Valuated Methods for Removing Select Agent Samples from Biosafety Level 3 Laboratories

Alexandria E. Kesterson, John E. Craig, Lara J. Chuvala, Henry S. Heine

The Federal Select Agent Program dictates that all research entities in the United States must rigorously assess laboratory protocols to sterilize samples being removed from containment areas. We validated procedures using sterile filtration and methanol to remove the following select agents: *Francisella tularensis*, *Burkholderia pseudomallei*, *B. mallei*, *Yersinia pestis*, and *Bacillus anthracis*. We validated methanol treatment for *B. pseudomallei*. These validations reaffirm safety protocols that enable researchers to keep samples sufficiently intact when samples are transferred between laboratories.

The Federal Select Agent Program (FSAP), which is jointly administered by the Centers for Disease Control and Prevention and the US Department of Agriculture, designates high-risk organisms and guidelines for their safe handling. FSAP defines Tier 1 select agents as organisms that have the potential to be used as biological weapons (1). These organisms might infect humans, important agricultural species of plant and animal origin, or both. Various safety and security measures prevent these organisms from being inadvertently released into the environment or obtained by persons without authorized access. For example, researchers can handle these organisms only within Biosafety Level (BSL) 3 or -4 laboratories. Transfer of these agents into, out of, or between laboratories must be well-documented to ensure the safety of the public and research personnel (1, 2). In 2015, failures in the sample removal protocols led the US Army to inadvertently ship live *Bacillus anthracis* spores to several laboratories in the United States and other countries (3). *B. anthracis* is a Tier 1 select agent and therefore subject to the rules of FSAP. These samples were thought to have been inactivated by radiation, but lapses in protocol resulted in incomplete sterility (3). Afterward, the FSAP created additional regulations and guidance on how samples potentially containing select agents could be removed from BSL-3 and -4 laboratories.

FSAP requires that each inactivation or sterility method for sample removal be individually tested and validated, ensuring that these methods account for assay variability and technical limits of detection (2). The new guidance requires the entity developing the procedure to assess the risk that live material will remain in an inactivated sample (2). The FSAP recognizes that checking the sterility of all samples is impossible, but laboratories should minimize the risk for a viable select agent remaining within a sample believed to be inactivated. In addition, if an entity changes an already validated procedure, the entity must revalidate that procedure (2). Entities might develop their procedures from commonly accepted practices or from methods described in the literature (2). The entity must then use the appropriate controls to validate the effectiveness of the procedure. We defined the term validate to mean that a protocol, if followed exactly, renders select agent–containing samples sterile at the bacterial concentrations stated and that the sterility verification procedures identify protocol failures.

Our laboratory at the University of Florida (Orlando, FL, USA) evaluates therapeutics for the Tier 1 select agents *Francisella tularensis*, *Burkholderia pseudomallei*, *B. mallei*, *Yersinia pestis*, and *Bacillus anthracis*. We frequently conduct studies in which serum, plasma, bronchoalveolar lavage (BAL) fluid, or spent media must be transferred from the BSL-3 to the BSL-2 laboratory to conduct specific assays. These samples must be sufficiently intact so that we can evaluate drug, cytokine, chemokine, or enzyme levels and other host or bacterial components of interest. In most instances, chemical inactivation of the samples is not advisable. We selected 0.2-µm centrifuge filtration as the most effective method to sterilize small volumes of select agent–containing samples while maintaining other components in the samples. We describe and validate a standardized method using several different matrices.

Author affiliation: University of Florida, Orlando, Florida, USA

DOI: https://doi.org/10.3201/eid2611.191630
Measuring the intracellular levels of antimicrobial drugs in the BAL fluid is sometimes necessary to determine the amount of compound penetrating the site of infection within the cell. When it is necessary to measure the intracellular concentration, we treat the BAL cell pellet and the BAL fluid as 2 independent samples. Because the cell pellet sample cannot be filtered, we describe an additional procedure for removing BAL cell pellets from the containment laboratory.

Materials and Methods

Biosafety
We tested all protocols in a BSL-3 laboratory at the University of Florida, which is registered and licensed with the Centers for Disease Control and Prevention and the Animal and Plant Health Inspection Service, US Department of Agriculture, to conduct select agent research. The containment laboratory uses a high-efficiency particulate air filter to decontaminate discharged air. All staff must don facility-dedicated scrubs, Tyvek suits (Dupont, https://www.dupont.com), respiratory protection, double gloves, and shoe covers. All bacterial work is performed in a class II Biosafety cabinet, and all waste is removed using pass-through autoclaves.

Bacterial Strains and Growth Conditions
We used the following strains from the Biodefense and Emerging Infections Resource Repository: B. anthracis (Ames), Y. pestis (CO92), F. tularensis (SchuS4), B. pseudomallei (1026b), and B. mallei (China 7). We isolated B. anthracis spores according to Leighton and Doi (4) and maintained the spores in refrigerated sterile water at \( \approx 1 \times 10^{10} \) CFU/mL. We verified this concentration by serial dilution in sterile water onto sheep blood agar plates as previously stated (5).

We cultured Y. pestis CO92 from frozen stock on sheep blood agar (Becton Dickinson, https://www.bd.com) and incubated it for 48 h at 28°C. We then removed colonies from the stock plate and used them to inoculate sheep blood agar plates as previously stated (5).

We cultured B. mallei China 7 and B. pseudomallei 1026b from frozen stock vials on tryptic soy agar and incubated them at 35°C for 24–48 h to generate a stock plate of each strain. We selected 2–3 colonies from each incubated stock plate and inoculated them in brain heart infusion (BHI) broth (Becton Dickinson) overnight culture. We then incubated the cultures at 35°C with agitation for 16–20 h.

We also cultured F. tularensis SchuS4 from frozen stock onto chocolate agar (Becton Dickinson) and incubated it at 35°C for 48 h. We selected colonies from the agar plate and used them to inoculate a BHI culture containing 2% Isovitalex (Becton Dickinson). We incubated this culture for 18–20 h at 35°C with agitation.

Matrices
We tested the filtration protocol with murine lung BAL fluid, serum, plasma, and the listed culture mediums (Table 2). For the spore preparation, we used BHI as the culture media. We purchased the murine serum, plasma, and BAL from BioreclamationIVT (https://bioivt.com). We used mouse plasma from Balb/c mice collected in sodium citrate–containing tubes and pooled across sex. We also used mouse BAL and serum from Balb/c mice and pooled across sex.

Test Sample Preparation
All matrices had a final volume of 2 mL. We selected test sample starting concentrations that exceeded the maximum published bacterial concentrations (Table 1). We established a conversion factor for each species on the basis of serial dilution plate counts and optical density (OD) measurements at 600 nm (H. Heine, unpub. data). We used these conversion factors to determine the concentrations of overnight cultures and spore preparations. Y. pestis had a conversion factor of \( 5.34 \times 10^8 \) CFU/OD, B. mallei and B. pseudomallei 1.57 \( \times 10^9 \) CFU/OD, and F. tularensis 3.89 \( \times 10^9 \) CFU/OD.

Table 1. Maximum bacterial concentrations of select agents in tissues of infected mice*

| Agent (reference) | Source of samples, bacterial load | Lung, per g | Cell pellet, per mL BAL | Blood, per mL | Overnight culture, per mL |
|-------------------|---------------------------------|-------------|-------------------------|---------------|-------------------------|
| *Bacillus anthracis (5,6) | | \( <10^8 \) | Not tested | \( <10^8 \) | \( 10^8 \) |
| Yersinia pestis (7) | | \( <10^{10} \) | Not tested | \( <10^6 \) | \( 10^9 \) |
| Burkholderia mallei (8–11) | | \( <10^{10} \)† | Not tested | \( <10^4 \) | \( 10^9 \) |
| Burkholderia pseudomallei (11,12) | | \( <10^6 \) | \( 10^6 \)‡ | \( <10^8 \) | \( 10^9 \) |
| Francisella tularensis (13) | | \( 10^7 \) | Not tested | \( <10^5 \) | \( 10^9 \) |

*BAL, bronchoalveolar lavage.
†References (7) and (8) use a different strain of B. mallei
‡Value determined through in-house testing of lung samples.
For *B. anthracis* Ames strain, we prepared spores and spiked the different matrices. We used 20 μL of the spore preparation for BAL and culture medium samples. We diluted the spore preparation 1:1000 and used 20 μL of the diluted solution to spike each serum and plasma sample (Table 2).

We prepared test samples for *Y. pestis* from the incubated 100 mL broth culture. We took an OD reading from serially diluted broth culture and conversion factors to determine the culture concentration. We centrifuged 20 mL of this culture at 3,500 × g for 15 min. We then resuspended this pellet in 2 mL of BAL fluid (Table 2). We repeated the process for the culture medium. We inoculated serum and plasma samples with a centrifuged overnight culture (Table 2).

We prepared *B. mallei* test samples from the overnight broth cultures incubated previously. We prepared BAL fluid test samples by centrifuging 2 mL overnight broth culture at 3,500 rpm for 15 min and then resuspending the pellet in 2 mL of culture media. We inoculated serum and plasma samples with a centrifuged overnight culture (Table 2).

We prepared *B. pseudomallei* test samples for culture medium as stated for *B. mallei* and *Y. pestis* using the conversion factor. We prepared BAL fluid samples by adding 200 μL overnight culture to 1.8 mL BAL fluid (Table 2). We inoculated serum and plasma with 20 μL of overnight culture that had been diluted 1:10, then added 20 μL to each matrix (Table 2). We inoculated culture medium by centrifuging 20 mL of the overnight culture then suspending the pellet in 2 mL of culture media (Table 2).

We prepared *F. tularensis* samples for culture medium with a final concentration of 2% Isovitalex. We took an OD reading and used the conversion factor to concentrate samples appropriately. We centrifuged 20 mL of an overnight culture and resuspended it in culture medium with 2% Isovitalex. We spiked serum and plasma samples with 20 μL of an overnight culture that was first diluted 1:10 and inoculated BAL fluid with 20 μL of an overnight culture (Table 2).

### Methanol Test Sample Preparation

Test samples, positive controls, and the negative control of BAL fluid for the methanol treatment procedure all had a final volume of 500 μL. We used stock plates to grow bacteria, then selected colonies and suspended them in 3 mL of sterile water for injection (GE Healthcare, https://www.gehealthcare.com). We took an OD reading at 600 nm on a spectrophotometer (ThermoFisher Scientific, https://www.thermofisher.com) using a 1 cm² cuvette (ThermoFisher Scientific). We converted this value to an approximate CFU per milliliter value using a conversion factor as stated in test sample preparation. We calculated the total volume needed to spike each sample so that each sample would have 2 × 10⁶ CFU (Table 2).

### Filtration Procedure

We conducted all filtration test procedures in triplicate for each matrix type. For negative controls, we used un inoculated matrix samples. For positive controls, we used 100 μL of unfiltered inoculated test samples suspended in broth culture medium. We then placed 450 μL of each test sample into a clean 0.2 μm PALL Nanosep Bio-Inert centrifuge filter (Pall Corporation, https://www.pall.com) with a sterile microcentrifuge tube. In accordance with the manufacturer’s recommendations, we centrifuged the filters for 3 min at 14,000 g. We then transferred the filtrate to a clean tube and sealed it to prevent secondary contamination. We emphasize that the filtrate collection tubes should not be sealed with the same cap used to close the centrifuge filter before spinning.

### Table 2. Preparation of select agents in different matrices*

| Agent             | CFU/mL (matrix) | BAL fluid | Serum and plasma, μL | Culture | BAL cell pellet |
|-------------------|-----------------|-----------|----------------------|---------|----------------|
| *Bacillus anthracis* | 10⁹ (spore prep†) | 20 μL | 20§ | 20 μL | NT |
| *Yersinia pestis* | 10⁹ (overnight culture) | Resuspend pellet¶ | 20 | Resuspend pellet¶ | NT |
| *Burkholderia mallei* | 10⁹ (overnight culture) | Resuspend pellet¶ | 20** | Resuspend pellet¶ | NT |
| *Burkholderia pseudomallei* | 10⁹ (overnight culture) | 200 μL + 1.8 mL BAL | 20†† | Resuspend pellet §§ | 2 × 10⁹ CFU |
| *Francisella tularensis* | 10⁹ (overnight culture§§) | 20 μL | 20†† | Resuspend pellet §§ | NT |

†Sporas for aerosol challenge were maintained in sterile water and diluted to the nebulizer-challenge concentration of »1 × 10⁹ CFU/mL.
‡All broth cultures will require a 2% supplement with Isovitalex (Becton Dickinson, https://www.bd.com) to obtain growth of *F. tularensis*.
§§Dilute overnight culture 1:10 transfer 20 μL to serum or plasma.
##Dilute overnight culture 1:10 transfer 20 μL to BAL fluid.
††Centrifuge 20 mL of overnight culture, resuspend pellet in 2 mL BAL fluid or culture media.
‡‡Dilute overnight culture 1:100 transfer 20 μL to serum and plasma.
§§Dilute overnight culture 1:10 transfer 20 μL to BAL fluid.
NT, not tested.
because this cap could be contaminated with residual unfiltered sample and thus might yield false positive outcomes. We then suspended the filtrate in 4.5 mL BHI and incubated it at 35°C for 2 d. We incubated the positive controls in the same manner. After 48 h, we checked the tubes for turbidity and plated 5 × 200 µL samples onto the appropriate media. We incubated these samples at 35°C for an additional 7 d to ensure complete sterility. We considered this method to be validated only if all 3 replicates of all matrices were sterile in both broth and agar medium. Any failure, defined here as positive growth on agar or in broth media, prompted a review of the procedures. Once we determined the cause of the failure, we made the appropriate adjustments and reconducted the procedure in 3 replicates.

**Methanol Procedure**

We centrifuged BAL fluid for 5 min at 5,000 × g. We removed the supernatant and decontaminated it using the filtration procedure detailed in the previous section. We suspended the pellet in 500 µL of 80% methanol (ThermoFisher Scientific) and incubated it for 10 min. We placed 10% of this sample into 9.5 mL Dey-Engley neutralization broth (D/E media) (Becton Dickinson) and incubated it at 35°C for 24 h. We also observed that all samples remained sterile after plating on agar medium incubated for 7 d (Table 3). We determined that the test sample that had not been sterilized had sustained secondary contamination from the centrifuge filter unit cap. The PALL centrifuge filters are supplied as a filter and tube unit; they do not come with sterile secondary caps. To avoid secondary contamination, we transferred the filtrate to a clean tube immediately after spinning. We also observed that all samples were sterilized after treatment with 80% methanol and after incubation in broth culture for 5 d. The samples remained sterile on agar after an additional 2 d incubation.

**Discussion**

Validating sterility procedures is a time-intensive and costly necessity for removing select agent samples from BSL-3 laboratories. Researchers can streamline this process by publishing validated methods in peer-reviewed journals.

We described and validated reproducible procedures for select agent sample removal. However, researchers should ascertain that none of their sample is lost because of binding to the filter material. In this study, we checked 100% of the sample as a proof of concept, although we recognize the impossibility of incubating 100% of the sample to ensure sterility during actual experiments. Our laboratory now samples 10% of the filtrate to verify successful disinfection. We have found that these filters have an approximate failure rate of 0.1%; however, other researchers such as Dauphin et al. have found a failure rate closer to 3% (14). The differences in failure rates, variety of available filter membranes, and new methods of sterilization showcase the need for clear, detailed, and reproducible published methods.

**About the Author**

Ms. Kesterson is a doctoral candidate in biomedical sciences at the University of Florida. Her research interests include bacterial host pathogen interaction and antimicrobial countermeasures for biothreat pathogens and their associated immune responses.
References

1. US Department of Health and Human Services. Biosafety in microbiological and biomedical laboratories, 5th ed. Washington (DC): The Department; 2009.

2. Centers for Disease Control and Prevention; Animal and Plant Health Inspection Service. Guidance on the Inactivation or Removal of Select Agents or Toxins for Future Use. 2018 [cited 2019 Aug 4]. https://www.selectagents.gov/irg-intro.html

3. US Government Accountability Office. Actions Needed to Improve Management of DOD’s Biosafety and Biosecurity Program. 2018 [cited 2019 Aug 4]. https://www.gao.gov/products/GAO-18-422

4. Leighton TJ, Doi RH. The stability of messenger ribonucleic acid during sporulation in Bacillus subtilis. J Biol Chem. 1971;246:3189-95.

5. Heine HS, Shadowy SV, Boyer AE, Chuvala L, Riggins R, Kesterson A, et al. Evaluation of combination drug therapy for treatment of antibiotic-resistant inhalation anthrax in a murine model. Antimicrob Agents Chemother. 2017;61:e00788-17. https://doi.org/10.1128/AAC.00788-17

6. Heine HS, Bassett J, Miller L, Hartings JM, Ivins BE, Pitt ML, et al. Determination of antibiotic efficacy against Bacillus anthracis in a mouse aerosol challenge model. Antimicrob Agents Chemother. 2007;51:1373-9. https://doi.org/10.1128/AAC.01050-06

7. Heine HS, Chuvala L, Riggins R, Hurteau G, Cirz R, Cass R, et al. Natural history of Yersinia pestis pneumonia in aerosol-challenged BALB/c mice. Antimicrob Agents Chemother. 2007;57:2010–5. https://doi.org/10.1128/AAC.02504-12

8. Judy BM, Whitlock GC, Torres AG, Estes DM. Comparison of the in vitro and in vivo susceptibilities of Burkholderia mallei to Ceftazidime and Levofloxacin. BMC Microbiol. 2009;9:88. https://doi.org/10.1186/1471-2180-9-88

9. Mott TM, Johnston RK, Vijayakumar S, Estes DM, Motamedi M, Sbrana E, et al. Monitoring therapeutic treatments against Burkholderia infections using imaging techniques. Pathogens. 2013;2:383–401. https://doi.org/10.3390/pathogens2020383

10. Moustafa DA, Scarff JM, Garcia PP, Cassidy SKB, DiGiandomenico A, Waag DM, et al. Recombinant salmonella expressing Burkholderia mallei LPS O antigen provides protection in a murine model of melioidosis and glanders. PLoS One. 2015;10:e0132032. https://doi.org/10.1371/journal.pone.0132032

11. Lafontaine ER, Zimmerman SM, Shaffer TL, Michel F, Gao X, Hogan RJ. Use of a safe, reproducible, and rapid aerosol delivery method to study infection by Burkholderia pseudomallei and Burkholderia mallei in mice. PLoS One. 2013;8:e76804. https://doi.org/10.1371/journal.pone.0076804

12. Tan GG, Liu Y, Sivalingam SP, Sim S-H, Wang D, Paucod J-C, et al. Burkholderia pseudomallei aerosol infection results in differential inflammatory responses in BALB/c and C57Bl/6 mice. J Med Microbiol. 2008;57:508–15. https://doi.org/10.1099/jmm.0.47956-0

13. Heine HS, Chuaula L, Riggins R, Cirz R, Cass R, Louie A, et al. Natural history of Francisella tularensis in aerosol-challenged BALB/c mice. Antimicrob Agents Chemother. 2016;60:1834–40. https://doi.org/10.1128/AAC.02897-15

14. Dauphin LA, Bowen MD. A simple method for the rapid removal of Bacillus anthracis spores from DNA preparations. J Microbiol Methods. 2009;76:212–4. https://doi.org/10.1016/j.mimet.2008.10.009

Address for correspondence: Henry Heine, Institute for Therapeutic Innovation, University of Florida, 6550 Sanger Rd, Orlando, FL 32827, USA; email: henry.heine@medicine.ufl.edu

EID Podcast
Telework during Epidemic Respiratory Illness

The COVID-19 pandemic has caused us to reevaluate what “work” should look like. Across the world, people have converted closets to offices, kitchen tables to desks, and curtains to videoconference backgrounds. Many employees cannot help but wonder if these changes will become a new normal.

During outbreaks of influenza, coronaviruses, and other respiratory diseases, telework is a tool to promote social distancing and prevent the spread of disease. As more people telework than ever before, employers are considering the ramifications of remote work on employees’ use of sick days, paid leave, and attendance.

In this EID podcast, Dr. Faruque Ahmed, an epidemiologist at CDC, discusses the economic impact of telework.

Visit our website to listen: https://go.usa.gov/xfcmN

Emerging Infectious Diseases®

The COVID-19 pandemic has caused us to reevaluate what “work” should look like. Across the world, people have converted closets to offices, kitchen tables to desks, and curtains to videoconference backgrounds. Many employees cannot help but wonder if these changes will become a new normal.

During outbreaks of influenza, coronaviruses, and other respiratory diseases, telework is a tool to promote social distancing and prevent the spread of disease. As more people telework than ever before, employers are considering the ramifications of remote work on employees’ use of sick days, paid leave, and attendance.

In this EID podcast, Dr. Faruque Ahmed, an epidemiologist at CDC, discusses the economic impact of telework.

Visit our website to listen: https://go.usa.gov/xfcmN