Video Article

Principles and Practices for Safe Manipulation of Pathogenic Mycobacteria in a BSL-3 Environment Part 1

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URL: http://www.jove.com/video/3048
DOI: doi:10.3791/3048

Keywords: Biological safety, Mycobacterium tuberculosis, microbiology, molecular biology

Date Published: 6/15/2015

Citation: Brian, W., Vilchèze, C., Chen, B., Kim, J., Chen, M., Larsen, M.H., Jacobs, W.R. Principles and Practices for Safe Manipulation of Pathogenic Mycobacteria in a BSL-3 Environment Part 1. *J. Vis. Exp.* (), e3048, doi:10.3791/3048 (2015).

Abstract

Mycobacterium tuberculosis, one of the most successful bacterial pathogens, is transmitted by aerosol. Despite the prevalence of tuberculosis infection, estimated to include one third of humanity, our knowledge of the biology of the pathogen is limited. In order to hasten the development of new therapeutics, a deeper understanding of how tuberculosis is able survive and thrive in the host is urgently required. The safe experimental manipulation of *M. tuberculosis* requires the use of proper techniques in a BSL-3 laboratory.

In these three protocols we demonstrate the proper techniques required to safely perform microbiological and molecular biological experiments on *M. tuberculosis* in a BSL-3 laboratory. The core principles of minimization of the generation of infectious aerosols and redundant containment and decontamination are emphasized. Specific topics include the use of personal protective equipment, setting up the biosafety cabinet, inoculation of cultures, plating bacteria, picking bacterial colonies, centrifugation, sonication, electroporation, fixation of cultures, disposal of contaminated items, and decontaminating the biosafety cabinet.

Video Link

The video component of this article can be found at http://www.jove.com/video/3048/

Protocol

1. Introduction

This protocol will familiarize you with the principles and practices required for safe manipulation of pathogenic mycobacteria in a Biosafety Level 3 environment. The two core principles are:

1. Minimization of the production of infectious aerosols
2. Redundant containment of viable pathogenic organisms

2. Personal Protective Equipment

1. We begin with personal protective equipment (PPE), the final barrier between you and the pathogens you are studying, which will only be challenged if other redundant barriers have failed.
2. After entering the anteroom, label your tyvek suit with your name and the date.
3. Put on your suit, N95 respirator, hair cover, shoe covers – ensure the respirator forms a tight seal around your nose and face.
4. Prepare strips of tape to seal your gloves to your suit; put on gloves and seal to your suit with the tape; put on sleeves.
5. Before entering the containment lab, check the airflow indicator.
6. Enter the containment lab and immediately put on a second pair of gloves.

3. Setting up the Biosafety Cabinet

1. Check that items in the biosafety cabinet have been well separated for UV light treatment.
2. Open cabinet and cover the floor with absorbant pads; ensure that the pads cover the whole floor of the cabinet and do not block vents.
3. The primary disinfectant is a 1:100 solution of vesphene II SE in water, prepare the solution if necessary; fill a vesphene pot, spray bottle, and disposal bottle and place in the cabinet. Include some rubber bands in the pot and a blue towel for wiping items with disinfectant.
4. Prepare a red biohazard bag by labeling with your name and the date, prepare strips of tape, and attach the bag to the inside wall of the cabinet. Be sure to allow the bag to rest on the floor of the cabinet so it is not torn from the wall when it is used.
5. Prepare a disposal sleeve for serological pipettes; fill the bag with vesphene and place it in the sleeve.
6. Prepare vortex mixer by sealing in a biohazard bag with a twist tie and reinforcing the bag with a cross of tape.

7. Prepare a large biohazard waste bag by adding water and place in a waste barrel; label the barrel with your name and the date.
8. Make certain that you have properly prepared the cabinet and have placed all required materials (pipette tips, culture bottles, etc.) within before proceeding to work with live organisms.

4. Inoculating Cultures from Freezer Stocks

1. Identify the location of the desired freezer stocks; remove the appropriate box from the freezer and place in the cabinet on a non-porous surface.
2. Remove the rubber band and place in the vesphene pot, open the box and remove the desired tubes. Close box, wipe box, and replace rubber band.
3. Prepare a clean area by wiping and place box on it.
4. Next wipe and dispose of gloves; put on new gloves, wipe gloves, box, and remove box from cabinet.
5. The preceding procedure embodies the principles of double wipe and double contain. Live cultures, in this case the freezer stocks, are double contained, here within the tube and box, whenever outside the cabinet. Items leaving the cabinet are wiped twice, before and after putting on new gloves. It is critical that once you have put on new gloves that you only touch the vesphene soaked blue towel and the item you are wiping.
6. To inoculate the cultures begin by loosening caps of the culture bottles. It is best to prepare the culture bottles with the appropriate media before entering the BSL-3.
7. Adjust micropipette to appropriate volume; remove rubber band from pipette tip box and place in vesphene pot; open and arrange waste bottle.
8. Get pipette tip, draw up culture, and pause over vesphene pot for any drips.
9. Dispense culture avoiding the creation of bubbles; draw up vesphene, and dispose of tip in waste bottle.
10. As always, live cultures must be double contained to leave the cabinet. In this case, place the culture bottle in another sealed container for incubation.
11. Begin by wiping your gloves and creating a clean area. Wipe the bottles and place on the clean area. Wipe the interior of the box and wipe the bottles into the box.
12. If necessary, secure the bottles with Styrofoam and/or rubber bands to prevent them from overturning when the box is shaken during incubation.
13. Wipe the interior of the lid and seal the box. Re-wipe the clean area and wipe box to the clean area. Wipe and discard gloves. Put on new gloves, wipe gloves, wipe box, and remove the box from the cabinet.
14. Label the box with your name, the date, and the name of the strains.
15. Check that the boxes in the incubator are stable; open door and place your box in the incubator. Check that the temperature is correct.

5. Plating and Picking Colonies

1. Prepare the cabinet with spreaders, plates, and foil.
2. Wipe gloves; prepare dilution tubes with buffer.
3. Transfer culture into dilution tubes; be sure to pause over vesphene pot for any drops.
4. Briefly vortex dilution tube; continue with dilution series.
5. Ensure that plates are properly labeled; transfer suspension to plate.
6. Open spreaders and remove one; spread suspension until dry.
7. Transfer spreader to vesphene pot then waste bottle or pipette sleeve to discard.
8. Dispose of dilution tubes by adding vesphene, dipping in vesphene pot, and disposing in waste bag.
9. Wrap plates in foil and double wipe from the cabinet.
10. After removing plates from the cabinet, label with your name, date, and the strain.
11. To pick colonies, prepare culture bottle, plates, and wooden sticks.
12. Pick colony and place stick in culture bottle; remove stick to vesphene pot and seal bottle.
13. Dispose of sticks in waste bottle or pipette sleeve; wipe bag of sticks into plastic bag.

6. Disposing of Plates and Culture Bottles

1. Wipe foil-wrapped plates into waste bag.
2. Add vesphene to culture bottles, dunk in vesphene pot, and wipe to waste bag.

Discussion

The following are critical principles to which you should strictly adhere:
All viable cultures must be double contained before removal from the biosafety cabinet.
Once a viable culture is opened in the cabinet, the cabinet is “dirty" and all items must be double wiped before removal from the cabinet.
Outer "dirty" gloves are removed after the first wipe and no “dirty" item may be touched when entering with clean gloves for the second wipe, only the vesphene towel and the once wiped items.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was funded by HHMI and NIAID.

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