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A simple undergraduate laboratory exercise for teaching the role of sentinel-level clinical microbiology testing in biological threat identification

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ABSTRACT Undergraduate students in the biomedical sciences are mostly unaware of how clinical microbiology laboratories handle suspected agents of bioterrorism or emerging infectious diseases. The Public Health Security Bioterrorism Preparedness and Response Act of 2002 requires the US Department of Health and Human Services (HHS) to maintain a list of microbes that pose serious biological threats to human health and safety, including Tier 1 agents with the potential for use in bioterrorism. The Laboratory Response Network (LRN), founded by the Centers for Disease Control and Prevention, the Federal Bureau of Investigation, and the Association of Public Health Laboratories, coordinates the response of sentinel, reference, and national laboratories to these biothreats. The sentinel laboratories, which comprise most hospital-based and commercial laboratories, are the first to encounter a suspicious agent. For this reason, the LRN has published a series of testing guidelines to assist the sentinel laboratories in deciding whether a microbial isolate should be considered potentially hazardous and thus sent to a reference or national laboratory for further characterization. Here, we describe a simple laboratory exercise that teaches sentinel-level testing requirements in the context of an applied setting of a potential outbreak of anthrax that would require a sentinel laboratory to recognize a potential threat, attempt to rule it out, and refer to a national laboratory for identification.

KEYWORDS laboratory response network, sentinel laboratory, bioterrorism, diagnostic testing, biothreat identification, undergraduate teaching, medical microbiology, anthrax, *Bacillus anthracis*

Although students in undergraduate microbiology courses are familiar with what bioterrorism means, they are less familiar with the role of diagnostic microbiology laboratories in the protection against deliberate acts of bioterrorism or emerging infectious diseases. The Public Health Security Bioterrorism Preparedness and Response Act of 2002 was enacted to require the US Department of Health and Human Services (HHS) to assemble a list of those bacteria, viruses, fungi, and toxins that pose an elevated risk to human health and public safety (1). Within this list of "select biological agents and toxins" is the subcategory of "Tier 1 select agents," comprising organisms with intrinsic characteristics that make them particularly amenable to exploitation for bioterrorism, such as ease of aerosol transmission, a low infective dose, and the ability to cause a high rate of morbidity and mortality (2).

Clinical microbiology laboratories are on the front lines for detection of biothreat (BT) agents or emerging infectious diseases. The Laboratory Response Network (LRN) was established in 1999 by the Centers for Disease Control and Prevention (CDC), the Federal Bureau of Investigation (FBI), and the Association of Public Health Laboratories (APHL), to integrate the response of clinical laboratories to these high priority threats

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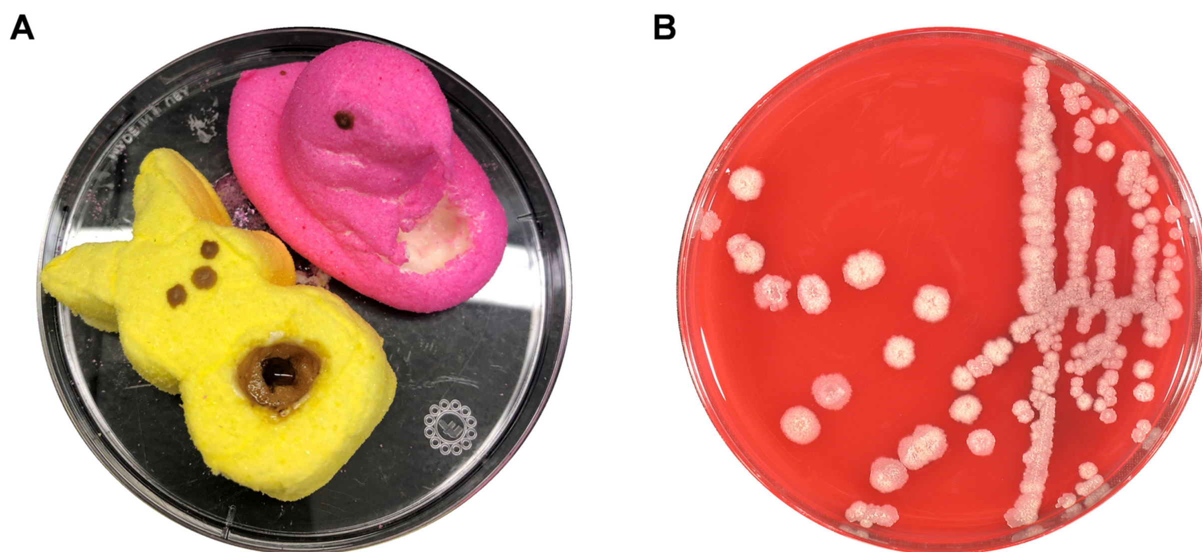

FIG 1 Surrogate materials to teach the role of sentinel-level laboratories in BT threat identification. (A) Marshmallow peeps prepared to display a fluid-filled "cutaneous ulcer" containing the suspect agent. The peep on the right is uninoculated. (B) A 24-h culture of *B. amyloliquefaciens* on a blood agar plate at 30°C, revealed a similar colony morphology to that of *B. anthracis*. For comparison to *B. anthracis*, the reader is directed to images held at the Public Health Image Library (PHIL) of the CDC (https://phil.cdc.gov/ImageidSearch.aspx). For example, image ID# 11752.

(3). The network consists of a three-tiered system encompassing sentinel, reference, and national laboratories. The sentinel laboratories, which include most hospital-based and commercial laboratories, are responsible for applying simple phenotypic tests to attempt to rule out the possibility that a suspicious microbial isolate is a BT agent, focusing on a limited number of easily accomplished procedures that minimize the expansion of potentially hazardous subcultures. If the sentinel laboratory is unable to rule out an isolate as a BT agent, the organism is submitted to State Department health laboratories for additional testing and, if necessary, large national laboratories such as the CDC.

Bacillus anthracis, the causative agent of anthrax, is considered a Tier 1 select agent because of its potential for causing a mass casualty event and its prior history of intentional use in bioterrorism (4, 5). Infections with *B. anthracis* are primarily zoonotic, affecting herbivores that encounter *B. anthracis* spores in contaminated soil (6). Humans may then become infected from direct exposure to livestock, or to contaminated animal products such as homemade cowhide drums that contain the bacterium or its spores. Infection can occur in one of three forms: gastrointestinal (GI), inhalational, or cutaneous disease. GI anthrax arises following ingestion of meat that is contaminated with *B. anthracis* spores, whereas the inhalational form is caused by spore inhalation. In either case, the germinating spores release exotoxins into the surrounding tissue, resulting in extensive tissue damage that is life-threatening. The majority (>95%) of human anthrax cases are the cutaneous form (7). Cutaneous anthrax is acquired when the spores enter through breaks in the skin and release at least two exotoxins as they germinate, resulting in a fluid-filled vesicle that ulcerates and develops a black necrotic center (eschar); edema factor toxin triggers pronounced edema and lethal factor toxin is responsible for the death of surrounding tissues (6). The LRN has established sentinel-level clinical laboratory guidelines to rule out the possibility that an isolate could be *B. anthracis*, regardless of which clinical form it was cultured from (8). The guidelines are based on the fact that *B. anthracis* can be differentiated from other Bacillus species using a panel of straightforward first-line laboratory tests. Following these guidelines, we have designed a simple laboratory exercise that teaches these basic microbiological tests in the context of an applied setting that would require a sentinel laboratory to recognize the threat, attempt to rule it out, and refer for identification.

PROCEDURE

Intended audience

The exercise was designed for undergraduate college students in years 2–4 of a medical sciences program. It has been implemented over the past 4 years as an applied exercise within a larger laboratory component of an elective microbiology course that teaches other microbiological principles and techniques.

Safety considerations

The course follows comprehensive Guidelines for Biosafety in Teaching Laboratories established by the American Society for Microbiology (ASM), using organisms that require Biosafety Level-1 (BSL-1) practices. However, BSL-2 guidelines are routinely followed to propagate a culture of laboratory safety (see supplementary materials for details). A discussion of these practices is also covered in a course lecture prior to the start of the laboratory.

Materials and methods

Automated identification systems such as Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) mass spectrometry are contraindicated for potential BT agents because of the hazards associated with specimen manipulation, combined with the possibility for misidentification (1). This exercise is designed to illustrate the approaches taken by a sentinel laboratory when an isolate, usually a *Bacillus* species, has a similar morphology to *B. anthracis*. There are 10 steps to the procedure:

- Step 1. Working in pairs, students are presented with a clinical scenario in which there is an outbreak of suspected cutaneous anthrax. Taking inspiration from a publication by Dahl and Gatlin (9), the "infected animal" is simulated by a marshmallow peep that is prepared in advance to reveal an edematous, fluid-filled vesicle consistent with cutaneous anthrax (Fig. 1). The vesicle has been previously inoculated with a culture of *Bacillus amyloliquefaciens*, representing a species that shares characteristics with *B. anthracis*. Students are instructed to streak the fluid for isolation onto a blood agar plate.
- Step 2. Rule-out criterion: colony morphology. *B. anthracis* colonies are described as non-hemolytic, forming large (2–5 µm), flat, off-white/gray colonies with a rough/ground glass surface and irregular borders (10, 11). Since these features are similar to *B. amyloliquefaciens*, and students are provided with control bacteria that form colonies that clearly differ from this description (see Table S1, supplementary materials), undergraduate students are unable to rule out *B. anthracis* at this point.
- Step 3. Rule-out criterion: Gram morphology. Students are instructed to Gram stain their isolate as part of a larger class exercise on Gram stain identification. Both *B. anthracis* and *B. amyloliquefaciens* form large, Gram-positive rods, often in chains, preventing rule out.
- Step 4. Rule-out criterion: catalase activity. Students are instructed to evaluate their isolate for catalase activity, using control strains that give positive and negative reactions for comparison. Although this is performed on the bench, students are informed that this, and other high-risk steps, would be performed in a biosafety cabinet to ensure the safety of laboratory personnel. Both *Bacillus* species are catalase positive, preventing a rule out.

- Step 5: Rule-out criterion: differential and selective media. Students are instructed to test their suspect isolate for growth on MacConkey agar as part of a laboratory exercise exploring the utility of differential and selective media, using other organisms as controls. Neither *B. anthracis* nor *B. amyloliquefaciens* grow on MacConkey agar, preventing a rule out.
- Step 6: Rule-out criterion: Antibiotic susceptibility testing. Students are instructed to examine their suspected isolate for susceptibility to penicillin G as part of a more comprehensive laboratory exercise on antibiotic susceptibility profiling. Both *B. anthracis* and *B. amyloliquefaciens* are sensitive to penicillin G, preventing a rule out.
- Step 7: Rule-out criterion: Phage typing. Since the specificity of gamma phage for *B. anthracis* is very high (>95%), susceptibility to the lytic action of this phage can be exploited as a diagnostic method for *B. anthracis* (12). The technique involves the spotting of a solution of gamma phage onto a lawn of the suspect bacteria. If the bacteria are susceptible, a zone of clearance will appear in the spotted area due to the lytic action of the phage on susceptible bacteria. Since phage typing is not available in some undergraduate laboratories, we took inspiration from a surrogate phage typing exercise designed by Khan and Read in which a lawn of bacteria is spotted with an alternative compound that kills the bacteria in the spotted area, creating a zone of inhibition similar to what is observed with lytic phage (13). After spotting the surrogate "phage" (a solution of ampicillin) onto the surface of a lawn of *B. amyloliquefaciens* and incubating overnight, a clear zone is evident around the spotted area that is morphologically indistinguishable from the effects of a lytic phage, thereby demonstrating the principle of gamma phage susceptibility and eliminating a rule out.
- Step 8. Rule-out criterion: Motility testing. Students are instructed to evaluate their isolate for motility as part of a class exercise on microbial motility, using motile and non-motile bacteria as controls. *B. amyloliquefaciens* is motile, whereas *B. anthracis* is not, allowing the students to finally eliminate the possibility that their isolate is *B. anthracis*. From the perspective of the clinical laboratory, this point of the analysis would remove any concern that the agent is *B. anthracis*, allowing them to proceed with further identification procedures if needed.
- Step 9: MALDI-TOF mass spectrometry in diagnosis. In addition to monitoring for BT agents, sentinel laboratories are charged with identifying emerging infectious diseases. Although the suspected isolate in this exercise was phenotypically ruled out as *B. anthracis*, its association with the cutaneous lesion outbreak in step 1 makes it a potential source of an emerging infectious disease. MALDI-TOF is increasingly used to identify microbial pathogens in the clinical diagnostic laboratory (14). In this technique, a microbial colony is embedded within a matrix and a laser is used to ionize the sample, resulting in particles that can be separated according to their mass-to-charge ratio and measured by the time it takes for those ions to travel to a detector at the end of a time-of-flight tube. The resulting spectral output is then matched to spectra from known microbes, and the machine uses an algorithm to indicate the level of confidence in the identification. After anthrax is ruled out by the application of the simple laboratory tests outlined in this exercise, clinical laboratories that are equipped with a MALDI-TOF mass spectrometer would proceed with this technology to identify the unknown isolate, or to refer to an outside laboratory that is capable of doing so. For step 9 in this exercise, students are told that the peep microbe has been submitted for MALDI-TOF analysis, and they are provided with the output from a *B. amyloliquefa-*

ciens MALDI-TOF analysis (Fig. S2). The result was unable to identify the species beyond a *Bacillus* group identification, which is a well-known limitation of this technology for highly related species (15).

- Step 10: sequencing in diagnosis. Since MALDI identification was not successful, the organism was submitted for rDNA sequencing. Students are provided with the output of a 16S rDNA sequence from *B. amyloliquefaciens* and challenged to use a BLAST search at the National Center of Biotechnology Information to identify the organism.

Assessment

At the conclusion of each of the first eight steps, students are directed to complete an online quiz that asks two questions:

1. Were you able to rule out the possibility that the suspected agent is *B. anthracis* based on the result of your test?
2. Explain your answer to question (1) and upload a photograph of your data to back up your conclusion.

For step 9 (MALDI-TOF mass spectrometry) and step 10 (rDNA sequencing), the students are challenged to use the literature to answer the following questions:

- How does MALDI-TOF identify microbes?
- Why was MALDI able to identify the *Bacillus* group, but not the species?
- What do the E-value and % identity values represent in the BLAST search results?
- What is the organism identification?
- Is the identified organism a common human pathogen? Where is it found in nature?
- How is the PCR technique able to amplify 16S rDNA from all species within a genus, yet still able to discriminate between species using the amplified product?

Potential modifications

Potential modifications include the following:

1. The exercise was designed to be an applied exercise within a laboratory sequence that complements a course in medical microbiology. The modular nature of the exercise's content allows for flexibility in terms of spreading out the procedures across multiple weeks, as we have done, or by combining the various tests into fewer laboratory sessions.
2. For microbiology majors, add a level of complexity by beginning with a polymicrobial infection, and/or adding a rule-out criterion exercise based on the APHL guidelines for Gram-negative bacilli/coccobacilli with biothreat potential (8).
3. At the conclusion of step 10, challenge the students to design an experiment using Koch's postulates to determine whether the organism is the causative agent of the cutaneous lesion outbreak, following the educational exercise developed by Dahl and Gatlin (9).

4. If an instructor wishes to build upon the theory and application of mass spectroscopy, the section on MALDI can be expanded, following a published educational exercise on MALDI-based microbial identification (16).

CONCLUSION

This exercise consists of a series of low-risk, inexpensive laboratory tests designed to raise student awareness of the central role of sentinel laboratories in biothreat protection, as well as to reinforce key principles in clinical microbiology such as colony morphology, hemolysin production, differential and selective media, catalase production, gram morphology, antibiotic susceptibility, phage typing, motility, MALDI-TOF mass spectrometry, and rDNA sequencing.

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AUTHOR CONTRIBUTIONS

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1 (jmbe00106-23-s0001.pdf). Expected result for surrogate phage typing procedure.
Fig. S2 (jmbe00106-23-s0002.pdf). MALDI-TOF output.
Supplemental material (jmbe00106-23-s0003.pdf). User-ready presentation of the lab exercise.

REFERENCES

1. Public Law 107-188. 2002. Public Health Security and Bioterrorism Preparedness and Response Act of 2002. Available by searching "Public Law 107-188" or by pasting the following into a browser. Available from: https://www.govinfo.gov/content/pkg/CRPT-107hrpt481/pdf/CRPT-107hrpt481.pdf
2. Pillai SP, Fruetel JA, Anderson K, Levinson R, Hernandez P, Heimer B, Morse SA. 2022. Application of multi-criteria decision analysis techniques for informing select agent designation and decision making. Front Bioeng Biotechnol 10:756586. https://doi.org/10.3389/fbioe.2022.756586
3. Villanueva J, Schweitzer B, Odle M, Aden T. 2019. Detecting emerging infectious diseases: an overview of the laboratory response network for biological threats. Public Health Rep 134:16S–21S. https://doi.org/10.1177/0033354919874354
4. Chugh T. 2019. Bioterrorism: clinical and public health aspects of anthrax. Curr Med Res Pract 9:110–111. https://doi.org/10.1016/j.cmrp.2019.05.004
5. Mondange L, Tessier É, Tournier J-N. 2022. Pathogenic *Bacilli* as an emerging biothreat. Pathogens 11:1186. https://doi.org/10.3390/pathogens11101186
6. Moayeri M, Leppla SH, Vrentas C, Pomerantsev AP, Liu S. 2015. Anthrax pathogenesis. Annu Rev Microbiol 69:185–208. https://doi.org/10.1146/annurev-micro-091014-104523

7. Kamal SM, Rashid AKMM, Bakar MA, Ahad MA. 2011. Anthrax: an update. Asian Pac J Trop Biomed 1:496–501. https://doi.org/10.1016/S2221-1691(11)60109-3
8. Association for Public Health Laboratories. 2018. "Recognize. rule out. refer. Biothreat agent bench cards for the Sentinal laboratory. Available by searching APHL.Org for "bench cards" or by pasting the following into a Browser". Available from: https://www.aphl.org/aboutAPHL/publications/Documents/2018_BiothreatAgents_SentinelLab_BenchCards_WEB.pdf
9. Dahl JL, Gatlin W III. 2018. "A microbiology teaching lab: using koch's postulates to determine the cause of "peep pox" in marshmallow peeps". Am Biol Teach 80:676–679. https://doi.org/10.1525/abt.2018.80.9.676
10. Beesley CA, Vanner CL, Helsel LO, Gee JE, Hoffmaster AR. 2010. Identification and characterization of clinical *Bacillus* spp. isolates phenotypically similar to *Bacillus anthracis*. FEMS Microbiol Lett 313:47–53. https://doi.org/10.1111/j.1574-6968.2010.02120.x
11. Spencer RC. 2003. *Bacillus anthracis*. J Clin Pathol 56:182–187. https://doi.org/10.1136/jcp.56.3.182
12. Abshire TG, Brown JE, Ezzell JW. 2005. Production and validation of the use of gamma phage for identification of *Bacillus anthracis*. J Clin Microbiol 43:4780–4788. https://doi.org/10.1128/JCM.43.9.4780-4788.2005
13. Khan LB, Read HM. 2018. A simple exercise for teaching bacteriophage concepts in the undergraduate laboratory using commercially available disinfectant. J Microbiol Biol Educ 19. https://doi.org/10.1128/jmbe.v19i2.1527
14. Evangelista AJ, Ferreira TL. 2022. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in the diagnosis of microorganisms. Future Microbiol 17:1409–1419. https://doi.org/10.2217/fmb-2022-0067
15. Muigg V, Cuénod A, Purushothaman S, Siegemund M, Wittwer M, Pflüger V, Schmidt KM, Weisser M, Ritz N, Widmer A, Goldenberger D, Hinic V, Roloff T, Søgaard KK, Egli A, Seth-Smith HMB. 2022. Diagnostic challenges within the *Bacillus cereus*-group: finding the beast without teeth. New Microbes New Infect 49–50:101040. https://doi.org/10.1016/j.nmni.2022.101040
16. Ng W. 2013. Teaching microbial identification with matrix-assisted laser desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS) and bioinformatics tools. J Microbiol Biol Educ 14:103–106. https://doi.org/10.1128/jmbe.v14i1.494