painted wallboard, 28 for carpet, 19 for concrete		Petrifilm aerobic count plate	105 CFU/m^2 for stainless steel and carpet, 102 CFU/m^2 for painted wallboard, 160 CFU/m^2 for concrete
3.3 ml for wet sampling, 16.1 ml for dry sampling	1 ml	11.3 for wet sampling, 18.4 for dry sampling		Trypticase soy agar	42 ± 6 CFU/m^2 for wet sampling, 100 ± 10 CFU/m^2 for dry sampling

using those from former tannery sites. Agarwal et al. (2002) (2) reported that the immunofluorescence assay was more sensitive when spores were spiked into sand (10^3) rather than into garden soil (10^4). For the IM-ECL method, Bruno and Yu (1996) (18) reported differences due to different strains, with Sterne (10^5) being more sensitive in the assay than Ames (10^6) and Vollum B1 (10^7).

Air. There were only two studies of the evaluation of aerosolized *B. anthracis* spores collected by an air sampler and extracted for detection. The ELISA-biochip system coupled with a portable bioaerosol collection system collected aerosolized spores at an air sampling rate of 150 liters/min for 2 min into 5 ml of PBS. The ELISA-biochip system consisted of an ELISA for antibody-based identification in combination with the biochip detection instrument. The environmental limit of detection of the ELISA-biochip system was 17 CFU/liter. For the ELISA-biochip system, the efficiency of the air sampler was reported as approximately 50%, but the distribution was not fully described (66). The anthrax smoke detector collected aerosolized spores using a bioaerosol collection system at a rate of 15 liters/minute for 1 min onto a glass fiber filter tape. The detection of the spores using the lifetime-gated fluorimeter occurred after a thermal lysis and addition of $TbCl_3$. The environmental limit of detection of the anthrax smoke detector was 50 CFU/liter (47).

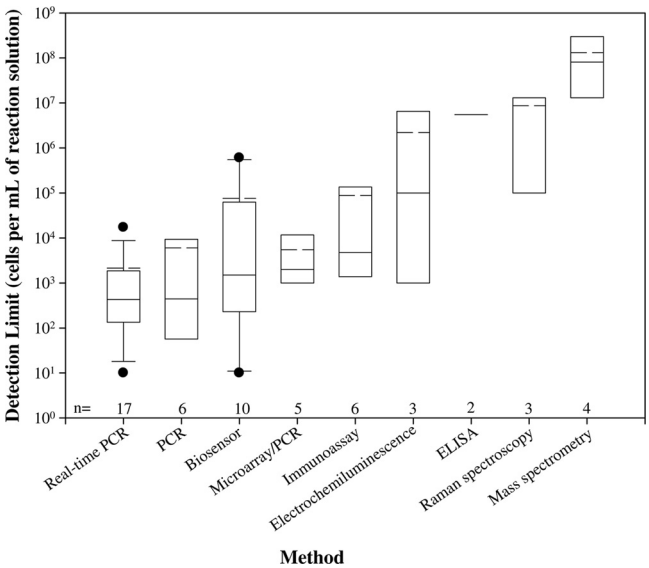

FIG. 1. Distribution of the instrument limits of detection for various methods. On each box plot, the solid line represents the median result, and the dashed line represents the mean result. The box plot whiskers above and below the box indicate the 90th and 10th percentiles, respectively. The solid circles represent the outlying limits of detection, and *n* represents the number of journal articles available on each detection method for *Bacillus anthracis*.

Fomites. Spores were seeded on fomites (stainless steel, plastic, wood, glass, etc.), recovered, extracted, and detected by cultivation. The environmental limit of detection was evaluated from stainless steel fomites ranging in surface area from 10 cm^2 to 1 m^2 (Table 2). In 2007, Brown et al. (14–16) also evaluated the environmental limit of detection on painted wallboard. In addition, the vacuum filter sock study tested porous fomites, carpet, and concrete (14).

The sampling methods evaluated by the articles were use of a macrofoam swab, cotton swab, polyester-rayon blend gauze wipe, rayon swab, vacuum filter sock, and biological sampling kit (BiSKit) (Table 2). Sampling methods such as use of cotton, macrofoam, polyester, and rayon swabs were all tested by Rose et al. in 2004 (59). It was concluded that the cotton and macrofoam swabs produced the highest recovery when the swabs were premoistened rather than dry. Similarly, in 2004, Buttner et al. (20) tested the BiSKit, cotton swabs, and foam swabs. The BiSKit was designed to do wet and dry sampling of large surfaces for bacteria, viruses, and toxins. The BiSKit resulted in the highest recovery out of the three methods. Using a wetting agent to recover spores from the surfaces enhanced the recovery and environmental limit of detection. Brown et al. (2007) (15, 16) used sterilized deionized water (except when using the vacuum filter sock), Buttner et al. (2004) (20) used potassium phosphate buffer with 0.05% Tween 20, and the other two authors used PBS with 0.04% Tween 80 (PBST).

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According to the CDC, the recommended wetting agents were sterile water, a sterile saline solution, or a sterile phosphate-buffered solution (http://www.bt.cdc.gov/Agent/Anthrax/environmental-sampling-apr2002.asp).

The detection method used for all fomite studies was cultivation; however, a different agar was used in each study. The focus of the Rose et al. (2004) (59) article was achieving the best recovery, and the study did not determine an environmental limit of detection. From the information given in the article, the environmental limit of detection was calculated by using the initial suspension concentration, the surface area, and the lowest recovery reported. The calculated environmental limit of detection was approximately 20 CFU/cm^2.

The recovery efficiencies for all the fomite studies ranged from 10 to 50%, and the extraction efficiencies ranged from 75 to 99%. Recovery of *B. anthracis* spores from fomites depends on many parameters, such as fomite type, sampling procedure, and sampling processing for detection. The recovery efficiency from the sampling method was primarily the controlling factor in determining the limit of detection and secondarily the efficiency from the extraction method.

Interestingly, in survival studies using cultivation as the detection method on fomites, surface characteristics, relative humidity, and temperature were the most important contributors to viability (62). It was not clear whether recovery and limit of detection changed with time in the environment, as this was difficult to differentiate from survival/degradation of the target. However, this distinction could be made by adding a marker along with the biological agent that does not degrade. For environmental monitoring, the separate time dependence of survival and recovery will be critical to define in future studies. Only the articles from Brown et al. in 2007 (14–16) reported and maintained the relative humidity and temperature in the fomite studies at 30% ± 10% and 25 ± 2°C, respectively. Determining and maintaining the relative humidity and temperature that are most optimal for viability may increase recovery efficiency. In addition, this information could be used at a contaminated site to inform first responders of the possible viability of remaining levels of the biological agent of concern.

Water. The spores were spiked into a volume of water, filtered through a 0.2- to 0.45-μm-pore-size filter, extracted from the filter, and then detected by various methods. The main challenge for detection of *B. anthracis* in water was the ability to concentrate the sample. If the sample was too dilute, then the number of *B. anthracis* cells per liter of water could fall below the environmental limit of detection. When the sample is concentrated, some loss of the initial cells is likely.

There was only one article that evaluated the detection of *B. anthracis* in water; the lack of articles could be due to this matrix being less likely a vehicle for transmission (62). Perez et al. (2005) (54) spiked *B. anthracis* spores into tap and source water in volumes ranging from 0.1 to 10 liters. Sample concentrations were detected using sheep red blood cell agar plates, *B. anthracis* chromogenic agar plates (R&F Laboratories), PCR, or nested PCR.

Cultivation was used to determine the viability of the organisms in the sample, and PCR was used to confirm the identities of any suspect colonies. When using the cultivation approach for the source water samples (Chesapeake Bay and Patuxent River), overgrowth of nontargeted flora occurred in all studies. PCR was only successful for testing source water when the sample concentrations were at least 26 CFU/ml. The environmental limit of detection for tap water was reported as 10 CFU/10 liters using the cultivation methods, while for PCR-based methods, the environmental limit of detection decreased to 534 CFU/liter. Though the PCR-based methods have a rapid detection time compared to that of the cultivation methods (more than 24 h), in this case, PCR was less sensitive. Challenges, such as loss of initial cells, could occur when concentrating large sample volumes (i.e., 10 liters) into 5 to 10 μl for the PCRs.

Quantifying limits of risk estimates. Five risk scenarios using instrument limits of detection and environmental limits of detection for C_{air} were evaluated. Log-transformed PCR and real-time PCR instrument limits of detection were normally distributed (Lilliefors test, P of 0.65 for PCR, P of 0.78 for real-time PCR) and were not significantly different (analysis of variance, P of 0.94). Therefore, the PCR and real-time PCR instrument limit of detection distributions were combined to increase the data set for the real-time PCR instrument limit of detection. With the assumption of 100% recovery, the median risk when C_{air} equaled the median real-time PCR instrument limit of detection was 0.006. When C_{air} was modeled with a lognormal distribution of real-time PCR instrument limits of detection, the estimated risk was 0.0062. The median risk of death from the inhalation of the entire dose of *B. anthracis* at the environmental limit of detection in air was 0.22 at the lower reported environmental limit of detection and 0.52 at the upper environmental limit of detection. Assuming that the environmental limit of detection would have a similar distribution as the instrument limit of detection (lognormal) and ranged from 17,000 to 50,000 spores/m^3, the median risk of death was 0.32 (Table 3). This assumption should be further evaluated with environmental studies to confirm that the environmental limit of detection would have the same distribution as the instrumental limit of detection. These risk estimates assumed that 100% of the spore sample was inhalable. Risk estimates were also reported for the percentages of 66.5%, 10%, and 1% of spores in the sample that were inhalable or respirable (Table 3). Approximately 70% of inhaled air volume actually contacts alveoli in the lungs, allowing spores to enter the body (71). In addition, the 5th and 95th percentiles of each risk distribution were used to define a 90% confidence interval for each risk estimate (Table 3).

A sensitivity analysis of the risk model was generated by Crystal Ball 7.3.1 (2007; Oracle) for each of the five risk scenarios. For the real-time PCR instrument limit of detection lognormal distribution, the limit of detection (79.4%) was the most sensitive factor in determining risk, followed by the exposure time (11.7%) and breathing rates (8.4%). The dose-response function parameter k (0.5%) had the least impact on the risk estimates. Similarly, for the assumed environmental limit of detection lognormal distribution, the analysis resulted in the exposure time (45%) being the most significant factor, followed by the limit of detection (27.5%), breathing rates (26.1%), and the k parameter (0.9%). The median real-time PCR instrument limit of detection and the two environmental limit of detection (lower and upper) scenarios resulted with the exposure time being the dominant factor in determining risk, followed by breathing rates and the k parameter. C_{air} values in

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TABLE 3. Risk estimates using the instrument limit of detection and environmental limit of detection scenarios

Risk scenario	Analyzed limit of detection	Percentile	Estimates of risk for % of sample inhaled			
			100%	66.5%	10%	1%
Real-time PCR median instrument limit of detection	429 cells/ml	5th	0.007	0.0047	0.0007	0.00007
		Median	0.006	0.0042	0.00063	0.000063
		95th	0.037	0.025	0.0038	0.00038
Real-time PCR instrument limit of detection	10–34,300 cells/ml	5th	0.0001	0.000067	0.00001	0.000001
		Median	0.0062	0.0041	0.00062	0.000062
		95th	0.28	0.19	0.032	0.0032
Lower environmental limit of detection in the air	17,000 CFU/m^3	5th	0.026	0.017	0.0026	0.00026
		Median	0.22	0.15	0.025	0.0025
		95th	0.78	0.63	0.14	0.015
Upper environmental limit of detection in the air	50,000 CFU/m^3	5th	0.075	0.051	0.0078	0.00078
		Median	0.52	0.39	0.071	0.0073
		95th	0.998	0.98	0.46	0.06
Assumed environmental limit of detection in the air	17,000–50,000 CFU/m^3	5th	0.03	0.02	0.003	0.00031
		Median	0.32	0.23	0.038	0.0038
		95th	0.94	0.85	0.25	0.028

these scenarios are point estimates rather than a distribution; therefore, the limit of detection was not a measured parameter in the sensitivity analysis.

Even assuming perfect sample collection and processing (no loss in initial concentration), the estimated risk at the instrument limit of detection was far above the commonly used 1:10,000 level. Environmental limits of detection increase due to the imperfect efficiency of sample collection and processing, increasing the risk at these higher detectable concentrations. These risk estimates show that, using current techniques reported in the literature, even allowing for all possible improvements in collection technology, any detectable *B. anthracis* constitutes an unacceptable risk. Moreover, these estimates define the lowest risk that could be determined from measurement, quantifying the risk that can exist even when no *B. anthracis* was detected.

Finding significant risk at *B. anthracis* limits of detection suggests that direct measurement will rarely be adequate for declaring a contaminated site as "clean," and alternative approaches (e.g., extrapolating from demonstrated log reductions) are needed. For fomites, soil, and water, further work is needed regarding the probability of infection by ingestion and contact before one can adequately address limits of detection and risk estimates. Direct measurement could, at best, reveal a catastrophic failure of decontamination. With respect to preventative monitoring, these estimates showed that significant risk was posed by undetectable concentrations of *B. anthracis* spores. This means that a low-concentration *B. anthracis* release would be more likely to be detected by the symptoms in exposed humans rather than by current sampling technology. Where there was danger or suspicion of a *B. anthracis* release, close monitoring of human health would be needed, in addition to environmental sampling in order to ensure timely medical treatment. Health monitoring alone may be preferred where resources are limited.

The risk assessment approach presented here could be further improved if an experimental probability distribution of the estimated dose was available. However, such a probability distribution was not available even for the most common matrix (soil). To obtain such a distribution, a large number (e.g., 30) of different true doses must be spiked in the environmental matrix of interest, and the sample must be processed through an entire protocol. This time-consuming process has not yet been reported.

Conclusion. Instrument and environmental limits of detection are necessary for QMRA when evaluating exposure to human pathogens in a contaminated environment. Due to the lack of pertinent data on the detection of *B. anthracis*, the environmental limit of detection could not be represented as a distribution. These distributions were necessary for estimating the risk at the environmental limit of detection. Even so, it was clear that environmental samples may be expected to have broad distributions due to the many challenges in sample processing that affect the limit of detection. More environmental detection studies need to be conducted in order to produce distributions similar to those of the instrument limit of detection. This will improve the risk assessment and improve the applicability of the information in regard to survival and cleanup goals, providing valuable information for first responders.

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