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About the Cover

A wide variety of experiments are conducted by astronaut-scientists in the International Space Station (ISS). Some of the experiments involve work with pathogens, tumor cells, and recombinant proteins and molecules. Many of the safety challenges can be addressed using proven biosafety practices that are employed in microbiological and biomedical laboratories on Earth. However, working in a microgravity environment provides unique challenges. To learn more about safety measures used at the ISS see the article entitled “Biosafety Onboard the International Space Station” by Wing C. Wong et al. on pages 158-162. Featured on the cover are images of the ISS as it orbits Earth and an astronaut performing a scientific experiment inside the Microgravity Science Glovebox in the ISS. Images are courtesy of NASA and all its International Partners.

Applied Biosafety Founding Editor

*Applied Biosafety* founding Editor, Melvin W. First passed away June 13, 2011, at 96, after a short illness. A U.S. Army WWII Veteran, graduate of MIT and Harvard University, Dr. First was a Professor of Environmental Health Engineering, emeritus, at Harvard School of Public Health. His contributions to biosafety and science were many, and included an intensive course open to the biosafety community on biosafety cabinet certification and use. In 1994, Dr. First proposed at the business meeting that ABSA move from producing a newsletter to producing a peer-reviewed journal. His enthusiasm was infectious and, after some debate, the motion passed. Dr. First served as the first Editor of the *Journal of the American Biological Safety Association* from 1996-1998. Described in his obituary as a “beloved husband, family man, scientist, mentor and friend to many”; we also remember him as an enthusiastic contributor to the practice of biological safety.
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A Workshop to Anticipate the Biosecurity Challenges of the Global Expansion of High-Containment Biological Laboratories

Adel Mahmoud1, Alison K. Hottes2, and Benjamin Rusek2*

1Princeton University, Princeton, New Jersey and 2U.S. National Academy of Sciences, Washington, DC

Although high-containment biological laboratories—equivalent to U.S. Centers for Disease Control and Prevention Biological Safety Level (BSL) 3 and 4—are needed to isolate and characterize highly dangerous pathogens, they are complex systems with inherent risks. On July 10-13, 2011, the U.S. National Academy of Sciences (NAS) in partnership with the Turkish Academy of Sciences held an international workshop on “Anticipating Biosecurity Challenges of the Global Expansion of High-Containment Biological Laboratories” in Istanbul, Turkey. The U.S. Department of State’s Biosecurity Engagement Program sponsored the workshop. Sixty-eight scientists and technical experts from 32 countries, including representatives from countries experienced in operating laboratories as well as individuals from countries, particularly in the developing world, that are contemplating or undertaking the construction of new facilities, participated in the workshop.

The workshop was designed to examine issues related to the design, construction, and operation of high-containment biological laboratories and to generate practical and implementable guidance for countries considering constructing or expanding their high-containment facilities. Attendees also addressed the public health benefits and financial, safety, and security considerations associated with high-containment laboratories and areas where current biosafety practices are not well-matched to actual safety or security needs.

The workshop began with a session that outlined the factors driving and restraining the expansion and creation of new and existing laboratories and examined the costs, benefits, and safety and security concerns associated with the expansion. Speakers reminded the participants that lab practices are intended to keep the worker safe, buildings are designed to keep the community safe, and that there are often many ways to achieve those objectives.

A significant amount of time was invested early in the workshop on characterizing the processes that countries and corporations use in deciding what types of labs to construct, where and when to build labs, and assessing how well the resulting labs meet their owners’ goals. First, speakers provided an overview of assessments by describing the analytical processes employed and the purposes they serve, as well as who should be involved in the process and what information might need to be included in the analytical process.

After considering assessments broadly, speakers then used specific labs as case studies to examine the degree to which original objectives are achieved and to share lessons they learned during design, construction, commissioning, and operation. They discussed how well particular labs are fulfilling their original research and public health goals, whether initial and ongoing costs and funding have been as expected, and challenges with ongoing biosafety, biosecurity, and maintenance efforts.

In addition, some participants developed and presented country studies that described in detail their country’s high-containment biological facilities, capabilities, and regulations as well as past accidents and safety and security issues.

On the following day, speakers examined the spectrum of molecular, immunological, and culture-based assays available to labs to fulfill their research and public health goals as well as the associated costs, effectiveness, and biosafety requirements for both human and animal diseases. Other speakers discussed available resources, regulations, and sources of guidance; measures for encouraging a culture of responsible conduct; design and operational options for improving sustainability, biosafety, and biosecurity; and the degree to which commonly used biosafety and biosecurity precautions are necessary and appropriate.

A special session was devoted to the unique challenges associated with BSL-4 facilities that addressed: associated construction and maintenance costs; biosecurity issues; environmental risks (especially for pathogens affecting domestic animals); training; strategies to manage an individual who may become infected with a BSL-4 agent; community relations; whether existing and planned networks are adequate; how much and what kinds of “surge capacity” are ideal; whether existing facilities can be re-tasked during a crisis; if BSL-4 labs can be safely constructed in a less technology-intensive manner or in ways that make them easier to sustain; and how many BSL-4 labs are needed in a country or region.

Participants also heard a keynote address from Dr. Peter Palese (Mount Sinai School of Medicine, New York,
National Toxicology Program (NTP) Issued 12th Report on Carcinogens (RoC)


The report is a congressionally-mandated, science-based public health document that identifies agents, substances, mixtures, and exposure circumstances that are known or reasonably anticipated to cause cancer in humans. The 12th RoC includes updated or new listings for five chemicals that are actively used in workplaces, one banned pesticide (captatof), and two botanical supplements (aristocholic acids and riddeline [not the pharmaceutical Ritalin]). The five laboratory chemicals with updated information are:

- **Formaldehyde**—Now listed as a known human carcinogen, previously listed as reasonably anticipated to be a human carcinogen.
- **Styrene**—Now listed as reasonably anticipated to be a human carcinogen, previously unlisted.
- **ortho-Nitrotoluene**—Now listed as reasonably anticipated to be a human carcinogen, previously unlisted.
- **Cobalt-Tungsten Carbide: Powders and Hard Metal**—Now listed as reasonable anticipated to be a human carcinogen, previously unlisted.
- **Certain Glass Wool Fibers (Inhalable)**—Listing unchanged—reasonably anticipated to be a human carcinogen. Petitioned for delisting.

OSHA has updated its web site based on the changes in the RoC, including updates to the OSHA’s Safety and Health Topics pages for Carcinogens, Formaldehyde, and Styrene.

NAS Review of EPA’s Assessment of Formaldehyde

The National Academy of Science (NAS) reviewed the EPA assessment of formaldehyde and found that the evidence “supports the conclusions that formaldehyde can cause irritation to the eyes, nose, and throat; lesions in the respiratory tract; and genetic mutations at high concentrations,” and that “the evidence is sufficient for EPA to conclude that formaldehyde exposures are a cause of cancers of the nose, nasal cavity, and upper throat.” However, NAS comments that EPA “has not adequately supported its conclusions that formaldehyde causes other cancers of the respiratory tract, leukemia, or several other non-cancer health outcomes.” The full report and a PDF summary are available free online at www.nap.edu/catalog.php?record_id=13142

Acknowledgments

Adel Mahmoud is a Professor in Molecular Biology and Public Policy at Princeton University and the Chair of the Workshop Organizing Committee. Benjamin Rusek is a Program Officer at the U.S. National Academy of Sciences (NAS), Committee on International Security and Arms Control and is the Responsible Staff Officer for the workshop. Alison Hottes was supported by a NAS Christine Mirzayan Science and Technology Policy Graduate Fellowship. The workshop was funded by the U.S. Department of State’s Biosecurity Engagement Program.

*Correspondence should be addressed to Benjamin Rusek at brusek@nas.edu.

Guest Editorial

National Toxicology Program (NTP) Issue


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Einstein Contained Aerosol Pulmonizer (ECAP): Improved Biosafety for Multi-Drug Resistant (MDR) and Extensively Drug Resistant (XDR) Mycobacterium tuberculosis Aerosol Infection Studies

Bing Chen¹, Torin R. Weisbrod¹, Tsungda Hsu¹, Vasan Sambandamurthy¹, Delia Vieira-Cruz², Anthony Chibbaro², Dan Ghidoni³, Todd Kile⁴, W. Emmett Barkley⁵, Catherine Vilchêze¹, Cody Colon-Berezin², David S. Thaler¹, Michelle H. Larsen¹, A. Willem Sturm⁶, and William R. Jacobs, Jr.¹*

¹Howard Hughes Medical Institute, Bronx, New York, ²Albert Einstein College of Medicine, Bronx, New York, ³The Baker Company, Sanford, Maine, ⁴University of Wisconsin—Madison, Madison, Wisconsin, ⁵Howard Hughes Medical Institute, Chevy Chase, Maryland, and ⁶University of KwaZulu-Natal, Congella, Durban, South Africa

Abstract

A new apparatus enhances the biosafety of containment (biosafety level 3 [BSL-3]) and provides experimental reproducibility for aerosol infection experiments with MDR and XDR Mycobacterium tuberculosis. The methods are generally applicable to the study of airborne pathogens.

Introduction

The study of emergent pathogens requires special care as antibiotic resistance and new mechanisms of pathogenesis may be present. While aerosol infection studies are necessary, they carry the greatest potential risk of any laboratory procedure. In the course of our work with Multi-Drug Resistant (MDR-) and Extensively Drug Resistant (XDR-) Mycobacterium tuberculosis, we have developed a new apparatus that enhances the biosafety of aerosolization experiments. This apparatus and the associated protocols were developed and validated for TB; however, they are likely to be of wider use.

Several different systems have been developed to generate aerosol infections in mice, guinea pigs, and rabbits (Druett, 1969; Hartings & Roy, 2004; Henderson, 1952; Louveau et al., 2005; Rosebury, 1947; Schwebach et al., 2002; Smith, 1970; Tsenova et al., 2006). The systems differ in their level of safety, ease of operation, level of required maintenance, adaptability to different animal models, number of animals that can be accommodated simultaneously, and quantitative repeatability of delivered dosage. Smith and colleagues developed the Madison Aerosol Exposure Chamber (MAEC), an excellent stand-alone system that is easy to use, has low maintenance requirements, is quantitatively reproducible, and can be used to infect large numbers of mice, guinea pigs, or rabbits in a single run (Smith, 1970). However, the Institutional Biosafety Committees of the Albert Einstein College of Medicine and the Howard Hughes Medical Institute (HHMI) have raised three serious concerns about MAEC safety: 1) when loading and unloading the inoculum culture to/from the stand-alone MAEC, the culture is open to the lab air—a potential source of contamination to the operators; 2) whole body aerosol-exposed mice, which can harbor spreadable pathogens on their external surfaces, are transiently open in the laboratory air during transfer from the exposure chamber to their housing cages; and 3) the stand-alone MAEC lacks back-up protection against operator exposure due to failure of the door gasket, exhaust air tubing system, or HEPA filter leak. These redundancies are important in BSL-3 safety. Biosafety concerns became most critical in the context of proposed aerosol infection studies with MDR- and XDR- M. tuberculosis. A new apparatus, the Einstein Contained Aerosol Pulmonizer (ECAP), was designed and constructed. The ECAP and its integration into a series of biosafety cabinets (BSCs) connected by airtight tunnels add redundant layers of safety to the stand-alone MAEC. With this new apparatus, the proposed studies have, in fact, been safely completed and others are in progress (Ashiru et al., 2010; Ioerger et al., 2009).

The ECAP joins the MAEC to a Baker Class III ventilated and negative pressure glovebox (Figure 1). The ECAP incorporates the excellent aerosolization protocols developed by Dr. Don Smith and Dr. David McMurray (Smith, 1970). This combined apparatus and its associated protocols for enhancing safe operation and maintenance were integrated into our BSL-3 laboratory (Figure 2A). The glovebox exposure chamber is connected to a Class II Type A2 biosafety cabinet (BSC) by an airlocked tunnel. Mice never leave a BSC as they are moved from pre-exposure housing to the aerosol exposure chamber to post-exposure housing. At appropriate times, mice are sacrificed in a Class II Type A2 BSC, then transferred through an airlock into another Class II Type A2 BSC where organs are dissected, homogenized, and spread.
on media for bacterial counts. Safety designs, implementations, and animal protocols were comprehensively reviewed and approved by both the Einstein and HHMI safety offices.

The traditional stand-alone MAEC diameter is 2' wide and 3' long. During operation, an 18-cage carousel is placed inside of the MAEC and the unit’s front door is locked. Each of the individual cages in the carousel is a perforated cylinder, 3" in diameter and 8.5" long; up to five mice can be exposed in each cage (Figure 1A). To create the new integrated assembly, the MAEC was joined to a Baker Class III ventilated glovebox (Figures 1B, 2A), so that the inside of the chamber could be accessed only through the glovebox. This results in approximately a 3.5' working distance inside the ventilated and negative pressure glovebox. Loading and unloading of caged experimental animals, aerating the cages and the animals, and setting up the nebulizer are all performed within the ventilated glovebox. The opposite side of the ventilated glovebox is joined, via an airtight wall tunnel,

**Figure 1**

A. Madison Aerosol Exposure Chamber (AEC) opened showing carousel with cylindrical mouse-cages. 
B. Einstein Contained Aerosol Pulmonizer (ECAP), integrates a Madison Aerosol Exposure Chamber (MAEC) with a Baker Class III ventilated glovebox, and a control box.

**Figure 2A**

Coupled hood series in the BSL-3 suite. The arrow indicates the direction of airflow between the rooms. The BSL-3/4 aerosol room has the most negative air pressure in the suite. A. Madison AEC; B. Baker Class III glovebox; C. animal changing hood; D. airtight wall tunnel; E. doors between rooms; F. BSL-3 suite room partition walls.
to a Class II Type A2 BSC (Figure 2A) in the animal holding area. A knife-edge gasket was substituted for the flat gasket of the MAEC chamber to better seal its door. The Collison nebulizer (BGI, Inc., Waltham, MA) mounted on the airtight door of the MAEC introduces the aerosolized infectious agent. The nebulizer is activated by HEPA-filtered compressed air. Secondary airflow from the ambient air inlet tube is drawn from the HEPA-filtered air already in the glovebox. All MAEC air is exhausted by a vacuum pump via two HEPA filters in series. This MAEC exhaust is HEPA-filtered as it vents into the glovebox exhaust system that is also HEPA-filtered. This system of two separate, successive filters provides an additional layer of safety and operator protection. As is normal inside BSL-3 facilities, air pressure is lower than in the outside corridor. Inside the BSL-3 facility, the aerosol delivery system is inside a smaller room (section 4) that has lower air pressure than the rest of the BSL-3 area (BSL-3/4). Air pressure is lowest of all inside the aerosol exposure chamber.

The internal MAEC and the glovebox were individually tested and each passed a pressure decay test, holding an internal overpressure against a 3" water column for 30'. The apparatus was then tested biologically both for containment and for the reproducibility of mouse exposures. The non-pathogenic species M. smegmatis mc²155 (Jacobs et al., 1987; Snapper et al., 1990) as well as three different strains of fully drug-sensitive and virulent M. tuberculosis—H37Rv, CDC1551, and Erdman—were used for tests. In brief, mycobacterial strains were prepared as previously described (Schwebach et al., 2002). For biocontainment tests, four sentinel plates were placed on top of the ventilated glovebox during operation, four around the tunnel connecting the Class III glovebox to the Class II Type A2 cabinet, and four inside the ventilated glovebox immediately after mice were removed post-aerosol exposure. In each set of four plates, two contained only Middlebrook 7H10 (Difco, Detroit, MI), 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD), and the other two were supplemented with cycloheximide at 100 µg/ml. Plates were left open overnight, then closed, incubated at 37°C for 6 weeks, and carefully examined for M. tuberculosis colonies. For M. smegmatis experiments, plates were read after 7 days.

For infection studies, female C57BL/6 mice were exposed to aerosolized mycobacteria for 20 minutes, followed by a clean air purge of 30 minutes inside the ECAP and then an additional 30 minutes outside the exposure chamber but inside the glovebox. Infected mice were sacrificed 3-4 hours for M. smegmatis and 24 hours post-aerosol infection for M. tuberculosis. Lungs were dissected, removed, placed in a stomacher bag containing 4.5 mls of PBS-0.05% Tween, and then homogenized using the stomacher apparatus (Seward Lab Systems, Northampton, UK) at high speed for 4 minutes. Serial dilutions were plated on Middlebrook 7H10 plates (Difco, Detroit, MI), supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD), and incubated for 3 weeks at 37°C before M. tuberculosis colonies were counted. Figure 2B shows the results of M. smeg-

Figure 2B

M. smegmatis was prepared at 4 different concentrations based on Optical Density (OD_{600}) readings before aerosol delivery, using C57BL/6 mice from NCI. We used 5 mice for each of 4 aerosol delivery runs, with input dosages at the level of n = 5, 6, 7, 8 (log increase). This figure shows a direct correlation between the input dosage and total bacterial load in lungs (measured in CFUs) measured at 24 hours post exposure. Error bars show standard deviation from CFUs recovered from 5 mice in each group.
matis mc²155 at four different infection dosages, with four groups of five mice for each dosage level. The consistency of dosage in each of the four groups is shown by the (small) standard deviation at each input dosage level. Table 1 compiles the results of experiments with *M. tuberculosis* CDC1551, Erdman, and H37Rv at different input dosages with groups of five mice for each dosage level; four dosage levels were used for Erdman and H37Rv and three levels for CDC1551. Again, the consistency of dosage is manifest in the (small) standard deviation at each input dosage level. Day-to-day variation was also small (data not shown). Mice were sacrificed and dissected. The lungs were homogenized and the cells diluted and plated. Plates were incubated at 37°C for 3-7 days for *M. smegmatis* or 3-4 weeks for *M. tuberculosis* and colonies counted in Class II Type A2 biosafety cabinets within the BSL-3 facility.

Over 100 additional experimental studies, consisting of more than 250 separate aerosol runs with over 7,000 mice, further confirmed the ability of the ECAP to deliver the intended dosage levels both accurately and consistently. More than 10 different strains of mice and over 15 different mycobacterial strains were used, including MDR- (Figure 3) and XDR- *M. tuberculosis* (data not shown). Sentinel plates showed no untoward microbes on any part of or around the integrated apparatus and no releases of contaminated aerosol or exposure of laboratory personnel has been detected throughout these experiments, which were conducted over a period of five years at the Albert Einstein College of Medicine. The ECAP allows up to 90 mice per run, with the same consistent dosage as the stand-alone MAEC. The ECAP design has been retrofitted into existing BSL-3 facilities and incorporated into new ones, notably the Human Vaccine Institute at Duke University and the K-RITH facility, a collaboration between the Howard Hughes Medical Institute and the University of KwaZulu-Natal in South Africa. Since the design and manufacture of this prototype, The Baker Company has sold several units.

### Table 1

Dose estimate at time of infection as determined by Optical Density (OD₆₀₀), input dosage as determined by Colony Forming Units (CFU; because of the slow growth of *M. tuberculosis*, colony counts become available only one month after exposure), and bacterial loads in mice infected with 3 different strains of virulent *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD°/ml</th>
<th>CFU***/ml</th>
<th>avg/lung</th>
<th>std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDC1551</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>6</td>
<td>8x10⁵</td>
<td>2x10⁶</td>
<td>107</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>8x10⁶</td>
<td>1x10⁷</td>
<td>588</td>
<td>58</td>
</tr>
<tr>
<td>8</td>
<td>8x10⁷</td>
<td>1x10⁸</td>
<td>3146</td>
<td>381</td>
</tr>
<tr>
<td><strong>Erdman</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1x10⁵</td>
<td>1x10⁴</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>1x10⁶</td>
<td>4x10⁴</td>
<td>60</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>1x10⁷</td>
<td>2x10⁶</td>
<td>650</td>
<td>84</td>
</tr>
<tr>
<td>8</td>
<td>1x10⁸</td>
<td>3x10⁸</td>
<td>2383</td>
<td>801</td>
</tr>
<tr>
<td><strong>H37Rv</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3x10⁵</td>
<td>3x10⁴</td>
<td>8</td>
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</tr>
<tr>
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<td>2x10⁵</td>
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<tr>
<td>8</td>
<td>6x10⁷</td>
<td>1x10⁷</td>
<td>690</td>
<td>257</td>
</tr>
</tbody>
</table>

Note: For each of the four strains tested, and for each of the four dosage levels (5, 6, 7, 8), absolute values are shown for OD dose, CFU dose, average bacterial load recovered from lungs, and standard deviation of that load. For each strain at each dosage level, 5 mice were used, for a total of 75 mice in all.

* The input concentration of *M. tuberculosis* for each experiment was set via OD₆₀₀; CFUs were estimated using the heuristic that 1 OD₆₀₀ represents 3 x 10⁸ CFU/ml.

** At the same time, a sample of the input culture was also diluted and plated for actual CFUs that were counted 3-4 weeks later.

*** This dosage level was not used for the CDC1551 strain.
Acknowledgments

John Kim helped with experimental aerosol studies. Joseph Vinciguerra and Patrick McGuire, Facilities Engineering at Einstein, assembled the ECAP and modified the BSL-3 rooms to accommodate it and the associated series of cabinets. We thank Harris Goldstein for the acronym “ECAP.” Lester Hoffman provided comments on drafts of the manuscript. This work was supported by U.S. National Institutes of Health grant AI26170 and the Einstein Center for AIDS Research grant AI051519. At the time of the work: Todd Kile was employed by the College of Engineering Shops, University of Wisconsin—Madison; W. Emmett Barkley and Vasan Sambandamurthy were employed by the Howard Hughes Medical Institute, and now are President of Proven Practices, LLC, and Group Leader for AstraZeneca, respectively.

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References


Comparison of Multiple Systems for Laboratory Whole Room Fumigation

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Abstract

Fumigation of high-containment microbiology facilities is an international requirement and in the United Kingdom this process is still commonly undertaken using formaldehyde vaporization. Formaldehyde usage is simple and inexpensive, but concerns exist over its toxicity and carcinogenicity. Alternative fumigants exist, although independent, parallel comparison of these substances is limited. This study determined the level of biocidal efficacy achievable with formaldehyde and compared this with other commonly used fumigants. Three different hydrogen peroxide-based fumigation systems were evaluated (two vapor and one dry-mist methods), along with true gas systems employing ozone and chlorine dioxide. A range of challenge microorganisms was used at different room locations to assess the efficacy, usability, and safety of the fumigation equipment. These microorganisms included Geobacillus stearothermophilus, Clostridium difficile, Mycobacterium fortuitum, and Vaccinia virus. Only chlorine dioxide and formaldehyde fumigants gave consistently high levels of antimicrobial efficacy across all bacterial challenge tests (typically greater than a 5-log reduction). All systems performed similarly against Vaccinia virus, but variable results were noted for Geobacillus, C. difficile, and M. fortuitum for the hydrogen peroxide- and ozone-based systems. The study also revealed inconsistencies in system reliability and reproducibility, with all fumigant systems aborting mid-cycle on at least one occasion. In contrast, formaldehyde fumigation was confirmed as extremely reliable, largely because of its simplicity (liquid plus hot plate). All the fumigants tested have UK workplace exposure limits of 2 ppm or less, yet residual fumigant was detected for the formaldehyde and hydrogen peroxide systems following cycle completion, even after room aeration.

Introduction

A number of characteristics have been proposed for the ideal fumigant, and these deserve consideration when planning gaseous fumigation work (Joslyn, 2001). High on the list of desirable qualities is product safety, with the ideal fumigant being effective in its application but non-toxic to the user. The reality is that most gaseous fumigants are potentially harmful to people and animals and require containment to remain both effective and safe during use. At best, they may constitute a serious respiratory and mucosal irritant; while at worst; they may be highly toxic, even at low exposure levels. Joslyn (2001) describes the ideal fumigant as one that should leave no residues or should be capable of rapid removal to safe levels following fumigation. For a number of fumigant products this remains a challenge, and one that was considered within this study, alongside the important issue of efficacy.

Formaldehyde vapor was of particular interest in view of its historical significance and continued use. The earliest reports of its use as a fumigant date back to the 1880s (Lach, 1990), and it has remained the chemical of choice for laboratory fumigation for decades (Dreyfus, 1914). Formaldehyde is typically delivered by heating formalin (35%-40%) with an appropriate amount of water in a thermostatically controlled unit (Jones, 1995). As such, this study sought to draw a comparison between formaldehyde and other alternative products that might also be used for fumigation within the laboratory setting. The most commonly used of these, or at least the chemical receiving most attention in the literature, is hydrogen peroxide. This is available in vapor and dry-mist forms and has been evaluated in healthcare, laboratory, security, and food sector environments, often using the vaporized form. Hydrogen peroxide vapor can be delivered with different levels of associated humidity, depending on the brand of equipment used (French et al., 2004; Hall et al., 2007; Kähnert et al., 2005; Krause et al., 2001, McDonnell et al., 2002; Rudnick et al., 2009). A more recently developed approach, using a dry mist of hydrogen peroxide, is also available and uses a lower source hydrogen peroxide concentration (typically 5%) with silver cations (Andersen et al., 2006; Bartels et al., 2008; Grare et al., 2008; Shapey et al., 2008). In addition, both chlorine dioxide gas and ozone gas have been used effectively in fumigation applications (Pan et al., 1992; Rastogi et al., 2009; Sy et al., 2005; Wilson et al., 2005). Although other fumigants do exist, the above products were chosen for this study because they have received commercial attention as a result of their reported antimicrobial qualities and, for some, their alleged greater safety compared to formaldehyde. Some of these technologies have benefited from intelligent and strong marketing initiatives by their manufacturers, which have raised their profile above that of some other available systems.

All of these described fumigants have been available for room and vehicular use for a relatively short time, compared with the many decades that formaldehyde
has been in use. Despite its historical use, formaldehyde is now restricted to facilities that can be completely sealed and have some degree of ventilation control in place to minimize the risk of human exposure. This is because formaldehyde is known to be a human sensitiz-er and carcinogen and can leave undesirable residues if its vapor is poorly delivered or not evacuated from the treated area within a defined period of treatment (Cheney & Collins, 1995; Nelson et al., 1986).

Due to its toxicity, formaldehyde has a workplace exposure limit (WEL) of 2 ppm for both short- and long-term exposure (Health and Safety Executive [UK], 2006 & 2007). Decisions at the European Union (EU) level, under the Biocidal Products Directive (Directive 98/8/EC, 1998), may eventually lead to restrictions on its use, though it is unclear whether this will affect laboratory fumigation. Because formaldehyde remains relatively stable compared with some other available fumigants, its persistence presents both benefits and added risks. De- spite the risks, formaldehyde remains an effective and easy-to-use fumigant, and available data suggest it is difficult to match in terms of broad antimicrobial efficacy.

Few independent data exist comparing the efficacy of multiple fumigation systems in the controlled laboratory setting, although some machine comparison work has been undertaken recently with viral challenges (Pottage et al., 2009). The aim of the current study was to investigate the alternatives to formaldehyde now available and to assess their efficacy within a contained microbiological facility, using bacterial and viral challeng-es. High levels of fumigant delivery were possible because the room could be properly sealed and ventilated. A controlled environment such as this presents an opportunity for fair comparison among the various fumi-gants, while applying consistent room conditions and microbial challenges. This information was required by Great Britain’s Health and Safety Executive (HSE) to inform and advise inspection activity in this area and to enable HSE to provide accurate advice for its duty hold-ers. With this in mind, the related experimental aims of this work were:

- To initially confirm the levels of biocidal efficacy achievable with formaldehyde and to use these data points for comparison testing of other fumigants
- To evaluate alternative methods of fumigation and the systems used to deliver them, using a range of chal-lenge microorganisms located at various room positions
- To assess equipment usability and related safety measures in use for these test fumigation systems when evaluated in the controlled air chamber (CAC) of Great Britain’s Health and Safety Laboratory (HSL) and in a Containment Level 3 (CL3) facility with scaled-up conditions
- To report the findings, with appropriate interpreta-tion, to HSE for its use in advice or guidance provisions for end-users of fumigation.

### Materials and Methods

#### Working with Chosen Bacterial Strains and Culture

This work involved preparation of seeded steel discs, using high-titre microbial challenges, which then required their placement, recovery, and cultivation. In view of the amount of sample-handling required and the need to deploy these discs within a chamber environ-ment, HSL followed principles recommended by HSE in The Control of Substances Hazardous to Health (COSHH) Regulations (2002) to control and so minimize any con-tamination risk to those performing experimental work. There is a primary duty under COSHH to prevent expo-sure of laboratory staff to biological agents during planned research (HSE-ACDP, 2005), by either avoiding their use or substituting with a safer alternative. For some types of laboratory work, such as diagnostic work, this may not be possible. However, it can be achieved for other types of work, such as planned experimental test-ing. With this in mind, the following cultures and meth-ods were used for fumigation testing:

**Clostridium difficile.** NCTC 11209, a widely used reference strain, was used as a surrogate for epidemic *C. difficile*. Cultures of *C. difficile* were grown anaerobi-cally in cooked meat broth (Oxoid Ltd., Cambridge, Eng-land) using a shaking incubator at 37°C for 48 hours. Oxoid-cooked meat broth is designed to promote anaero-bic growth conditions in a sealed flask without the need for additional anaerobic controls. However, as an addi-tional precaution, all culture flasks were placed in anaer-obic jars containing anaerobic gas packs (Anaerogen, Oxoid Ltd.). Liquid culture was agitated to ensure a uni-form suspension before decanting 50 ml of the suspens-ion. This was centrifuged at 3,000 rpm for 10 minutes, and the pellet was then resuspended in 5 ml fresh-cooked meat broth to concentrate the bacteria. The combination of using meat-broth culture and drying the seeded residues onto discs effectively induced sporula-tion. Spore concentrations were confirmed by staining the dried suspensions with malachite green. This pro cess showed between 85% and 90% spore formation for each independent experiment (data not shown). Fifty microliters of *C. difficile* stock typically contained ap-proximately 10^6 to 10^7 cells. This volume was seeded onto sterile stainless steel discs in quadruplet (triplicate rep-licas for exposure to fumigant, plus a single comparative positive process control for each room location). Seeded discs were dried for 1.5 hours prior to fumigation and then positioned in predetermined chamber locations prior to the start of the test fumigation.

**Mycobacterium fortuitum.** NCTC 10394, a fast-growing, non-tuberculous *Mycobacterium* species, was used as a safe surrogate of *Mycobacterium tuberculosis*. *M. fortuitum* has the added advantage of growing as quantifiable colonies on standard agar plates, unlike other members of this genus, which require growth on
M. fortuitum was grown aerobically in Middlebrook 7H9 broth containing 10% Middlebrook ADC enrichment broth and 1% Tween and was maintained on Middlebrook 7H10 agar plates (Becton Dickinson, Franklin Lakes, NJ) at 37°C for 4 days. To prepare a sample for fumigation experiments, 10 ml was removed from a 4-day-old broth culture of M. fortuitum and pipetted into a universal tube containing two glass beads. The sample was vortexed for 1 minute to minimize cell clumping, and the resulting suspension was used to seed steel discs in 50-μl aliquots, as for C. difficile. The 50-μl aliquots of the M. fortuitum stock typically contained approximately 10^6 to 10^7 cells.

Commercially prepared spore discs of Geobacillus stearothermophilus 7953, at a concentration of approximately 10^6 spores/disc (ATI Atlas, West Sussex, UK), were used as an additional control and point of comparison. Recovered G. stearothermophilus was grown on Tryptone Soya Agar (TSA-Oxoid, Cambridge, England) at 55°C overnight. This bacterium has been used extensively for the evaluation of disinfection and sterilization methods and was included here as a recognized standard within the industry. The steel disc preparation method was used for this organism, rather than the cellulose strip process, because the steel discs are designed for fumigation assessment.

Virus and Cell Culture

Vaccinia virus was chosen as a surrogate for Variola virus, the causative agent of smallpox. The Vaccinia virus (a vaccine strain that was previously adapted for growth on Vero E6 cells from the UK’s National Collection of Pathogenic Viruses) was obtained from the European Collection of Cell Cultures (ECACC). The Vero E6 cells were also obtained from ECACC and cultured as directed by the supplier. High-titre stocks of virus were grown on cultured Vero E6 cells as previously described by Cann (1999) with the exception that virus was incubated for 3 days and staining was performed using a second overlay of agarose-containing neutral red (Knipe et al., 2001).

The titre of virus used for seeding stainless steel discs was measured using a standard plaque assay as described by Cann (1999) and Knipe et al. (2001). Six-well plates were seeded with 5x10^6 Vero E6 cells per well grown in 3 ml of Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units), and streptomycin (100 μg), GlutaMAX™ (2 mM), nonessential amino acids (0.1 mM), referred to hereafter as complete EMEM. Cells were incubated at 37°C with 5% CO₂ overnight. The medium was removed from the wells, and the cells were washed with phosphate buffered saline (PBS) followed by infection medium (complete EMEM medium with 2.5% FCS instead of 10%). Dilution series of viral stocks were prepared using infection medium as the diluent. Infection medium was removed from the six-well plates and 1-ml aliquots of the virus samples were transferred to the wells. Plates were gently rotated for 30 seconds to ensure even viral adhesion, then incubated for 1 hour at 37°C, 5% CO₂. The cells were then washed with PBS before adding 2 ml of overlay medium (1:1 ratio of complete EMEM and pre-warmed LMP agarose, prepared in distilled water). Once the overlay had set (20 minutes at room temperature), plates were incubated at 37°C, 5% CO₂ for 72 hours. Plates were stained with neutral red using a second agarose overlay of 2 ml as described above, with the addition of 3% neutral red. The numbers of plaque-forming units (pfu)/ml were then calculated by taking an average of the numbers of plaques counted from the highest dilutions showing between 1 and 50 plaques. The average number of plaques identified was then multiplied by the dilution factor. A 50-μl aliquot of a 10^7 (pfu)/ml Vaccinia virus culture was used for final challenge preparations.

Preparation of Samples for Fumigation

Stainless steel discs (surgical grade, 15 mm diameter) were prepared in the workshop at HSL. These were cleaned and pre-sterilized by autoclaving prior to use as a seeding surface. For each test location and for process controls, discs were prepared in a Petri dish in a biological safety cabinet and a 50-μl aliquot of each bacterial or viral culture was pipetted onto the surface of the discs. For the bacterial samples, an aliquot of liquid culture containing approximately 1 x 10^6 colony forming units (cfu)/ml was prepared on triplicate test discs, with additional (unexposed) controls prepared from the same stock suspension. For the virus samples, an aliquot of working viral stock containing approximately 2 x 10^5 (pfu)/ml was used. Test and control discs were prepared in the same manner as the bacterial discs. The discs were then left to air-dry for approximately 1.5 hours or until visibly dry.

Simulated, small-volume spills were set up in flat-bottom, multi-well plates. An aliquot of 750 μl of culture (using the same source culture as prepared for dried-down samples) was placed in 1 well of a 6-well plate, covering the bottom of the well. For bacterial samples, triplicate wells were set up, and for viral samples, a single well was set up. A single well of each sample was set up in a separate 6-well plate as a (unexposed) control.

Setup of Fumigation Experiment

Controlled Air Chamber (CAC)

The HSL’s CAC had an internal volume of 35 m³ and was set up as a mock laboratory for this series of fumigation experiments by adding basic laboratory furniture and equipment (Figure 1). Wherever possible, the fumigation machines were placed in one designated position within the chamber. Dried discs were placed on the open bench top, on the floor under a cupboard, and inside a
partially open centrifuge. The simulated spill plate was placed at floor level, under the front edge of a cupboard unit. Initial experiments showed that some fumigants were able to penetrate even double-sealed control samples that were intended to remain unexposed to the fumigant. In view of this, unexposed process controls of dried discs and simulated spills were prepared in an identical fashion to exposed samples, but were then kept in a separate laboratory of comparable temperature and relative humidity (RH), avoiding any exposure to the fumigant. Prior to the start of each experiment, the CAC room conditions were set to a room temperature of 23°C and RH of 40%, to give a consistent starting point for the different experiments.

**Containment Level 3 Laboratory (CL3)**

Further fumigation experiments were carried out within HSL’s CL3 laboratory that had an internal volume of 105 m³. In this laboratory, the location of test samples was chosen to be as consistent as possible with those used for earlier CAC experiments; dried discs were placed on the open bench top, in a partially open centrifuge, on the floor under the front edge of a cupboard, and inside a Class I biological safety cabinet (Figure 2). As for the CAC-based experiments, the simulated spill was placed at floor level, under the front edge of a cupboard. Dried control discs and a simulated spill were placed in a separate laboratory of equivalent temperature and RH and, therefore, were not exposed to any fumigant. It was not possible to control the starting temperature or relative humidity in the CL3, and ambient conditions prevailed.

For all fumigation experiments in the CAC or the CL3, the air supply to the room was isolated before commencing fumigation. Following fumigation, the room was vented to remove any residual fumigant. Prior to re-entry, levels of fumigant were checked using a PortaSens II hand-held monitor containing the appropriate calibrated fumigant sensor (GemLog Controls Limited, Pulborough, UK). Personnel did not re-enter the room until the level of fumigant was below the WEL.

**Processing Samples Following Fumigation**

### Bacterial Recovery from Steel Discs

Following exposure to the fumigant, each disc was placed in a tube containing two 5-mm sterile glass beads and 10 ml of PBS; the mixture was vigorously agitated for 1 minute by vortexing. The tubes were left at room temperature for 30 minutes and then vortexed again to ensure efficient rehydration and removal of the dried bacterial sample from the disc.

The simulated spills were recovered from the wells and each transferred into a sterile tube. In some cases, particularly following overnight exposure or venting, the liquid in the spill wells had evaporated partially or completely. In these cases, the sample was reconstituted to
its original 750-µl volume using PBS before transfer to a sterile tube.

The samples were serially diluted, 10-fold, and 100-µl aliquots of each dilution were plated onto duplicate agar plates; mycobacteria were recovered on Middlebrook 7H10 agar plates (Becton Dickinson), clostridia on Columbia blood agar, and Geobacillus on TSA. Following appropriate incubation, the plates were counted and the number of bacterial colonies recovered from each tile or spill well was calculated. Exposed samples were compared with unexposed control samples to determine the percentage kill and the log_{10} reduction.

**Viral Samples**

**Virus Recovery from Steel Discs**

Sterile swabs, moistened with sterile PBS, were used to gently wipe the viral load from each steel disc. The swab was immersed in a 2.5-ml cryovial containing 1 ml of cell culture medium and gently rotated to dislodge some of the virus from the swab into the medium. The nib of the swab was cut from the shaft, and the samples were refrigerated overnight to soak, releasing the maximum amount of virus into the medium. The swabs were then removed, taking care to remove as much medium as possible by pressing the swab against the side of the tube. Samples from simulated spill wells were recovered into cryovials. All samples were stored at -80°C until they could be analyzed by plaque assay, as previously described.

**Formaldehyde Fumigation**

Formaldehyde fumigation was carried out using aqueous solutions of 37% formaldehyde (formalin) and additional water appropriate to the volume of the room, as described by others (Cheney & Collins, 1995; Lach, 1990). All runs were carried out using a theoretically calculated level of 600 ppm formaldehyde. Within the CAC and CL3 facilities, this equated to using 60 ml and 180 ml of formalin, respectively. The amount of formalin to added water was typically 1:20; this was higher than the 1:9 ratio used by some but was typical of the ratio used in our own facility at the time. This higher water component did not cause room condensation problems in either room facility. The formalin/water mixture was placed in an electrically operated wok, which effectively functioned like a hot plate, and was controlled from outside the room being decontaminated. The solution was
heated until all the liquid had evaporated. This typically required 40 and 60 minutes, respectively, for the CAC and CL3 facilities.

**Other Fumigation Devices Evaluated in this Study**

Fumigation devices were provided by five different companies for use in this project (see below). Each manufacturer was informed of the nature of the tests and the microorganisms being used in the study, and each advised on the most effective decontamination cycle for its machine, based on the room volumes. At least three fumigation runs for each system were performed in each facility. The exception to this was the chlorine dioxide system, which could not be used within the CL3 without a major door re-design. This additional cost was beyond the scope of the study, so this system was evaluated in the CAC only. If the results from an initial run demonstrated that the fumigation device had been less effective than expected, the supplier was given the opportunity to suggest changes to the cycle program prior to further work. These additional fumigation systems were as follows:

**Hydrogen Peroxide—System 1 (H2O2-1)**

This supplier has developed a machine that attempts to deposit an even layer of “micro-condensation” of hydrogen peroxide vapor over all surfaces. The manufacturer claims that this system generates an even spread of hydrogen peroxide vapor, due to a high-velocity gas distribution system that includes nozzles and fans. The system uses AnalR grade 30% hydrogen peroxide, and the decontamination cycle consists of four phases: conditioning; gassing; dwell; and aeration. Current uses of this technology include the healthcare sector, food manufacturers, and defense and life science laboratories.

The supplied system is not normally available for purchase and is usually operated by trained engineers as a decontamination service. This system consisted of a vapor generator with a gas distribution nozzle and fan, with approximate dimensions of 1.5 m high by 0.5 m wide by 0.5 m deep. The fumigant delivery system is used in conjunction with an aeration unit, an instrumentation module, and a computer module. Only one aeration unit was supplied, but the supplier would normally provide additional linked units, as necessary. For testing purposes here, a decision was made to use the existing room air purge to aid aeration, rather than to add more units to the room, which might possibly hinder experimental procedures. The first supplied machine tested at HSL had a laptop control unit, but this unit was reclaimed by the supplier following initial CAC testing, as the company needed it to fulfil commercial obligations. A second, identical unit was later provided that had new control software. The computer (laptop) was located outside the room being fumigated and was connected to the fumigant delivery system via computer cables.

Generally, an engineer would monitor the performance of the machine throughout the decontamination procedure, but for ease of use, a pre-programmed cycle was employed that had been prepared by the supplier for the type and volume of the rooms being fumigated. Although this supplier has other available bio-decontamination units using this technology, this unit was chosen because it is still widely used by the company and by those who have purchased this technology in the past.

**Hydrogen Peroxide—System 2 (H2O2-2)**

This system tested by HSL uses a biocide containing 5% hydrogen peroxide and less than 50 ppm silver cations (supplied as silver nitrate). Fumigant delivery from this system is by “dry mist” technology, where a fine spray is generated from a single nozzle containing electrically charged droplets in the 8 µm to 12 µm range. The supplier claims this size range enables the disinfectant to be dispersed onto all surfaces during treatment. The system is pre-programmed to deliver a fumigation cycle based on the volume of the room. The machine is 1.1 m high by 0.5 m wide by 0.5 m deep. This was the smallest and lightest of all the specialist machines, though its three wheels (one front and two rear) meant that care was required when wheeling the device over distances or uneven surfaces. This technology is used in a number of National Health Service trusts across the UK and has been reported to have some effectiveness against a range of bacteria.

**Hydrogen Peroxide—System 3 (H2O2-3)**

This system maintains the vapor level below its condensation or dew point. The hydrogen peroxide vapor concentration in the room during fumigation is generally 0.1 mg/l to 3 mg/l with this system. This technology has been used in the healthcare sector, pharmaceutical and manufacturing industries, and life science and defense laboratories.

The supplied machine is not typically available for purchase, but a range of similar machines is available commercially. According to the manufacturer’s information, this machine was designed for decontamination of areas up to 85 m², though advice from the supplier’s engineer confirmed that it has been successfully used for larger room volumes during research work. The decontamination cycle consists of four phases: dehumidification; conditioning; sterilization; and aeration. Each phase of this cycle is programmed by the user and is dependent on the volume of the room.

The fumigation machine itself is 1 m high by 0.7 m wide by 0.4 m deep, and although heavy, it is highly manoeuvrable on its four wheels. The system uses 35% hydrogen peroxide provided by the supplier. A drying cartridge is required for operation of the fumigation machine, which must be dried on a customized cartridge.
regenerator for 6 hours or overnight. Alternatively, single-use-only drying cartridges can be purchased from the supplier.

**Ozone—System 4**

The ozone delivery system was designed to promote a reaction between ozone and water to generate biocidal-free radicals. This process, as described by the supplier, involves four stages: phase 1 is Ozone Debt Absorption, in which the latent contaminants in the room are addressed; phase 2 is the Build phase, in which ozone levels are raised by the generator; phase 3 is the Killing phase; and phase 4 is the Decay phase, in which residual ozone is converted to harmless by-products by a quenching agent.

The supplier has developed a number of ozone delivery systems and has only recently begun to market these commercially. This study was supplied with a fully functioning prototype machine, which was approximately 1 m high. It required a small oxygen cylinder and cans of quenching agent (formulation confidential). The system required the operator to input the ozone level and length of time required for the sterilization cycle; this information was optimized by the supplier prior to testing. Systems from this supplier are currently used in the food manufacturing industry, and its ozone technology is used widely in washer disinfectors.

**Chlorine Dioxide (ClO₂)—System 5**

HSL was supplied with a commercially available chlorine dioxide unit designed for the decontamination of rooms and buildings. The system has a maximum volume delivery capacity of up to 800 m³. The system requires a large supply gas cylinder, and gas is typically supplied from compressed gas cylinders containing a mixture of 2% chlorine gas in 98% nitrogen (inert carrier). This allows production of ClO₂ at point of use. The ClO₂ is passed in a controlled manner through catalyst cartridges containing a sodium chlorite mixture that results in a contained chemical reaction, producing pure ClO₂ gas. The gas is delivered to the target area via kynar tubing. The decontamination device must be situated outside the treated room. Gas is piped into the room via door or wall apertures that must be sealable. For this reason the equipment could not be tested in the CL3, as the scope of the project did not extend to modification of the CL3 door. A small humidifier and a desk fan were used within the treated area. This machine was the largest of the devices tested at nearly 2 m high by almost 1 m wide. Source gas bottles must be removed during transit.

The ClO₂ decontamination cycle consisted of five phases: Preconditioning, in which the relative humidity is raised and checks are made for leaks; Conditioning, in which the raised relative humidity is held; Charging, in which ClO₂ is generated and delivered; Controlled air, in which the required level of ClO₂ is maintained; and Aeration to finish. The user programs the cycle, which is dependent on the room volume. No aeration unit was supplied with the machine, although one could be fitted into the ventilation ducts of rooms that are frequently treated. The CAC room purge was used for fumigant removal on completion of the experiments. All lights had to be turned off in the room during decontamination, as ClO₂ is broken down in the presence of UV light.

**Statistical Analysis**

Specific questions were considered at the study outset, and both experimental design and statistical analyses were formulated to address these questions. The key outcome of interest from this study was the log reduction observed for microorganisms exposed to the fumigation process compared with unexposed controls. The data were not normally distributed; therefore, medians plus interquartile ranges were used to describe the data for each organism by fumigation system and location.

Mixed effects linear regression was used to compare fumigation systems and locations for each organism. Organism, fumigation system, and location of sample were entered as fixed/main effects, including all two- and three-way interactions (full three-factorial model). The ClO₂ system was used as the reference fumigation system, and the bench top was used as the reference location. The logarithm of the control concentration was also entered as a main effect to adjust for the different control concentrations observed. The experiment number was entered as a random effect to take into account possible clustering. This controls for factors that could affect all observations from an experiment, such as the settings of the fumigation system or the room in which the experiment was conducted. Jackknife estimation [a method of estimating the standard errors [and therefore confidence intervals] that does not rely on the underlying distribution of the data] with clustering on experiment number was used to estimate standard errors and construct 95% confidence intervals. The Wald test was used to test the overall significance of variables (significance, \( p \leq 0.05 \)). Testing different components of the interaction terms (using the Wald test) provided a statistical test of interaction between fumigation system and location for each organism—that is, it tested whether the differences in log reduction between locations varied significantly from fumigation system to fumigation system. All analyses were conducted in Stata/SE 10 for Windows.

**Results**

**Formaldehyde Level Used for Fumigations**

Initial experiments were completed to assess the efficacy of formaldehyde as a fumigant, using Geobacillus stearothermophilus spores only. Experiments in the
HSL CAC facility initially employed a level of 1,400 ppm formaldehyde. This reflected the levels of formaldehyde used in our own CL3 safety procedure, for emergency fumigation, and is similar to the formaldehyde concentration described by others (Lach, 1990). However, following further literature searching, it was decided to reduce the level of formaldehyde delivery as far as possible, while still seeking to achieve a reliable biocidal effect, to provide a fairer comparison with other fumigation technologies. Based on this principle, a level of 600 ppm was found to be the threshold at which any breakthrough (survival) from 10⁶ Geobacillus stearothermophilus spores was prevented (data not shown). This concentration of fumigant was therefore used as the threshold value for subsequent formaldehyde tests; with the caveat that repeated failure to achieve effective killing would result in a review of the amount of formaldehyde used. Subsequent data confirm the effectiveness of this chosen formaldehyde level, and these are presented below.

**Comparison of the Efficacy of Different Fumigation Systems**

At least three fumigation runs were completed within the CAC with each system. A similar number of runs were completed within the higher room volume of the CL3 laboratory, with the exception of the ClO₂ system, which could not be trialled in the CL3 for reasons previously described. The data presented represents overall data from two room locations, where this was available, or reflect the CAC data only for ClO₂. Because no biological safety cabinet was available in the CAC, cabinet data are not available for the ClO₂ system (Figures 2-4).

**Overall Results by Room Location, Challenge Organism, and System Type**

Efficacy trends are evident from data plots, allowing a comparison between what might be reasonably expected of any disinfection process, as indicated by a dashed line 4-log reduction, and what was actually achievable. Figures 2, 3, and 4 shows the observed median log reduction by fumigation system and location for *C. difficile*, *M. fortuitum*, and Vaccinia virus, respectively. Clear differences were observed in overall efficacies among fumigation systems, sample locations, and challenge organisms.

Figure 2 shows results against *C. difficile*, with formaldehyde, hydrogen peroxide vapor systems, and ClO₂ all demonstrating spore kill potential of up to 6- to 7-log for most room locations. Formaldehyde and ClO₂ provided the most consistent data against *C. difficile*, both for the dried residues and simulated spill. Exceptions were noted. For the simulated 750-μl spill, hydrogen peroxide delivered by system H₂O₂-1 appeared to be less effective (overall 1.5-log reduction). For the penetrative test of the partly open centrifuge, hydrogen peroxide delivered by system H₂O₂-3 appeared to be less effective than against dry residues (approximately 1.6-log reduction). Another important observation from these data concerns the variation in performances for individual systems among their identical repeat cycles. The interquartile ranges (Table 1) indicate that considerable variation was seen for hydrogen peroxide-based systems, compared with the more consistent results seen for formaldehyde and ClO₂; this is particularly visible in Figure 2, where the error bars actually represent the interquartile ranges. Ozone and dry mist hydrogen peroxide/silver systems were the least effective.

**Table 1**

<table>
<thead>
<tr>
<th>System</th>
<th>Geobacillus*</th>
<th>C. difficile</th>
<th>M. fortuitum</th>
<th>Vaccinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂-1</td>
<td>7 (5.30 – 5.79)</td>
<td>31 (0.12 – 6.20)</td>
<td>31 (0.76 – 4.22)</td>
<td>31 (2.78 – 4.41)</td>
</tr>
<tr>
<td>ClO₂</td>
<td>3 (4.85 – 5.96)</td>
<td>11 (5.85 – 6.24)</td>
<td>11 (6.44 – 7.38)</td>
<td>12 (3.30 – 4.64)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>6 (2.27 – 5.55)</td>
<td>36 (5.30 – 6.33)</td>
<td>36 (6.24 – 7.19)</td>
<td>17 (2.06 – 3.76)</td>
</tr>
<tr>
<td>H₂O₂-2</td>
<td>9 (0.85 – 5.17)</td>
<td>40 (0.00 – 1.24)</td>
<td>40 (0.05 – 0.79)</td>
<td>25 (2.35 – 3.88)</td>
</tr>
<tr>
<td>H₂O₂-3</td>
<td>7 (2.27 – 5.69)</td>
<td>37 (3.24 – 6.55)</td>
<td>37 (0.51 – 5.35)</td>
<td>37 (2.38 – 3.30)</td>
</tr>
<tr>
<td>Ozone</td>
<td>7 (0.52 – 3.03)</td>
<td>30 (0.00 – 0.07)</td>
<td>31 (0.08 – 0.71)</td>
<td>26 (1.37 – 4.18)</td>
</tr>
</tbody>
</table>

Data are number of observations—median log reduction plus interquartile range (in parentheses)—by fumigation system and organism.

* Geobacillus placed only at one location (the bench).
effective for *C. difficile*, with both achieving less than 0.5-log reductions overall when using the standard cycle settings provided by the suppliers (Table 1).

Challenging the systems with *M. fortuitum* again showed that the most consistently efficacious results were for formaldehyde and ClO₂ (6- to 7-log reductions; Figure 3; Table 1). The overall performance of both hydrogen peroxide vapor systems (H₂O₂-1 and H₂O₂-3) was more variable against this bacterium, with reductions typically on the order of 5-log, at best (Table 1). For both these hydrogen peroxide systems, the penetrative centrifuge test resulted in a reduced efficacy of only about 1.7-log. Mycobacterial spill test results were particularly poor for these hydrogen peroxide vapor systems, with each producing less than 0.5-log reduction against *M. fortuitum* in 750 μl of challenge broth. The dry mist hydrogen peroxide/silver system and ozone fumigation again gave a low level of efficacy against this bacterium, with less than 1-log reduction overall, for all room locations (Figure 3). Individual data for the dry mist hydrogen peroxide/silver system indicated potential reductions of up to 2-log, though this was not typical (Table 1).

Observed results for Vaccinia virus challenges are given in Figure 4. Seeding levels in the order of 10⁶ pfu of Vaccinia virus were typically dried onto tiles. The air-drying process (approximately 1.5 hours) caused a 2- to 3-log reduction in viable virus in the absence of any fumigant exposure, despite the protective presence of viral infection medium (data not shown). This meant that the remaining viable challenge for each test was reduced to levels of approximately 10³ to 10⁴ pfu, depending on the extent of viability loss. So, although Figure 4 indicates log reductions in the range 10² to 10⁴ for Vaccinia virus, this actually equated to an elimination of all remaining virus in most cases. While differences in viability loss post-drying were noted among fumigation cycles (a minimum of three cycles were undertaken for each fumigation system and lab setting), viable virus reduction occurring on individual samples within individual experiments was consistent. Replicate samples were used for each location within each experiment, with the exception of simulated spills, which maintained a single sample per experiment. For these low-volume liquid challenges, the viability of virus was retained more effectively, leading to 5- to 6-log reductions. This equated to elimination of all viable virus for most fumigation systems. The exception was the ozone system, which appeared to have the least penetrative ability with the liquid challenge, achieving less than a 2-log reduction against the Vaccinia virus spill (Figure 4).

**Figure 3**
Observed median Log reduction by fumigation system and location for *M. fortuitum*.

![Figure 3](image-url)
Efficacy Results Related to Individual Challenges and Their Room Locations

A good insight into each system’s maximum efficacy can be provided by assessing log reduction for samples located on the open bench (i.e., in one of the most exposed sites within the treated room) and comparing these data to less exposed room locations. Table 2 presents the differences in log reduction through mixed effects linear regression. There were statistically significant differences among fumigation systems for G. stearothermophilus, C. difficile, and M. fortuitum for samples taken at the open bench (all Wald test p-value << 0.0001). For G. stearothermophilus, the formaldehyde, $\text{H}_2\text{O}_2$-2, and ozone systems all had statistically significantly lower log reductions than the ClO$_2$ system. For C. difficile, the $\text{H}_2\text{O}_2$-2 and ozone systems had statistically significantly lower log reductions than the ClO$_2$ system. For M. fortuitum, the $\text{H}_2\text{O}_2$-1, $\text{H}_2\text{O}_2$-2, $\text{H}_2\text{O}_2$-3, and ozone systems all had statistically significantly lower log reductions than the ClO$_2$ system. There were no statistically significant differences among systems for samples of Vaccinia virus located on the bench, with all achieving similar efficacy (Wald test p-value = 0.262).

Next, differences in log reduction by fumigation system and location were analyzed for each organism. Results for G. stearothermophilus are not reported since this was placed only on the bench.

Table 3 shows differences in log reduction for C. difficile estimated using mixed effects linear regression. For C. difficile, differences in log reduction due to location differed among fumigation systems (overall p-value for this interaction, < 0.0001). There were no statistically significant differences among locations for the $\text{H}_2\text{O}_2$-1, ClO$_2$, and $\text{H}_2\text{O}_2$-2 systems (all Wald test p-values > 0.05). However, in the remaining three systems, there were statistically significant differences in log reduction among locations (p-values ≤ 0.05). For the formaldehyde system, a spill had a lower log reduction than the bench; for the $\text{H}_2\text{O}_2$-3 system, the centrifuge location gave a lower log reduction than the bench; and for ozone, a spill had a lower log reduction than the bench.

Table 4 shows differences in log reduction for M. fortuitum estimated using mixed effects linear regression. For M. fortuitum, differences in log reduction due to location differed among fumigation systems (overall p-value for this interaction, p-value < 0.0001). There were
no statistically significant differences among locations for the ClO$_2$ and formaldehyde systems (both Wald test p-values > 0.05). There were statistically significant differences in the log reduction among locations in the remaining systems (p-values ≤ 0.05). For the H$_2$O$_2$-1 system, the cabinet, centrifuge, and spills all had lower log reduction than the bench; for both the H$_2$O$_2$-2 and H$_2$O$_2$-3 systems, the centrifuge and spills had a lower log reduction than the bench; and for the ozone system, the cabinet and spills had a lower log reduction than the bench.

Table 5 shows differences in log reduction for Vaccinia virus estimated using mixed effects linear regression. For Vaccinia virus, differences in log reduction due to location differed among fumigation systems and location (p-value for this interaction, p-value ≤ 0.01). There were no statistically significant differences among locations for the H$_2$O$_2$-1, ClO$_2$, formaldehyde, and H$_2$O$_2$-3 systems (all Wald test p-values > 0.05). For the H$_2$O$_2$-2 system, the centrifuge had a statistically significantly lower log reduction than the bench (p-value ≤ 0.05), and for the ozone system, spills had a statistically significantly lower log reduction than the bench (p-value ≤ 0.05).

**Discussion**

This study evaluated available alternatives to formaldehyde for the fumigation of CL3 and CL4 laboratories. The work involved an investigation of system efficacy and usability in a laboratory setting. Other factors also need to be evaluated when considering alternative fumigants for such facilities, including reproducibility and reliability of system performance. The safety of the system, how easy it is to use, its cost, and time taken to fumigate are also important to the end-user and are considered below.
Efficacy

A priority when evaluating alternative fumigants to formaldehyde is the efficacy of other available systems, i.e., how effective is a particular fumigant at significantly reducing or eliminating microorganisms? Within the healthcare environment at least a 4-log microbial reduction is required before a disinfection process is regarded as effective. However, reduction on that scale may be somewhat less than needed for the laboratory environment, where higher levels of efficacy may be necessary if high titres of pathogens are in use. In this situation, there is a requirement for an effective emergency fumigation procedure to eradicate these high-level contaminants should that become necessary.

Formaldehyde was shown to be an effective fumigant, giving up to 6-log reductions for all organisms tested. This included efficacy for the simulated spill, though it was marginally less effective than ClO\textsubscript{2} when used against C. difficile. The 600-ppm level chosen for comparison with alternative systems did not deliver the maximum efficacy achievable for formaldehyde, and some laboratories may be using 1,200 ppm to 1,400 ppm for their whole-room treatments. However, the higher levels of formaldehyde used by some may exceed the necessary dose required and the findings here indicate that many laboratories could almost certainly reduce their overall use of formaldehyde. This lower-level formaldehyde demonstrated efficacy and consistency across the full range of experimental challenges.

The ClO\textsubscript{2} system demonstrated efficacy against all challenge organisms and was therefore used as the reference category for statistical analysis. Its performance

Table 3

<table>
<thead>
<tr>
<th>Location</th>
<th>H\textsubscript{2}O\textsubscript{2-1}</th>
<th>ClO\textsubscript{2}</th>
<th>Formaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diff.</td>
<td>(95% CI)</td>
<td>Wald Test</td>
<td>Diff.</td>
</tr>
<tr>
<td>Bench</td>
<td>(ref)</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td>Cabinet</td>
<td>1.67</td>
<td>(-0.03, 3.37)</td>
<td>0.00</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>-0.15</td>
<td>(-0.42, 0.13)</td>
<td>0.00</td>
</tr>
<tr>
<td>Floor</td>
<td>-0.05</td>
<td>(-0.14, 0.05)</td>
<td>0.00</td>
</tr>
<tr>
<td>Spills</td>
<td>-0.91</td>
<td>(-2.15, 0.32)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The p-value for the overall fumigation system by location interaction was < 0.0001. The Wald tests show whether there were any statistically significant differences in Log reduction between locations for each of the fumigation systems. Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.

Ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
N/A: not applicable
* significant at p≤0.05
** significant at p≤0.01
against \textit{C. difficile} and \textit{M. fortuitum} was especially notable, with 6- to 7-log reductions overall. The ClO\textsubscript{2} method was capable of killing these bacteria in a simulated spill as well as in dry residue form. Although good levels of efficacy were observed for hydrogen peroxide vapor systems, these exhibited greater variation in performance across location and sample type and did not consistently achieve the same efficacy levels as the ClO\textsubscript{2} system. 

Overall, formaldehyde, ClO\textsubscript{2}, \textit{H\textsubscript{2}O\textsubscript{2}}-1, and \textit{H\textsubscript{2}O\textsubscript{2}}-3 systems were effective for \textit{C. difficile}, but efficacy observed with the \textit{H\textsubscript{2}O\textsubscript{2}}-2 and ozone systems gave significantly lower log reductions. With \textit{M. fortuitum}, \textit{H\textsubscript{2}O\textsubscript{2}}-1, \textit{H\textsubscript{2}O\textsubscript{2}}-2, \textit{H\textsubscript{2}O\textsubscript{2}}-3, and ozone systems provided significantly lower log reductions than ClO\textsubscript{2}, with formaldehyde not being statistically significantly different to ClO\textsubscript{2}. 

For Vaccinia virus, all fumigation systems providing 3- to 4-log reductions in viable virus and no statistically significant differences in efficacy were observed among the different systems. Most viruses begin to lose viability once removed from their host cell culture, and the viral challenges used here (10\textsuperscript{7} (pfu)/ml, ~ 10\textsuperscript{6} (pfu)/disc) meant that typical levels presented for fumigation treatment were in the order of 10\textsuperscript{3}-10\textsuperscript{4} (pfu)/disc. The observed 3- to 4-log reduction, therefore, represented close to complete eradication of remaining virus. 

Some of the differences in efficacy among organisms may be due to the physical properties of the microorganisms, such as the presentation of \textit{C. difficile} and \textit{G. stearothermophilus} as spores, making them more resistant to killing by external agents. The target organism
ences were particularly evident for posed locations, such as the cabinet, centrifuge, and spills for penetration of the fumigant, with locations such as the bench top. These differences were particularly evident for C. difficile, where penetration of the centrifuge sample was limited for the H2O2-3 system (less than 2-log) when compared to other locations for this same machine (about 6-log). This same system performed well in challenging spill tests with C. difficile (6- to 7-log reduction). Conversely, the H2O2-1 system performed well against dried C. difficile in the centrifuge location and in other locations (6-log typical), but less effectively in the spill test (less than 2-log). These data, observed for a single bacterial species, underline the variability that may be observed for similar fumigants delivered in different ways. Penetrative ability against recesses and liquids, therefore, remains a challenge for fumigation technologies. The partially open centrifuge presented such a challenge and the data indicate that some fumigants penetrated less effectively here when compared to open-bench locations. This is

<table>
<thead>
<tr>
<th>Location</th>
<th>H2O2-1 Diff. (95% CI)</th>
<th>Wald Test p</th>
<th>ClO2 Diff. (95% CI)</th>
<th>Wald Test p</th>
<th>Formaldehyde Diff. (95% CI)</th>
<th>Wald Test p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench</td>
<td>(ref)</td>
<td>(ref)</td>
<td>N/A</td>
<td>p=0.0592</td>
<td>p=0.1350</td>
<td></td>
</tr>
<tr>
<td>Cabinet</td>
<td>-0.59 (-1.18, 0.01)</td>
<td></td>
<td></td>
<td></td>
<td>-0.14 (-0.52, 0.23)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge</td>
<td>-0.04 (-0.33, 0.25)</td>
<td>0.00</td>
<td>(-0.00, 0.00)</td>
<td>0.00</td>
<td>(-0.00, 0.00)</td>
<td></td>
</tr>
<tr>
<td>Floor</td>
<td>0.20 (-0.18, 0.58)</td>
<td>0.00</td>
<td>(-0.00, 0.00)</td>
<td>0.00</td>
<td>(-0.00, 0.00)</td>
<td></td>
</tr>
<tr>
<td>Spills</td>
<td>0.40 (-0.88, 1.69)</td>
<td>0.53</td>
<td>(-0.02, 1.08)</td>
<td>1.03</td>
<td>(-0.08, 2.15)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location</th>
<th>H2O2-2 Diff. (95% CI)</th>
<th>Wald Test p</th>
<th>H2O2-3 Diff. (95% CI)</th>
<th>Wald Test p</th>
<th>Ozone Diff. (95% CI)</th>
<th>Wald Test p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench</td>
<td>(ref)</td>
<td>(ref)</td>
<td>(ref)</td>
<td>(ref)</td>
<td>(ref)</td>
<td>(ref)</td>
</tr>
<tr>
<td>Cabinet</td>
<td>-0.01 (-0.66, 0.64)</td>
<td>-0.26</td>
<td>(-0.5, 0.001)</td>
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<td>Centrifuge</td>
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<td>-0.18</td>
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<td>(-0.85, 0.24)</td>
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</tr>
<tr>
<td>Floor</td>
<td>0.17 (-0.34, 0.68)</td>
<td>-0.11</td>
<td>(-0.22, 0.01)</td>
<td>-0.00</td>
<td>(-0.00, 0.00)</td>
<td></td>
</tr>
<tr>
<td>Spills</td>
<td>-0.68 (-3.45, 2.09)</td>
<td>0.90</td>
<td>(0.11, 1.70)*</td>
<td>-3.55</td>
<td>(-6.44, -0.65)*</td>
<td></td>
</tr>
</tbody>
</table>

The p-value for the overall fumigation system by location interaction was < 0.01. The Wald tests show whether there were any statistically significant differences in Log reduction among locations for each of the fumigation systems. Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.

ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
N/A: not applicable
* significant at p≤0.05
** significant at p≤0.01
likely to be due to the physical qualities of the fumigant and its resulting concentration within different parts of the treated room.

These observations underline the need for end-user optimization of the chosen system, based on individual laboratory requirements. These data also showed that antimicrobial efficacy of some systems, including the H\textsubscript{2}O\textsubscript{2}-1, H\textsubscript{2}O\textsubscript{2}-2, H\textsubscript{2}O\textsubscript{2}-3, and ozone systems were reduced for liquid spills compared with dry residues on the open bench. This may have been due to a localized dilution effect at the point of contact and corresponding difficulty in fumigant penetration of the liquid. However, this would require further investigation to confirm.

Reproducibility

For CL3 and CL4 fumigations, there is a need for confidence that the chosen system will give reproducible reductions in viable microorganisms every time it is used. Formaldehyde and Cl\textsubscript{2}O\textsubscript{2} gave consistently good results in all experiments in this study. The simplicity of formaldehyde delivery may have contributed to its reproducible efficacy, and it demonstrated the ability to kill microorganisms of all types. The Cl\textsubscript{2}O\textsubscript{2} system gave similarly consistent results for all microbial challenges and conditions under which it was used.

Some systems demonstrated less reproducibility and reliability than formaldehyde and Cl\textsubscript{2}O\textsubscript{2}. In particular, a marked difference was shown between the performances of two machines of the same type (H\textsubscript{2}O\textsubscript{2}-1); the first machine HSL tested had to be returned at the supplier’s request and was replaced soon after by an identical model. The replacement system performed better than the first and it was thought that a software control upgrade might have improved the performance of the second system. With the H\textsubscript{2}O\textsubscript{2}-2, H\textsubscript{2}O\textsubscript{2}-3, and ozone systems, more variation was observed among experiments than would be acceptable for routine, laboratory-based fumigation procedures.

Reliability

Formaldehyde fumigation is an inherently reliable approach and uses a simple method of delivery (a hot-plate approach). All the other systems tested in this study involved more complex machines and various reliability problems were encountered with each system. All alternative systems aborted on at least one occasion during use. If any of these alternative technologies is to be used in laboratories, they will require improved reliability.

The greatest number of problems was encountered with the H\textsubscript{2}O\textsubscript{2}-2 system, where narrow-bore tubing delivering fumigant to the nozzle became pinched between control valves after standing unused for longer than a week, limiting further use. Such weaknesses would need to be overcome for laboratories that do not perform regular weekly fumigations.

The H\textsubscript{2}O\textsubscript{2}-3 system was extremely reliable in tests performed in the CAC. However, in the larger CL3 lab, problems were encountered with the performance of the system’s desiccant cartridge. This cartridge contains a silicone-based desiccant that aids moisture removal at the end of the fumigation cycle. This needs to be dried (re-charged) between uses and problems were experienced in completely re-drying the cartridge. This may have related to the larger room size of the CL3 facility, although the supplier indicated that the system could handle the room volumes used for the tests.

With the H\textsubscript{2}O\textsubscript{2}-1 system, machine problems arose in relation to fumigant delivery and fan rotation, with both faults requiring supplier intervention.

The ozone system aborted on several occasions because the required ozone level in the room was not being reached. A problem was identified with the ozone sensors on the machine and required supplier intervention to rectify.

The Cl\textsubscript{2}O\textsubscript{2} machine experienced several aborted runs when it failed to reach its target humidity level within the room. The supplier had to visit HSL on only one occasion to remedy this.

Safety

Formaldehyde is a toxic chemical and has been classified as a Group 1 human carcinogen. For this reason, a choice of alternative, effective fumigation technologies is desirable. However, none of the fumigants evaluated here are harmless, and all have workplace exposure limits (WELs).

Hydrogen Peroxide

Three of the systems tested use hydrogen peroxide-based fumigant, which is an irritant and can be corrosive. Airborne residues of the fumigant must be checked prior to room re-entry to avoid inhalation and eye exposure. The need to open a bottle of concentrated H\textsubscript{2}O\textsubscript{2} (25% to 30% H\textsubscript{2}O\textsubscript{2}(aq)) did arise during testing and this increased potential exposure to this substance, which can burn skin and damage clothing. Suitable personal protection must therefore be worn. The H\textsubscript{2}O\textsubscript{2}-2 system used a lower 5% H\textsubscript{2}O\textsubscript{2} source from a sealed cartridge; these were sometimes difficult to install, although no spills occurred. Unwanted condensation of H\textsubscript{2}O\textsubscript{2} vapor (e.g., below cold windows or walls) poses a risk of damaging the fabric of the room and equipment upon repeated fumigations. However, serious condensation effects with H\textsubscript{2}O\textsubscript{2} were not observed during or after testing in the current study.

Ozone

The ozone system used an oxygen cylinder (industrial grade O\textsubscript{2}) to generate ozone, and this required supplier training to install and change, as well as an appropriate location to store spare bottles. This sys-
tem also used an aerosol quenching agent, which is generated at the end of ozone treatments and reacts with the ozone to assist its removal. This process generates by-products, including acetic acid residue, which have an obvious odor. The supplier reported that these residues are non-toxic, as demonstrated by independent testing. Odor levels were reduced during testing by improved quencher control, though some level of aeration was required after ozone quenching to assist in odor removal prior to room re-occupation.

**Chlorine Dioxide**

The ClO₂ system requires a supply of stabilized chlorine gas, provided by twin gas cylinders containing 2% chlorine gas in nitrogen under pressure (2,000 PSIG); gas data provided by supplier. As chlorine is a strong oxidizing agent and irritating to skin and mucosal membranes, a safety procedure, including personal protective equipment (PPE) or some type of gas-containment system, must be in place prior to use. Like other powerful oxidizing agents, ClO₂ may cause progressive damage to room surfaces and equipment that is not impervious to fumigation. The above information is well described within the training provided with the ClO₂ system.

During tests in the CL3 laboratory, all machines had to be operated from within the room, since we could not allow cables or pipes to extrude from the room via door seals. This would have compromised the ability to seal the CL3 laboratory. Unfortunately, the ClO₂ system supplier could not sanction the use of its machine within the CL3, so ClO₂ test data are not available for this location. Technical solutions allow other suppliers to provide control of their systems from outside the treated area, while their machines remain within the treated room. These include delays in start function on the machine or wireless operation of the system from outside the room. Although such systems allow for easier remote control and may provide useful information via remote screens during fumigation, they remain dependent on the ability to fully expose the machine to its own fumigant. This was not a preferred option for the ClO₂ system at the time of testing and was the only real limitation to this machine’s use.

**Fumigant Monitoring Prior to Room Re-entry**

This study revealed, without exception, that levels of fumigant were above the WEL at the end of fumigation cycles and required further air purging prior to room re-entry. This would not typically pose a risk of exposure in a containment laboratory, as it is always possible to use the room air-handling system to clear fumigant, but it is something users should be aware of, particularly those planning to use such systems in other settings (e.g., healthcare). A recommendation is that all systems be sold with a device for monitoring fumigant levels at the end of a cycle. Examples of portable monitors include the Portasens II (GemLog Controls Limited) and Dräeger hand-held systems.

**Ease of Use**

The vaporization of formaldehyde is a simple process. All the other systems involve more complex machines that require initial training to use safely and effectively. During this study, some of the machines required re-programming, and the H₂O₂-2 machine was the easiest to use, despite the increased complexity when modified cycles were used. The H₂O₂-3 system was the most difficult to program and its operation found to be non-intuitive. The ozone and ClO₂ machines had clear touch-screens, taking the user logically through the programming procedure. Although the H₂O₂-1 machine has multiple units to connect, it would typically be operated by trained operators from the supplier, and the company markets other systems that may be easier to operate but were not evaluated here.

Door modification would be essential for the ClO₂ unit, though it might not be desirable to have such a large machine located immediately outside a CL3 main door (e.g., in a lobby area). Some of the other machines are not normally operated from within the room being fumigated but were able to be operated effectively once installed there by the supplier. For example, the ozone machine is typically positioned outside the room, mainly to preserve the integrity of its ozone sensors, with ozone being piped into the room. Similarly, the H₂O₂-1 machine normally has a computer outside the room (connected via PC cables) for its operation, although the machine itself can reside within the room. For some of these mobile machines, the manufacturers need to consider their use in sealed rooms and to adapt them accordingly to make their use as straight-forward as possible within these higher-risk areas. In addition, the authors did have some problems with a machine setting off a particulate sensor fire alarm (dry mist system H₂O₂-2). This may be a problem for other users who switch from formaldehyde to alternative fumigants, since this problem was not associated with formaldehyde vapor alone.

For formaldehyde fumigation, a hot plate is in place for use in an emergency. Each of the alternative technologies involves a large machine, which would need to be stored between uses and maneuver into place for fumigation. This could be difficult in an emergency situation. In the case of newly built laboratories, some of these new technologies could be integrated within the fabric of the room, which should make treatment delivery easier. Alternatively, mobile machines could be located within facilities such as CL3 laboratories, but they would need to have the system operated remotely from outside the laboratory area. Such remote operation should, for safety reasons, include the ability to cancel and restart cycles in the event of machine failure.
Cost

While cost was not a primary consideration for this study, it is likely to influence user choice of alternative fumigation technologies. Formaldehyde remains a cost-effective method for fumigation, involving only the cost of purchasing formalin and a hot plate (probably less than £100 [$160 USD] for up to 1 year’s treatment, including the cost of a simple hot plate [e.g., a wok]). All the alternative systems tested involve a large initial outlay of thousands of pounds (£15K to £50K [$25,000-$80,000 USD] at the time of testing) to purchase the equipment, as well as servicing and repair costs. The consumables required for these systems vary in price but tend to be higher where there is the requirement for a custom-made product (e.g., the need to purchase dedicated cartridges to operate the H2O2-2 system, or the recommended H2O2-3 active product sold for that system). In other cases, the consumables are relatively inexpensive; for example, the H2O2-1 supplier recommended the purchase of good quality, 30% hydrogen peroxide from any chemical supplier. Industrial oxygen supplies for the ozone system were approximately £16 ($16 USD) per 10 kg bottle, and this was generally enough for three fumigation cycles of the CAC facility, though only one complete cycle could be reliably completed for the larger-volume CL3. Piped oxygen, if available, would probably be less expensive but also less versatile if the system needed to be used elsewhere.

Length of Time to Fumigate

Formaldehyde fumigation takes at least 6 hours with formalin/water first heated and vaporized, left to dwell in the room for a minimum of 4 hours, and then removed by a process of room venting. This is routinely done overnight in most laboratories for convenience, with final purging of the room using mechanical ventilation. All of the alternative technologies evaluated here are considerably faster for fumigation and often also have a defined fumigant removal step, although this was frequently found to be less effective than stated by the supplier. All CL3 and CL4 rooms have a ventilation facility for purging the air, and by using this to speed up removal of the fumigant, most systems allow re-entry to the room within 3 to 4 hours after the start of fumigation. The duration of room treatment may be an important consideration for laboratories that carry out regular (e.g., weekly) fumigations, but less of a priority for laboratories that carry out whole room fumigations only in emergency situations or prior to major maintenance work.

Geobacillus stearothermophilus as a Fumigation Test Organism

Geobacillus stearothermophilus is often used as a test organism to validate fumigation, and this role has evolved historically from its extensive use for the testing of steam sterilizers. In this study, variable levels of log reduction were observed for different microorganisms, and some were more difficult to eliminate than G. stearothermophilus, despite its use here in spore form. This underlines the need for system validation within the room to be fumigated, using relevant challenge organisms appropriate for that work area. Based on the findings here, it is concluded that a fumigation system can only be used with confidence when its efficacy is confirmed in this way.

Final Recommendations

This study has highlighted that differences in performance exist among the various fumigation systems, and that efficacy against different microorganisms can vary for one machine. The following recommendations are made from this study:

• Validation is important. Supportive data should be requested on the efficacy of the fumigation system, with particular attention to the type of microbial challenges likely to be faced by the end-user. Ideally, individual laboratories need to find appropriate surrogates (test challenges) if considering replacing formaldehyde with an alternative fumigation system, since not all fumigants are as widely effective as formaldehyde;

• When considering major equipment purchases, a full working demonstration of the chosen system prior to purchase or hire is recommended. Certain systems will be better suited to some room environments than others;

• Consider the logistics and ease-of-use of fumigation equipment as well as outright efficacy. Look at more than one available system, if possible, to allow comparison.

• Equipment purchases should not be made in isolation; include the views of appropriate partners (e.g., scientific staff, occupational health and safety advisors, risk management staff);

• When considering the cost of new fumigation equipment, ask for information on service provision as well as outright purchase (if options exist). Ensure that consumable costs will be acceptable as a long-term commitment.

• Health and safety are of paramount importance when using fumigation equipment, and comprehensive information and advice should be available prior to delivery and use of the system. This should include:
  ○ Effective, onsite training if a system is to be operated by the purchaser's own staff and not by the supplier
  ○ Handling of chemicals in line with Control of Substances Hazardous to Health regulations for transportation, handling, and storage
  ○ A risk assessment, identifying who might be at risk from fumigant exposure and how any risk can be mitigated
  ○ Checks for residual levels of fumigant after use of the machine, usually performed with an appropriate hand-held monitoring device

Finally, manufacturers of all the machines tested in this study need to address the issue of poor reliability. This is especially important for emergency fumigations,
where reliability would be paramount. It would also be helpful if manufacturers investigated the increased use of remote control of their systems for use within sealed rooms.

Acknowledgments

None of the authors involved in this research or in the preparation of this manuscript has any commercial affiliations or consultancies, stock, or equity interests, or patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted manuscript. © Crown Copyright. Health and Safety Laboratory, 2011. *Correspondence should be addressed to Alan J. Beswick at alan.beswick@hsl.gov.uk.

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Free Training Tools Available on the Web

*Applied Biosafety* readers may be interested in new training tools which are now freely available on the web.

- **Introduction to Biosafety and Biocontainment for Non-Laboratorians** is a 1-hour introduction to biocontainment laboratories designed for the general public. The basic safeguards in BSL-1, 2, 3, and 4 laboratories are graphically described. The curriculum was developed by Richard Green and is accessible at www.frontlinefoundation.org under “training.”
- **Autoclave Safety: The Proper Use of an Autoclave to Decontaminate Biohazardous Waste** is available on You Tube. This is a Laboratory Safety Project produced by The National Biosafety and Biocontainment Training Program, The National Institutes of Health, and Dartmouth College. Versions are available in English, German, Dutch, and Arabic.
Biosafety Onboard the International Space Station

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Abstract

In a typical microbiological or biomedical laboratory, potentially harmful biological hazards are contained by laboratory practices and techniques, safety equipment (primary barriers and personal protective equipment), and facility design. Despite the unique and restrictive environment in space, sound biosafety principles and practices that are commonly used on the ground, such as adequate medical surveillance, thorough risk assessment, sufficient level of containment, effective remedial controls, and the use of proper personal protective equipment, can still be applied in space to mitigate the potential risks that biohazardous materials present to the astronauts and to the internal environment of the International Space Station (ISS).

Introduction

In 2005, the National Aeronautics and Space Administration (NASA) Authorization Act designated the U.S. segment of the International Space Station (ISS) as a national laboratory (NASA, 2005). An increasing number of payload experiments from the U.S. and International Partners involving potentially harmful biological hazards are used in scientific investigations aboard the ISS (Figure 1).

Potentially biohazardous materials that can be found in space-bound payload experiments include bacteria, fungi, animals, plants, toxins, recombinant DNA, and mammalian cell lines. Sound biosafety principles and practices that are commonly practiced on the ground, such as adequate medical surveillance, thorough risk assessment, sufficient level of containment, effective remedial controls, and the use of proper personal protective equipment, are applied in space to mitigate the potential risks associated with the biohazardous materials.

Medical Surveillance for Astronauts

Close contact among astronauts onboard a closed environmental system such as the ISS raises the likelihood of transmission of infectious agents when compared to most ground-based environments. To minimize the transmission of potential infectious pathogens, the following medical surveillance practices have been implemented per NASA’s Flight Crew Health Stabilization Program (NASA JSC, 2005; NASA JSC, 2006).

Quarantine

Astronauts participate in the Johnson Space Center (JSC) Flight Medicine Program which has its own flight surgeons, examination rooms, and equipment. This separation from the general employee Occupational Health Clinic and its physicians limits the astronauts’ exposures to transmissible diseases. The astronaut crew resides in the Crew Quarantine Facility for 7 days before launch. Essential support personnel in contact with the crew during this period must undergo medical evaluation and show no symptoms of disease before they are allowed to be in close proximity with the crew.

Vaccinations

All active astronauts are current on the following immunizations: diphtheria and tetanus, polio, hepatitis A, hepatitis B, measles/mumps/and rubella, and varicella. The astronauts are also tested for tuberculosis annually by either a Purified Protein Derivative skin test or a Quantiferon gamma-interferon test to ensure that they are free of tuberculosis.

Health Assessments

For ISS long-duration flight astronauts, pre-flight health assessments are performed at the astronaut’s annual medical examination, at 45 to 30 days before scheduled launch (L-45/30), and at 10 days before scheduled launch (L-10) (NASA JSC, 2006). A post-flight health assessment is also performed upon landing. Astronaut health assessments include a clinical nutritional assessment, dental examination, ophthalmology exam, audiograph, physical examination, psychological evaluation, functional neurologic assessment, neurocognitive assessment, functional fitness assessment, resting ECG, maximum and sub-maximum heart rate testing, aerobic capacity testing, pulmonary function testing, bone densitometry, and clinical laboratory assessments such as complete blood counts, blood chemistry, urinalysis, and methicillin resistant Staphylococcus aureus (MRSA) nasal screening.

Biological Payload Experiments

Risk Assessments

All payloads destined to the ISS are assessed by the Payload Safety Review Panel (PSRP) at JSC. The payload safety review process includes the identification of hazards (toxicological, biological, physical, and flammabil-
ity), the definitions of hazard controls, and the verifications of hazard controls (NASA JSC, 2007). All ISS-bound payload experiments containing biological materials are assessed by the Biosafety Review Board (BRB) at JSC in support of the PSRP’s overall safety assessment (NASA, 1998). The JSC BRB is the equivalent of an Institutional Biosafety Committee (NIH, 2011). The BRB is composed of a team of biosafety professionals, microbiologists, cell biologists, physicians, industrial hygienists, and safety professionals to assess the wide range of biohazardous materials encountered.

Payload experiments containing bacteria, fungi, animals, plants, toxins, recombinant DNA, and mammalian cell lines are routinely flown to the ISS. Thorough risk assessments to identify and implement the appropriate hazard controls for biohazardous materials encountered onboard the ISS are performed. The criteria for assessing biohazardous materials include but are not limited to the identity of the agent, the nature of the agent, the infectious dosage, the amount of the agent present, the route of infection, the medical consequences of infection, and the hazards associated with the experimental protocols.

Infectious agents are typically categorized into risk groups based on their relative risk (NIH, 2011; WHO, 2004). Biosafety level refers to the laboratory practices and techniques, safety equipment, and laboratory facilities that are utilized to contain infectious materials in the laboratory environment (U.S. Department of Health and Human Services, 2007). Risk groups correlate with but do not necessarily equate to biosafety levels. Risk assessment determines the degree of correlation between an agent’s risk group classification and biosafety level (U.S. Department of Health and Human Services, 2007). All identified biohazardous materials are assigned a JSC biosafety level (BSL). The BSL designation is used by the PSRP to assign level(s) of control or containment necessary for spaceflight. The ultimate goal is to prevent any biohazardous incident that may endanger flight crews and vehicle integrity. The BRB at JSC has modified, for its own purposes, the Biosafety in Microbiological and Biomedical Laboratories (BMBL) guidelines (U.S. Department of Health and Human Services, 2007) and categorized biohazardous materials into five groups as listed in Table 1.

Biohazardous materials that have been assessed as NASA JSC BSL-3 or BSL-4 are not allowed to be flown on any payload experiment. These are potentially lethal agents that cause disease by airborne transmission and/or direct contact. As a result of this restriction, in order to provide a greater risk distinction for assessing biohazardous materials that fall under the NASA BSL-2 category, the BRB at JSC has divided the category into two groups: BSL-2 Moderate and BSL-2 High. BSL-2 High agents typically have lower infectious doses and carry higher aerosolization risks during the experimental process.

Containment

In the ISS environment, the containment of biological hazards in payload experiments faces spaceflight-unique constraints such as the fluid dynamics in microgravity and the lack of a full-sized class II or III biological safety cabinet.

In microgravity, fluids flow freely in the cabin, thus increasing the exposure risk to the crew should there be a containment breach. A Microgravity Science Glovebox (MSG) is available for biological experiments onboard the ISS. The MSG (Figure 2) offers 255 L (9 ft$^3$) of work area sealed and held at negative pressure, accessible to the crew through glove ports. The limited availability and small working surface of the MSG create an added challenge to contain biohazardous materials in all of the biological payload experiments.

Given these unique constraints, basic containment principles such as appropriate practices and techniques,
safety equipment (primary barriers and personal protective equipment), and facility design can still be applied in space, but the emphasis is placed on hazard minimization and engineering controls designed to minimize the possibility of a breach of containment and reduce the astronauts’ exposure to hazardous biological materials. As a general rule, the experiment safety review process promotes a “design for minimum hazards” and “elimination/minimization of hazard potential” approach whenever possible. That is, given the choice between a lower or higher risk biological/toxicological substance that would produce similar end results, the principal investigator (PI) should always attempt to use the lowest risk substance if it can accomplish the same scientific objective. Included within this philosophy is attention to the selection of design features that isolate against potential hazards and may include dissimilar features and protection against common-cause failure modes.

### Table 1

NASA JSC BRB’s classification of biological hazards.

<table>
<thead>
<tr>
<th>JSC Biosafety Level (BSL)</th>
<th>Description</th>
<th>Level of Containment/Control Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Well-characterized agents not known to cause disease in healthy adult humans, and of minimal potential hazard to the environment.</td>
<td>1</td>
</tr>
<tr>
<td>2 Moderate</td>
<td>Agents that are associated with human disease and are of moderate risk to humans and/or the environment. The infectious doses of these agents are higher and the likelihood of aerosolization during the experimental process is remote.</td>
<td>2</td>
</tr>
<tr>
<td>2 High</td>
<td>Higher risk agents associated with human disease. Risk is increased by lower infectious dose, likelihood of aerosolization during the experimental process, and other factors.</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Agents that can cause serious or potentially lethal disease as a result of airborne transmission.</td>
<td>Not allowed to fly</td>
</tr>
<tr>
<td>4</td>
<td>Agents that can cause life-threatening disease as a result of airborne transmission and/or direct contact.</td>
<td>Not allowed to fly</td>
</tr>
</tbody>
</table>

### Figure 2

Microgravity Science Glovebox
that may remove multiple controls in one off-nominal event. For example, three concentric o-ring seals in a single container may have a common-cause failure mode should the structure itself be compromised from a material flaw/imperfection in the container wall; thus, the design must be thoroughly qualified and tested not only at the individual seals, but also the strength/integrity of the construction materials of the walls must also be verified. Operational hazard controls and reliance upon astronaut procedures/training are minimized, whenever possible, in favor of robust equipment design features. Human error must always be a serious consideration in development of a thorough hazards analysis and must be included as part of the experiment’s failure modes and effects analysis/fault tree construction.

With the lack of a full-sized primary barrier such as a biosafety cabinet onboard the ISS, all biohazardous materials are controlled or securely contained in a designated-level(s) of control or containment, respectively. The level(s) of control or containment is designated by the PSRP at JSC based on the biosafety level assessments performed by the BRB and other hazardous factors such as chemical, radiation, electrical, and thermal hazards associated with the payloads. For containment, biohazardous materials assessed by the BRB as BSL-1, BSL-2 Moderate, and BSL-2 High typically require 1, 2, and 3 level(s) of containment, respectively. Typically, the number of level of containment increases as the risk increases. Each level of containment assigned by the PSRP has to pass various stringent validation tests prior to launch. Such tests may include (pre-flight) depressurization/re-pressurization testing of the assembled hardware in vacuum chambers with before/after weight comparisons to confirm “leak-tightness” under reduced pressures, bubble leak tests (submerging the hardware underwater to a certain depth for gross leak checks), and/or higher-fidelity helium leak testing to confirm acceptable leak rates for assembled equipment.

Other hazard control verification discussions conducted at the safety review panel include crew handling loads, repetitiveness of levels of containment, cycling and manipulation of polymeric containment barriers, and the ability to re-seal/re-verify safety barriers if opened/re-closed on-orbit (such as ziploc bags, vials with screw-on/off caps and/or o-ring seals). Materials compatibility is also crucial. A typical deliverable to the safety panel might include an analysis by the hardware provider documenting the expected life of the seals, given worst-case conditions of temperatures, pressures, and assumed continuous fluid/gas contact between the materials of construction and materials of study, and data to show that the safety-critical seals do not degrade for the expected duration of the hardware on-orbit. Finally, the safety panel will conduct detailed reviews of the hardware’s mechanical testing regime to verify its ability to survive worst-case shock and vibration loading, which could cause structural failure of containment barriers. Such testing programs usually include qualification testing of the hardware to much higher than expected loading conditions, with acceptance level testing to flight loads for verification of workmanship.

**Practices and Techniques**

Principal Investigators flying payload experiments are aware of the constraints in-flight. As a result, procedural steps such as vortexing and pipetting are engineered out of the protocol and are sometimes replaced by automated processes where the involvement of the astronaut is reduced to the push of a button. Astronauts are trained to perform the payload experiments prior to launch. In-flight protocols are also uploaded to the ISS to assist the astronauts in performing the experiments. Appropriate hazard level labels addressing biosafety, toxicity, and flammability are implemented onboard the ISS. Specific control protocols corresponding to the assigned hazard levels are developed as part of the safety assessment process for the cleanup of biohazardous materials.

Disinfectants are carefully scrutinized with respect to flammability or potential toxic side effects when used in such a restricted environment. Alcohol-based disinfectants are not allowed in-flight due to an ISS water recovery system requirement that limits the release of volatile organic compounds. Disinfectants that are available for the cleanup of biohazardous spills include hydrogen peroxide-based and quaternary ammonium chloride-based wipes. Disinfectants in spray bottles are prohibited in-flight due to the free floating nature of mists and droplets in microgravity, which increases the risk of mucous membrane exposure to the crew.

All blood and body fluids collected in-flight are stored in leak-proof containers and transported back to ground for post-flight analysis.

**Safety Equipment (Personal Protective Equipment)**

Eye goggles, N-95 particulate respirators, gas and vapor respirators, air-supplied respirators, nitrile gloves, latex gloves, silver shield gloves, and protective clothing are readily available and are listed in experimental and cleanup protocols as needed.

**Facility Design**

With the interconnected modular design of the ISS, only a few of the traditional ground-based facility designs can be implemented on the ISS to minimize the transmission of infectious diseases. Most of the interior surfaces of the ISS are made of aluminum and stainless steel, which can be easily cleaned and decontaminated with the approved disinfectant wipes (quaternary ammonium chloride-based or hydrogen peroxide-based). The air treatment system includes high efficiency particulate air (HEPA) filters strategically placed at numerous return air vents onboard.
the ISS to reduce the transmission of airborne environmental contaminants between modules. Hygienic hand-wipes are located throughout the ISS for ease of hand cleaning. A closed eye wash system is also available onboard the ISS.

**Summary**

Despite the unique and restrictive environment in space, sound biosafety principles and practices commonly used on the ground, such as adequate medical surveillance, thorough risk assessment, sufficient level of containment, effective remedial controls, and the use of proper personal protective equipment, have been successfully implemented in the ISS to mitigate risks of exposure to biohazardous materials to astronauts and to the internal environment of the ISS. To date, no incidents of a complete breach of containment and no documented crew exposures to biohazardous agents used in any of the payload experiments have been reported. The ISS provides a safe environment for the astronauts to live in and to conduct meaningful biomedical research.

**Acknowledgments**

This study was not funded by any external agencies, organizations, or companies. *Correspondence should be addressed to Wing C. Wong at wing.wong-1@nasa.gov.*

**References**


**Training Announcements**

**Workshop**

The ABSA Affiliate and Biosafety Association Workshop will take place on November 3, 2011 at the Anaheim Marriott in Anaheim, California following the ABSA Annual Conference.

**Principles & Practices of Biosafety (PPB)**

The Principles & Practices of Biosafety is a comprehensive, interactive, 5-day course that introduces the essential elements of biosafety and provides extensive resource lists for use after the course. Interactive exercises are used throughout to provide hands-on experience and to encourage networking and problem-solving among participants and instructors. To register for the PPB at the Embassy Suites San Diego Bay-Downtown in San Diego, California from February 26 through March 2, 2012, go to www.absa.org/eduppb.html

**Webinars**

A “Call for Webinars” is posted on the ABSA web site at www.xcdsystem.com/absa2011/webinar.cfm
Survival of Microorganisms on HEPA Filters
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Abstract

High Efficiency Particulate Air (HEPA) filters are required to minimize the release of microorganisms from laboratories and other settings. This study was carried out to assess whether a range of microorganisms captured on HEPA filters would survive under normal operating conditions. Bacillus atrophaeus (NCTC 10073), Staphylococcus epidermidis (NCIMB 12721), MS-2 coliphage, Escherichia coli (NCIMB 9481), Brevundimonas diminuta (NCIMB 11091), and Aspergillus brasiliensis (ATCC 16404) were individually aerosolized using a Collison nebulizer and captured on HEPA filter material. Clean air was drawn through the loaded filters for 6 days at a constant rate (face velocity of 0.4-0.5 m/s) for all organisms and for 210 days for B. atrophaeus to simulate the use of a HEPA filter. Pre-packed sterile filters were also contaminated with B. atrophaeus which survived on the HEPA filter material for 210 days with no significant loss of viability. MS-2 coliphage and A. brasiliensis survived over the 6 days without any significant loss of viability. There was a 5-log reduction in viability of S. epidermidis over 6 days, while both Gram-negative bacteria, E. coli and B. diminuta, were not recoverable after 48 hours of exposure. This study highlights the need for risk assessments and rigorous guidelines on the use and handling of air filtration membranes exposed to resistant pathogenic agents to minimize the risks of occupational exposure.

Keywords
HEPA filters, air microbiology, aerosols, biosafety, filter material

Introduction

The use of HEPA filters in microbiological laboratory heating, ventilation, and air-conditioning (HVAC) systems is critical for the containment of airborne pathogens. The major role of HEPA filters in laboratories is to remove a range of microorganisms from the air and to prevent their release into uncontrolled areas (Abraham et al., 1998; Maus et al., 2001). The integrity and efficiency of HEPA filters are evaluated at regular intervals to determine that their performance is satisfactory. These evaluations involve an annual assessment of the integrity of the filter medium to determine that the filter is still retained in its housing and that the pressure drop across the filter is acceptable. When the filters are handled for assessment or removal, there is potential for exposure through re-aerosolization of microorganisms that may have been deposited onto the HEPA filter membrane surface during usage (Bearg, 1993; Neumeister et al., 1997).

This study set out to assess the survival of a range of microorganisms including bacteria, bacteriophage, and moulds on filter membranes. New HEPA filter materials were challenged with microbial aerosols under defined conditions and placed on a modular air-flow system to simulate use over a range of time periods.

A range of microorganisms (Bacillus atrophaeus, Staphylococcus epidermidis, Escherichia coli, Brevundimonas diminuta, Aspergillus brasiliensis, and MS-2 coliphage) were selected to represent spore formers, vegetative bacteria, moulds, and viruses that could present a challenge to filters (Fannin et al., 1985; Laitinen et al., 1994; Maus et al., 2001) currently used in HVAC systems.

Materials and Methods

Stock cultures of S. epidermidis (NCIMB 12721) and E. coli (NCIMB 9481) (stored at 4ºC) were grown in Trypticase Soy Broth (TSB) (100 ml) in a shaking water bath (130 rpm, 37ºC for 19 hours ± 1 hour) to a concentration of 1.17 x 10^10 cfu/ml (nomenclature for “cfu per ml”) and 3.15 x 10^9 cfu/ml, respectively.

Coliphage MS-2 (NCIMB 10108) was replicated using E. coli (NCIMB 9481) as the host. E. coli was sub-cultured from TSBA agar (37º ± 2ºC for 20 hours) into TSB (60 ml) and placed into a shaking incubator (120 rpm, 150 minutes at 37º ± 2ºC). Coliphage was then prepared by inoculating MS-2 stock into TSB which was aerated by shaking (37º ± 2ºC for 3 hours). The suspension was then centrifuged twice at 2,000 g (20 minutes) to remove the cell debris. The supernatant was transferred to a fresh flask and the concentration of phage was then determined.

An aqueous spore suspension (3.53 x 10^9 spores/ml) of B. atrophaeus (NCTC 10073), previously produced as a stock solution by Health Protection Agency (HPA) (Porton Production Division, Salisbury, UK) was used as the stock spore suspension for this study. Serial dilutions were prepared in phosphate buffer containing maniocul and antifoam (PBMA) and plated onto tryptic soy agar (TSA) plates that were incubated at 37º ± 2ºC for 24 hours.

A. *brasiliensis* (ATCC 16404) was grown on malt extract agar (MEA) (n=5) at 37º ± 2ºC for 48 hours. Spores were retrieved by pouring 10 ml of sterile distilled water onto each agar plate and then gently removing the growth, using a sterile L-shaped inoculating loop into a sterile container (50 ml), to achieve a spore suspension of 3.9 x 10^6 spores/ml^-1.

A stock culture (4ºC) of *B. diminuta* (NCIMB 11091) was grown in TSB (100 ml) in a shaking water bath (130 rpm, 30ºC for 19 hours ± 1 hour). The suspension was centrifuged at 2,000 g for 30 minutes. The pellet was washed three times, after which the supernatant was discarded and the cells re-suspended in sterile distilled water (60 ml) to achieve a suspension of 6 x 10^9 cfu/ml^-1.

**HEPA Filters**

Pre-packed hydrophobic pleated membrane HEPA filters (BB22-15, Pall, Portsmouth, UK) were aseptically dismantled. An area of the filter material (0.042 m in diameter) was removed and assembled in a sterile filter holder (16508 B, polycarbonate in-line 50-mm filter holder, Sartorius-stedim, France) which was connected to the Henderson Apparatus (Henderson, 1952) to challenge filters with the microbial aerosol (Druett, 1969).

Individual suspensions of *B. atrophaeus*, *S. epidermidis*, MS-2 coliphage, *E. coli*, *B. diminuta*, and *A. brasiliensis* were placed into the Collison nebulizer (BGI, Inc., Waltham, MA) to generate an aerosol that passed along the flow channel of the Henderson Apparatus (Druett, 1969). The centrifugal fan unit was used to draw conditioned room air through both filters at a constant flow rate, as measured by a mass flow meter (TSI 4040, TSI, Shoreview, MN) giving a face velocity ranging from 0.4-0.5 m/s^-1. This apparatus was used to simulate HEPA filters mounted within a laboratory over a period of 6 days. Following exposure, a section from each filter (0.016 m in diameter) was removed for each microorganism (in triplicate) and placed into PBMA (10 ml) containing sterile glass beads (4 glass beads, each 4 mm in diameter) and vortexed (1 minute). Serial dilutions were then prepared and were plated onto the appropriate medium (TSA for *S. epidermidis*, *E. coli*, *B. diminuta*; *B. atrophaeus* and MEA for *A. brasiliensis*) and incubated as described above.

Complete pre-packed sterile filters (Maus et al., 2001) were also used in this study to determine the recovery of *B. atrophaeus* over a longer time period. The filter cartridge was connected to the Henderson Apparatus and a suspension of *B. atrophaeus* was placed into the Collison nebulizer to challenge the pleated membrane as described above for the filter holder (Figure 1). The filter cartridges (Maus et al., 2001) were then removed from the Henderson Apparatus and connected to an airflow apparatus operated at room temperature. A centrifugal fan (ACI, Axminster, UK) was used to blow air through a H14 HEPA filter into a central column, onto which the test filters were fitted. This allowed filtered air to be blown through each filter simultaneously at a constant rate (0.4-0.5 m/s^-1 face velocity) for up to 210 days.

Filters were removed from the airflow apparatus and an area (0.016 m in diameter) of each filter material was aseptically removed from each monthly sample (in triplicate) and placed into PBMA (10 ml) containing sterile glass beads (4 glass beads, each 4 mm in diameter) and vortexed (1 minute). Each set of monthly filters were processed (in triplicate) and serial dilutions were plated onto TSA plates and incubated as described above.

**Figure 1**

Schematic of the microbial challenge system showing the Collison Nebulizer, Henderson apparatus, and filter assembly.
Plaque Assay

E. coli was sub-cultured from a TSA plate (37° ± 2°C for 20 hours) and transferred into sterile nutrient broth in a glass bottle, mixed and incubated (37° ± 2°C for 260 minutes). Soft phage agar was melted and held in a water bath at 60° ± 2°C. Prior to use it was allowed to cool to 40° ± 2°C for use in the assay. MS-2 suspension in PBMA (100 µl) and 60 µl of the E. coli suspension were added to the soft agar which was poured on a Tryptone Soya Broth Agar (TSBA) plate. Duplicate samples were prepared and incubated at 37°C ± 2°C overnight. Viral plaques were counted the following day and data from plates containing 30 to 300 plaque forming units (PFU) were recorded.

The microbial recovery from the membrane filters was calculated by multiplying the cfu/ml⁻¹ by the area of the membrane placed in the holder (1.38 x 10⁻³/m²) and dividing by the area of the portion of the membrane from the holder used for assaying the amount of colonies (2.01 x 10⁻⁴/m²) and by the area of whole membrane unit (0.07 m²) in the case of B. atrophaeus in the 210-day study.

Results and Discussion

The results indicated that survival times were different for the range of microorganisms deposited onto the HEPA filter (Figures 2 and 3). E. coli and B. diminuta were not recoverable after 2 days whilst S. epidermidis was not recoverable after 6 days (Figure 2).

B. atrophaeus and A. brasiliensis showed no significant loss of viability on the HEPA filters for up to 6 days (Figure 2) whilst MS-2 coliphage lost less than 1 log (80% of original sample) over 6 days. The additional experiments using the filter cartridges demonstrated that B. atrophaeus spores survived for 210 days with little loss in viability (Figure 3).

The results for A. brasiliensis and MS-2 coliphage (used as a viral surrogate) were comparable with B. atrophaeus. A. brasiliensis spores are known for their resistance to desiccation (Barg, 1993) and MS-2 virus, which is a non-enveloped virus, lacks a lipid bilayer, and has been shown to survive longer than enveloped virus (Rengasamy et al., 2010; Rutala & Weber, 2004) such as influenza.

These findings support other studies demonstrating that B. atrophaeus survives longer than S. epidermidis (Brosseau, 1994) on filters. Not surprisingly, increased survival of B. atrophaeus has been demonstrated on filters in the presence of nutrients (Wang et al., 1999). Whilst other studies have also demonstrated the survival of B. atrophaeus for up to 5 days (Maus et al., 2001) and A. brasiliensis for several weeks under air flow conditions on filters (Kemp et al., 2001), this current study demonstrated that B. atrophaeus can also survive on filters (with air flow) for more than 210 days (Figure 3) in the absence of contaminating nutrients. In addition, bacteriophage MS-2 was also capable of retaining viability for up to 7 days on filter surfaces and so may indicate that other viruses could also survive.

Figure 2

Survival of B. atrophaeus, MS-2 coliphage, A. brasiliensis, S. epidermidis, E. coli, and B. diminuta on the filter membrane over 6 days (bars represent means of triplicate counts and error bars represent standard deviations).
Whilst this study used a range of non-pathogenic microorganisms, it would be prudent to be cautious with HEPA filters removed from laboratories where pathogenic microorganisms are used, as this removal process may indeed present a situation where occupational exposure could occur. A risk assessment should be conducted to determine potential occupational exposure hazards that may be present during removal and disposal of filters. Based on the risk assessment, mitigation measures may involve decontamination of filters, use of personal protective equipment, or other methodologies prior to removal and disposal of HEPA filters.

Acknowledgments

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References


Abstract

Anesthetic gases such as halogenated gases used independently or in conjunction with nitrous oxide are utilized to sedate animals prior to surgery. Acute and chronic exposure to anesthetic gas poses a risk to human health; therefore, appropriate controls must be utilized to protect the health of employees handling anesthetic gases. Engineering controls and work practice controls were developed at the University of Pittsburgh Regional Biocontainment Laboratory (RBL) to protect workers from exposure to levels of waste anesthetic gases (WAG) that exceed the recommended exposure limit (REL). Additionally, a decontamination strategy for monitoring badges was developed so that the monitoring badges used in BSL-3 facilities to detect and quantify WAG exposure could be safely analyzed outside of the BSL-3.

Introduction

Anesthetic gases such as halogenated anesthetic gases (including isofluorane and sevoflurane) and nitrous oxide are utilized to anesthetize both humans and animals. Both acute and chronic exposures to halogenated anesthetic gases pose health risks to humans, including headache, nausea, ataxia, liver and kidney disease, and reproductive effects (Gardner et al., 1991; OSHA, 2000). The National Institute for Occupational Safety and Health (NIOSH) estimated that in 1975 over 200,000 employees were potentially exposed to anesthetic gases in the United States (NIOSH, 1977). This estimate included approximately 50,000 veterinarians and other veterinary employees who are at risk due to the use of anesthetic gases during animal surgeries. In 1977 NIOSH published a recommended exposure limit (REL) of 2 ppm for halothane over a 1-hour sampling period (NIOSH, 1977). Although the NIOSH REL has not been updated since then and does not specifically address some of the commonly used anesthetic gases such as isofluorane, sevoflurane, and desflurane, it is still informally applied to all halogenated anesthetic gases including isofluorane. The Occupational Safety and Health Administration (OSHA) has not established an exposure limit for halogenated anesthetic gases, although OSHA has provided more recent guidance, it is still based upon the NIOSH REL (OSHA, 2000).

The Regional Biocontainment Laboratory (UPitt RBL) is part of the University of Pittsburgh’s Center for Vaccine Research and supports development of vaccines and therapeutics to emerging infectious diseases as well as potential bioterrorism agents (NIAID, 2002, 2003a, 2003b). The facility has approximately 20,000 square feet of biosafety level 3 (BSL-3) and animal biosafety level 3 (ABSL-3) research space. Each of the four ABSL-3 suites contains an animal holding room, a procedure room, and a laboratory for animal support. Several assumptions were made during the design phase based upon the research anticipated at that time: 1) non-human primates would be the main type of animal utilized in research; 2) only minor survival surgery procedures such as phlebotomy and bronchoalveolar lavage would be performed in the animal suite procedure rooms; and 3) all major survival surgeries would be performed in the designated surgery suite. Therefore, the procedure rooms were outfitted with surgical lights, steel tables, and controlled drug boxes, but without air/oxygen/gas scavenging lines. Each animal suite laboratory was equipped with one 6-foot Class II type A2 canopy-connected biological safety cabinet (SteriGARD II, The Baker Company, Sanford, ME) to support processing of non-human primate (NHP) blood, serum, and tissues, and minute quantities of toxic and volatile chemicals needed for sample analysis (NSF, 2009). During the construction and commissioning phases, the focus of the UPitt RBL was expanded to include pathogenesis and vaccine studies for a variety of infectious agents and additional animal models as well as a pre-clinical imaging suite equipped with microPET (Siemens Medical Solutions USA, Inc., Malvern, PA) and computed tomography (NeuroLogica, Danvers, MA). The surgical suite was redesigned and constructed into the imaging suite, and the imaging suite was the last area commissioned prior to opening the facility to animal research.

Since opening in 2008, animal research studies utilizing mice, rats, ferrets, rabbits, and two species of non-human primates have involved the use of isoflurane. Isoflurane provides smooth muscle relaxation and, unlike other halogenated gases such as halothane and enflurane, does not cause cardiac arrhythmias, cardiac depression, or seizures (Stimpfel & Gershey, 1991) in the animal subjects. When used with a precision vaporizer, isoflurane allows the veterinarian to maintain the animal on a desirable plane of anesthesia until the completion of surgery, after which a rapid recovery time is
typically observed. Isoflurane anesthesia of rodents and ferrets has been performed in the thimble-connected Class II type A2 biological safety cabinets (BSCs) in the RBL animal suite labs and personnel monitoring has demonstrated that the use of the BSCs results in no detectable levels of anesthetic gases in the room (data not shown). This is not possible for larger animals such as rabbits because the room is relatively small and the BSC sash is too short (8 inches) to allow workers to safely manipulate the larger animal and perform phlebotomy via the marginal ear vein/artery or other common manipulations. Therefore, isoflurane anesthesia of a larger animal must be performed in the procedure rooms which lack built-in engineering controls for WAG. Although this is less preferred and may be less safe in terms of reducing personnel exposure to WAG, safety for personnel handling sharp devices with infected animals is perceived to increase by allowing them the full range of motion not permitted via the BSC sash.

Potential sources of WAG in the veterinary setting include the exhaust ports of passive gas-scavenging cannisters, leakage around the door/ seal of an induction box, leakage around the seal of the nose cone to the animal’s snout/nose, portable gas delivery systems that either lack scavenging systems altogether or that lack mechanized scavenging systems, difficult intubation of certain research animals, equipment such as Class II BSCs that recirculate air back to the room, and laminar flow equipment that directly pushes air across the work surface to the employee (Cooper et al., 1998; Gardner et al., 1991; Smith & Bolon, 2006). It was recognized that due to the lack of built-in engineering controls, the use of isoflurane in the UPitt RBL procedure rooms could lead to WAG levels in the room that exceed the REL. Additionally, due to animals’ rapid recovery time from isoflurane anesthesia, a continuous flow of isoflurane must be administered until the completion of the procedure, thereby increasing the level of WAG in the room. This article describes the development of work practice controls, engineering controls, and personal protective equipment (PPE) to reduce the level of WAG in the environment while maintaining safe sharps practices and to prevent occupational exposures to WAG. This article also describes the development of a method to inactivate any potential biological contamination on the monitoring badges used in the BSL-3 facility while preserving the integrity of the monitoring badge results.

Method

All animal studies were reviewed and approved by the University of Pittsburgh Animal Care and Use Committee (AAALAC accreditation #A3187-01). New Zealand white rabbits (Oryctolagus cuniculus, Myrtle’s Rabbity, Thompsons Station, TN) were vaccinated and bled weekly for 30 days then exposed to the challenge agent, after which bleeds were performed every 2-3 days for up to 28 days followed by euthanasia and necropsy. During the first phase of the study, pairs of rabbits were sedated with ketamine (80 mg/kg) and xylazine (8 mg/kg) via intramuscular injection, removed from their cages, and transported to the procedure room (approximately 20 m³) for phlebotomy on a stainless steel surgical table. The veterinary staff observed that ketamine used in insufficient quantities resulted in an insufficient recovery time, but that slightly increasing the dose induced an unexpectedly deep sedation and subsequent prolonged recovery time (T. Dunsmore, personal communication).

Because the technicians are required to observe the animals until they recover from sedation and regain consciousness and maintain sternal recumbency, either an additional pair of technicians would be required to process the next pair of animals, or the procedure would take an estimated 12-18 hours to complete for the entire cohort. Additionally, because the animals would be challenged with an infectious respiratory agent, there were concerns that unusually deep sedation could result in severely depressed respiratory function possibly leading to mortality (Inglis & Strunk, 2009). Attempts were made to titrate the dose down to place the animal on an appropriate plane of anesthesia to allow the ear bleed but provide a shortened recovery time; however, this optimal dose was not found. Therefore, the investigator gained approval to use isoflurane.

For subsequent phlebotomy, pairs of animals were sedated with xylazine via intramuscular injection (8 mg/kg) and, once observed to be lightly sedated and relaxed, were then given general anesthesia at a rate of 1%-5% isoflurane (Webster Veterinary, Devens, MA) until judged appropriately anesthetized for blood withdrawal. The anesthesia circuit consisted of a Matrix VIP 3000 anesthesia machine (Midmark Corp., Versailles, OH), a carbon dioxide absorbance chamber, a Matrix veterinary anesthesia ventilator (not used), a compressed oxygen cylinder, all corrugated tubing for a non-rebreathing circuit, and a nose cone equipped with a gasket seal. The animal was maintained on a stream of isoflurane during the procedure, and then the second animal was processed similarly. When both blood draws were complete, the animals were returned to their home cages in the adjacent holding room, and the process was repeated for the next pair. Twelve man-hours were required to complete the bleeds for the entire cohort (two veterinary technicians working for 6 hours).

Because the room lacked engineering controls to scavenge WAG, the veterinary facility manager and the biosafety officer mandated that all employees entering the room or performing the procedure were required to wear a combination charcoal-HEPA filter (GVP-441, 3M, Minneapolis, MN) on the powered air-purifying respirator (PAPR; model GVP-1, 3M). The veterinary facility manager and biosafety officer performed observations of the procedure to determine if the best work practice controls were being followed, as well as to monitor both the room.
level of WAG and the employee exposure to WAG. Monitoring and observations were performed twice prior to the introduction of a BSL-3 agent in the animal suite. Monitoring was accomplished using a ChemDisk monitor for halogenated anesthetic gas (Assay Technology, Inc., Livermore, CA). The monitor consisted of an activated carbon disk with PTFE (DuPont Teflon® fluoropolymer resin) binder, seated in a plastic housing with snap-lid. The recommended sampling time is 15 minutes to 8 hours; and a standard sampling time is 60 minutes (Assay Technology, Inc.). One badge was affixed to the overall suit of each veterinary technician (in the upper chest or shoulder area to represent the breathing zone) in order to sample the environmental level of WAG (Figure 1A). A second badge was affixed to the inside of each veterinary technician’s PAPR helmet in order to sample the level of WAG to which he/she was exposed. The charcoal/HEPA filter was in use at all times (Figure 1B). Each badge was opened and exposed to potential WAG, then closed, wiped with a disinfectant (Vesphene II, Steris Corporation, Erie, PA), and sent back to the vendor for analysis.

In the first monitoring session, levels of WAG exceeding the NIOSH-recommended exposure limit were detected in the room (Table 1). Levels of WAG under the NIOSH-recommended exposure limit were detected inside the technicians’ PAPR helmets, presumably due to the effective removal by the combination charcoal-HEPA filters on their PAPRs. In the second monitoring session, much higher levels of WAG (500 ppm) were detected in the room, and the level of WAG detected at the worker level had increased although not exceeding the NIOSH-recommended exposure limit of 2 ppm over 1 hour. This model of PAPR is considered to be a loose-fitting face-piece respirator with an assigned protection factor of 25 (CFR, 1998). Therefore, a room level of 500 ppm could theoretically mean that the PAPR would provide worker protection only to approximately 20 ppm. Although a much lower level of WAG was detected inside the PAPR for that particular sample, the much higher level of WAG

**Figure 1**
A. Photograph of a monitoring badge affixed to the exterior of the overall suit.
B. Photograph of a monitoring badge affixed to the interior of the PAPR helmet.

**Table 1**
Results of initial isoflurane monitoring in the animal suite procedure room during isoflurane anesthesia of rabbits.

<table>
<thead>
<tr>
<th>Session #</th>
<th>Technician #</th>
<th>Location of Monitor</th>
<th>Exposure to Isoflurane (PPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Inside PAPR helmet with charcoal/HEPA filter</td>
<td>ND*</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>On coverall suit</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Inside PAPR helmet with charcoal/HEPA filter</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>On coverall suit</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Inside PAPR helmet with charcoal/HEPA filter</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>On coverall suit</td>
<td>Not performed</td>
</tr>
</tbody>
</table>

*ND = Not detected; below the reporting limit of 0.6 ppm.
detected in the room indicated that additional controls were needed.

As a result, work practices were more closely examined, and it was determined that improvements could be implemented to attempt to reduce the level of WAG in the room. A request was made to repeat the personnel monitoring for the next round of ear bleeds; however, by this point the animals had been challenged with a BSL-3 agent. It is the policy of the UPitt RBL that tools and equipment may not be removed from a BSL-3 suite prior to being fully sprayed with or soaked in disinfectant, steam sterilized, or treated with vaporized hydrogen peroxide. Furthermore, because rabbit hair had become pervasively airborne throughout the holding room and procedure room and had coated most surfaces throughout the rooms during the study, there was a concern that various surfaces and equipment throughout the rooms could become contaminated with the infectious agent. Thus, the potential for the analyte disk to become contaminated with the infectious agent could not be ruled out. To complicate matters, the infectious agent in use is also regulated by the Department of Health and Human Services as a Select Agent and, therefore, it is the responsibility of the institution to prevent release of the agent outside of the approved facility (CFR, 2008a, 2008b, 2008c). The vendor of the monitoring badges was contacted to determine if there were a safe means of disinfecting the badges after exposure, while simultaneously preserving the analyte on the badge. The vendor advised that the badges could not be sprayed with disinfectants as this could destroy the analyte. To test this advice, prior to an infectious agent being used in the animal suite, one monitoring badge was exposed to the room during the use of isoflurane to anesthetize uninfected rabbits (thus presumably exposing it to a high level of isoflurane as was previously determined), sprayed thoroughly with 70% isopropanol, and then allowed to sit for 1 hour under the isopropanol before being sent to the vendor for analysis. No isoflurane was detected on the badge, suggesting that the isopropanol destroyed the analyte or washed it off the carbon disk (data not shown). The vendor endorsed decontamination with vaporized hydrogen peroxide to inactivate any infectious agents on the badge but acknowledged that this was an untested procedure.

The UPitt RBL utilizes vaporized hydrogen peroxide (VHP) to ensure full disinfection of rooms and equipment. VHP has been shown to be efficacious against a broad spectrum of microorganisms including spore-forming bacteria. The mechanism of action is the oxidation of proteins, lipids, and nucleic acids in the microorganism, resulting in death (Heckert et al., 1997; Kokubo et al., 1998; Rogers et al., 2005). VHP is compatible with many standard laboratory materials including electronic devices, if humidity and sterilant concentration are controlled properly (Krause et al., 2001). VHP deteriorates relatively quickly into oxygen and water, unlike formaldehyde gas which can polymerize into paraformaldehyde which must in turn be neutralized with ammonium carbonate and then the resulting residue removed via surface wipe-down (McDonell, 2007). VHP has been used successfully at the UPitt RBL to fully disinfect various types of equipment for release from the BSL-3 facility, and has been used to disinfect entire rooms which can subsequently be isolated from the rest of the facility and cleared for entry without protective equipment requirements.

To determine the effect of VHP on halogenated anesthetic gases adsorbed to the personnel monitors, four sets of ChemDisk monitors (Assay Technology, Boardman, OH) were exposed to isoflurane and halothane. The exposure circuit consisted of a Miller-Nelson HCS-501 atmosphere generator (Assay Technology, Boardman, OH) a Harvard syringe pump (Harvard apparatus, Holliston, MA), a solvent vaporizer, and a clear acrylic one-pass exposure chamber (30 x 10 x 10 cm). For the first two sets of test badges, a flow of 150 L/min (25 cm/sec) of air from the atmosphere generator was established (25°C, 50% relative humidity.) Using a micro-syringe and the syringe pump, liquid isoflurane and halothane anesthetics were continuously infused into the flowing air stream (through a septum mounted into a "T" in the delivery tubing) at a metered rate to attain nominal vapor concentrations, respectively, of 20-40 ppm (equivalent to 5-10 ppm for an 8-hour TWA (time weighted average). Five personal monitoring badges (Assay Technology item no. 574) were placed on a wire cage and into the acrylic chamber for 120 minutes. Upon completion, the badges were removed, and the exposure was repeated with five fresh badges and a concentration of isoflurane and halothane that was 50% higher than the previous experiment. For each experiment, three of the five badges were held in storage at the vendor and the remaining two badges from each experiment were sent to the UPitt RBL for VHP exposure. The experiment was repeated for two additional sets of badges exposed to relatively lower levels of halothane and isoflurane.

At the UPitt RBL, the highest concentration of VHP (800-1000 ppm) was observed during treatment of the Class III BSC (The Baker Company) in the aerosol challenge suite and, therefore, this cycle was selected for exposure of the test badges. In this cycle, the Class III BSC was maintained at 23°C and conditioned to a starting relative humidity of no more than 40%. VHP (35% hydrogen peroxide, Sigma-Aldrich Co., St. Louis, MO) was injected at a rate of 5 g/min for 10 minutes and then reduced to a rate of 3 g/min for 15 minutes, after which the VHP supply ceased and air was allowed to circulate in the enclosure for 2 hours prior to restoring normal air supply and exhaust. The level of VHP was monitored by observing the value shown on a two-wire gas transmitter equipped with a VHP sensor on a 25-foot cable (Flow
The peak level of VHP was measured at 814 ppm. During the VHP treatment of the third and fourth sets of test badges, the peak level of VHP was measured at 856 ppm (data not shown).

Table 2 shows the results of the first and second sets of test badges. The amounts in micrograms of both analytes detected (halothane and isoflurane) are shown. The means and standard deviations were calculated for the amounts of analyte detected and then compared using the student’s t-test (GraphPad Software, La Jolla, CA). There was no significant difference between the stored badges and the VHP-treated badges for either set of badges and for either analyte.

Table 3 shows the results of the third and fourth sets of test badges. The amounts in micrograms of both analytes detected (halothane and isoflurane) are shown. The mean and standard deviation were calculated for the amount of analyte detected and then compared using the student’s t-test (GraphPad Software). There was no significant difference between the stored badges and the VHP-treated badges for either set of badges and for either analyte, with the exception of the fourth set of test badges, for which only one badge had a detectable level of isoflurane. Because halothane was detected on that badge, it is assumed that the isoflurane ran out during loading of the test badges with the analytes, rather than assuming that the badge had been damaged by exposure to VHP, transport, or storage prior to analysis.

Concurrent with the analysis of the test badges was the development of safer work practices for isoflurane anesthesia of rabbits, as well as testing of a portable engineering control (Active Scavenge System, VetQuip, Queensland, Australia). Table 4 represents the critical action items within the SOP that was developed for safer isoflurane anesthesia of rabbits, including improvements in work practice controls as well as the use of the anes-
Figure 3
Concentration of vaporized hydrogen peroxide during the treatment of the first and second sets of test badges in the Class III biological safety cabinet.

Table 2
Comparison of anesthetic gas recovery from stored (untreated) and VHP-treated test badges for the first and second sets of test badges. Each set of badges was exposed to both halothane and isoflurane, then divided and either stored or sent for exposure to VHP. The results of the stored badges are shown in the first two columns, and the results of the VHP-treated badges are shown in the third and fourth columns. Additionally, the results for halothane are shown first, and the results for isoflurane are shown second.

<table>
<thead>
<tr>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
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<tbody>
<tr>
<td>Stored 1-Halo</td>
<td>220</td>
<td>VHP 1-Halo</td>
<td>240</td>
</tr>
<tr>
<td>Stored 1-Halo</td>
<td>240</td>
<td>VHP 1-Halo</td>
<td>260</td>
</tr>
<tr>
<td>Stored 1-Halo</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>233.33 +/- 11.55</td>
<td></td>
<td>250.0 +/- 14.14</td>
</tr>
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Student’s t-test: Two-tailed P-value = 0.2394

<table>
<thead>
<tr>
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<th>Amount Detected (µg)</th>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored 1-Iso</td>
<td>110</td>
<td>VHP 1-Iso</td>
<td>120</td>
</tr>
<tr>
<td>Stored 1-Iso</td>
<td>120</td>
<td>VHP 1-Iso</td>
<td>130</td>
</tr>
<tr>
<td>Stored 1-Iso</td>
<td>130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>116.67 +/- 5.77</td>
<td></td>
<td>125.0 +/- 7.07</td>
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Student’s t-test: Two-tailed P-value = 0.2394

<table>
<thead>
<tr>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored 2-Halo</td>
<td>320</td>
<td>VHP 2-Halo</td>
<td>310</td>
</tr>
<tr>
<td>Stored 2-Halo</td>
<td>360</td>
<td>VHP 2-Halo</td>
<td>360</td>
</tr>
<tr>
<td>Stored 2-Halo</td>
<td>360</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>346.67 +/- 23.09</td>
<td></td>
<td>335.0 +/- 35.36</td>
</tr>
</tbody>
</table>

Student’s t-test: Two-tailed P-value = 0.6769

<table>
<thead>
<tr>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored 2-Iso</td>
<td>160</td>
<td>VHP 2-Iso</td>
<td>150</td>
</tr>
<tr>
<td>Stored 2-Iso</td>
<td>170</td>
<td>VHP 2-Iso</td>
<td>170</td>
</tr>
<tr>
<td>Stored 2-Iso</td>
<td>170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>166.67 +/- 5.77</td>
<td></td>
<td>160.0 +/- 14.14</td>
</tr>
</tbody>
</table>

Student’s t-test: Two-tailed P-value = 0.4950
All employees were trained to follow the SOP and then observed by the veterinary facility manager and biosafety officer to ensure compliance. Monitoring was repeated four times and the monitoring badges were treated with VHP either in the Class III BSC or in a room that was undergoing disinfection before being sent back to the vendor for analysis. Table 5 shows the monitoring results subsequent to retraining on improved work practices. No WAG was detected inside the PAPR helmet when the combination charcoal-HEPA filter was worn. WAG was detected in the room at a significantly lower level than was detected prior to implementation of new work practice and engineering controls; the average exposure was 12.0 ppm with a range from not detected (reporting limit of 0.6 ppm) to 21 ppm. To verify that WAG were not scavenged by the additional HEPA filter on the PAPR, a mock PAPR helmet was connected to the motor-blower and HEPA filter, and a monitoring badge was affixed to the inside of the helmet in the same way that it would be affixed to the interior of a worker’s helmet. The PAPR was placed next to the anesthesia circuit and monitoring was performed for 60 minutes. WAG at 6.9 ppm was detected on this badge, verifying that the PAPR filter alone will not protect against exposure to WAG.

### Discussion

Upon recognizing that the room level of isoflurane was unacceptably high during the use of isoflurane to anesthetize rabbits, the availability of engineering controls to scavenge WAG was discussed. The initial moni-

---

**Table 3**

Comparison of anesthetic gas recovery from stored (untreated) and VHP-treated test badges for the third and fourth sets of test badges. Each set of badges was exposed to both halothane and isoflurane, and the relative amounts of each analyte were lower than those used for the first and second sets of test badges. Each set of badges was then divided and either stored or sent for exposure to VHP. The results of the stored badges are shown in the first two columns, and the results of the VHP-treated badges are shown in the third and fourth columns. Additionally, the results for halothane are shown first, and the results for isoflurane are shown second.

<table>
<thead>
<tr>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
<th>Badge Type</th>
<th>Amount Detected (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored 3-Halo</td>
<td>23</td>
<td>VHP 3-Halo</td>
<td>26</td>
</tr>
<tr>
<td>Stored 3-Halo</td>
<td>25</td>
<td>VHP 3-Halo</td>
<td>27</td>
</tr>
<tr>
<td>Stored 3-Halo</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>24.33 +/- 1.15</td>
<td></td>
<td>26.5 +/- 0.71</td>
</tr>
<tr>
<td>Student’s t-test: Two-tailed P-value = 0.1040</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stored 3-Iso</td>
<td>12</td>
<td>VHP 3-Iso</td>
<td>16</td>
</tr>
<tr>
<td>Stored 3-Iso</td>
<td>15</td>
<td>VHP 3-Iso</td>
<td>17</td>
</tr>
<tr>
<td>Stored 3-Iso</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>14.33 +/- 2.08</td>
<td></td>
<td>16.5 +/- 0.71</td>
</tr>
<tr>
<td>Student’s t-test: Two-tailed P-value = 0.2676</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stored 4-Halo</td>
<td>48</td>
<td>VHP 4-Halo</td>
<td>48</td>
</tr>
<tr>
<td>Stored 4-Halo</td>
<td>50</td>
<td>VHP 4-Halo</td>
<td>50</td>
</tr>
<tr>
<td>Stored 4-Halo</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>50.67 +/- 3.06</td>
<td></td>
<td>49 +/- 1.41</td>
</tr>
<tr>
<td>Student’s t-test: Two-tailed P-value = 0.5367</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stored 4-Iso</td>
<td>33</td>
<td>VHP 4-Iso</td>
<td>34</td>
</tr>
<tr>
<td>Stored 4-Iso</td>
<td>34</td>
<td>VHP 4-Iso</td>
<td>ND*</td>
</tr>
<tr>
<td>Stored 4-Iso</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>34.33 +/- 1.53</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Student’s t-test: Not performed as there is only one datum for the VHP-treated badges</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND = Not detected*
toring results showed that the rate of room air changes (at least 20/hr), although high for an average animal facility, was not adequate to scavenge WAG to levels below the REL. Ductless fume hoods were evaluated, but the institutional policy is that ducted engineering solutions are preferred (F. Pokrywka, personal communication). It was not possible to retrofit the ABSL-3 procedure room to accommodate a built-in engineering control such as a snorkel-type duct or a traditional chemical fume hood. The ABSL-3 procedure room is finished with fully sealed monolithic wall and ceiling panels (Arcoplast, Inc., St. Peters, MO) which cannot be penetrated to install an engineering control without full suite shut-down and disinfection. Furthermore, the ABSL-3 suites in the UPitt RBL were commissioned with relative tightness testing to serve as primary containment barriers for infectious agents (CFR, 2008b, 2008c). The relative tightness testing and additional re-commissioning activities would need to be repeated satisfactorily in order to reopen the suite for ABSL-3 work. This was determined to be time- and cost-prohibitive to ongoing research in the facility. Accurately predicting the full scope of research that may occur over the lifetime of a high-containment laboratory during the design phase is difficult. However, institutions may benefit from making every effort to incorporate as much flexibility into the facility as area and costs allow. For example, a minimum number of centrally-located chemical fume hoods or snorkel-type ducts could be installed to accommodate studies involving anesthetic gases, other hazardous chemicals, or radioisotopes.

Once it was determined that the ABSL-3 suite could not be retrofitted with engineering controls, the use of improved work practice controls as well as a portable engineering control were considered. The results indi-

### Table 4
Excerpt of critical procedures from SOP of isoflurane anesthesia of rabbits and other large animals.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protective Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Don combination charcoal-HEPA filter on PAPR.</td>
</tr>
<tr>
<td></td>
<td>• Post signage in PAPR room and procedure room that charcoal-HEPA filter is required.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Engineering Control Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Connect evacuation pump to exhaust line of anesthesia circuit.</td>
</tr>
<tr>
<td>• Connect absorbent charcoal canister to outlet of evacuation pump; ensure charcoal canister is new or has adequate capacity for procedure; ensure charcoal canister is placed on its side and not with the exhaust air port face-down.</td>
</tr>
<tr>
<td>• Turn on evacuation pump.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anesthesia Machine Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Ensure at least 500 psi of pressure in oxygen tank.</td>
</tr>
<tr>
<td>• Refill anesthesia machine with pouring spout attached to isoflurane bottle.</td>
</tr>
<tr>
<td>• Refill carbon dioxide absorbent chamber with fresh absorbent.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anesthesia Machine Leak Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Turn on oxygen and flow meter.</td>
</tr>
<tr>
<td>• Close pop-off valve.</td>
</tr>
<tr>
<td>• Place thumb where nose cone will attach, and press the flush button on the bottom of the flow meter.</td>
</tr>
<tr>
<td>• Observe the re-breathing bag filling.</td>
</tr>
<tr>
<td>• Verify manehelic gauge has reached 20-30 and is not losing pressure.</td>
</tr>
<tr>
<td>• At completion of leak test, place properly-sized nose cone with sealing gasket on supply end of circuit.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Sedate animal via IM injection.</td>
</tr>
<tr>
<td>• Place animal in physical restraint (cat sack).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Sedation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Turn oxygen to 2L/min.</td>
</tr>
<tr>
<td>• Place nose cone on animal, ensure proper fit, and only then turn anesthesia machine on.</td>
</tr>
<tr>
<td>• Reduce flow rate when animal appears to be properly sedated.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Completion of Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Turn off anesthesia machine when phlebotomy completed.</td>
</tr>
<tr>
<td>• Flush system by pressing purge button at least twice.</td>
</tr>
<tr>
<td>• Remove nose cone from animal.</td>
</tr>
<tr>
<td>• Return animal to holding box.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Completion at End of Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Turn off evacuation pump.</td>
</tr>
<tr>
<td>• Weigh the charcoal canister and record or dispose if at capacity.</td>
</tr>
<tr>
<td>• Report any problems to supervisor.</td>
</tr>
</tbody>
</table>
cate that the improved work practice controls, additional procedure-specific training, and the use of the anesthesia evacuation pump coupled with an absorbent cylinder greatly reduced the level of detectable WAG in the room from 500 ppm to less than 25 ppm. Re-educating technicians about the anesthesia circuit and how to inspect the circuit was also useful, as halogenated anesthetic gases can deteriorate rubber (Stimpfel & Gershey, 1991). Monitoring the level of isoflurane in the room as well as the level of employee exposure will be performed periodically. The RBL biosafety officer will continue to observe procedures to ensure that the veterinary technicians follow the revised SOP.

Although PPE is considered the last option in the hierarchy of safety controls (after administrative controls, engineering controls, and work practice controls), it still appears necessary for employees to wear the combination charcoal-HEPA filter on the PAPR in order to reduce exposure below the REL. Alternative methodologies for control of WAG in the room will be investigated in subsequent studies. For example, the investigator has proposed the use of sampling ports on the rabbits for rapid blood withdrawal without anesthesia or under only light injectable anesthesia.

Finally, verification that the VHP treatment process does not interfere with the analyte will allow for personnel and environmental monitoring of WAG for any infectious agent in use in the facility.

**Summary**

Unacceptable levels of isoflurane were detected during anesthesia of rabbits. A combination of work practice controls, engineering controls, and personal protective equipment were developed to significantly reduce the level of waste anesthetic gases in the room and to ensure that employees were not exposed above the recommended limit (NIOSH, 1977). Treatment of potentially contaminated monitoring badges with vaporized hydrogen peroxide was tested to ensure that monitoring badges inside BSL-3 facilities could be fully disinfected, removed from the BSL-3 facility, and safely returned to the vendor for accurate analysis. Monitoring will continue to ensure that engineering and work practice controls are being followed and that protective equipment remains effective.

---

**Table 5**

Results of monitoring after implementation of improved work practice controls and a portable engineering control.

<table>
<thead>
<tr>
<th>Session</th>
<th>Category</th>
<th>Exposure to isoflurane, PPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>ND*</td>
</tr>
<tr>
<td>1</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>Not performed</td>
</tr>
<tr>
<td>4</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>Not performed</td>
</tr>
<tr>
<td>4</td>
<td>Vet tech wearing combination charcoal-HEPA filter on PAPR</td>
<td>Not performed</td>
</tr>
<tr>
<td>4</td>
<td>On the tech’s coverall suit</td>
<td>15</td>
</tr>
<tr>
<td>1</td>
<td>On the tech’s coverall suit</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>On the tech’s coverall suit</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>On the tech’s coverall suit</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>On the tech’s coverall suit</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>On the tech’s coverall suit</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>On the tech’s coverall suit</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>On the tech’s coverall suit</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Mock PAPR with HEPA-only filter on PAPR</td>
<td>6.9</td>
</tr>
</tbody>
</table>

*ND = Not detected; below the reporting limit of 0.6 ppm.
Acknowledgments

The authors would like to thank Frank Pokrywka, CIH, and Douglas Noble of the University of Pittsburgh Department of Environmental Health and Safety for technical advice regarding control of waste anesthetic gases; Jacquelyn Bales of Pitt Center for Vaccine Research for assistance with data analysis; and Joan Hearing of Assay Technology, Inc. for analytical chemistry support. Lesley Homer, Dennis Heffin, and Kelly Stefano Cole are employed by the University of Pittsburgh and have no conflict of interest. C. R. Manning is employed by Assay Technology, Inc. *Correspondence should be addressed to Lesley C. Homer at LCE3@pitt.edu.

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Pokrywka, F., CIH, Industrial Hygienist, Department of Environmental Health and Safety, University of Pittsburgh, personal communication.


Quantifying Competency in Biosafety: Adaption of the Instructional Systems Design Methodology (ISD) to Biosafety and Laboratory Biosecurity

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Abstract

A competency training program for international public health laboratories was designed by detailing all biosafety tasks in the context of working laboratory procedures. The list of biosafety tasks was used to develop practical, laboratory-based training checklists that can be used to demonstrate individual competencies across multiple trainers. Because of the intensive nature of the training, in this program, we employed two types of trainers: The Biosafety Instructor and the Laboratory Trainer and we expanded the number of trainers to include a Laboratory Trainer in each laboratory. This Train-the-Trainer course defines a step-by-step process to establish competency training programs in a facility. In the Biosafety Instructor training program, biosafety officers and laboratory managers are trained alongside each other as trainers. This type of program establishes facility-specific, quality-controlled training programs that promote the transfer of biosafety working procedures from the classroom to the laboratory. The program design gives the Laboratory Manager, Biosafety Officer, and biosafety committee ownership in the biosafety program, establishing a role for the laboratory manager as a Laboratory Trainer and encouraging a culture of biosafety within the facility.

Introduction

Competency training is recognized as a necessary element in a biosafety program. The American Association for the Advancement of Science (AAAS, 2009), World Health Organization (WHO, 2004), and The European Committee for Standardization (CEN CWA 15793) call for, but do not provide, a methodology to assess individual biosafety and laboratory biosecurity competency. These documents suggest a wide array of topics necessary to assure establishment of a competent biosafety and laboratory biosecurity program, ranging from program management to ethical responsibility, highlighting the multi-disciplinary scope of biosafety training and program needs. Adding to the complex nature of the subject, each facility containing biological hazards has unique biosafety and laboratory biosecurity needs. Working from a task-based analysis of working procedures and practices in a clinical laboratory, this research suggests a definition for competency that can be applied in a flexible, facility-specific manner to biosafety and laboratory biosecurity training programs. The study details a method to quantitatively assess competency in biosafety in a clinical laboratory. Since the methodology is readily adapted to laboratory biosecurity in all biological research or clinical facilities, we further elaborate on necessary elements for quantification of competency in a laboratory biosecurity program.

This training program has been developed for application in a laboratory supporting an international infectious disease surveillance program. The targeted audience for training were biosafety officers and laboratory managers. The overall training was designed in an intensive 2-week format with 1 week dedicated to training of Biosafety Managers and the second to Biosafety Instructors, respectively. Although focused within these parameters, the Biosafety Officer Training Program methodology described lends itself to many different training designs. The material can be integrated into current classroom training and can be readily applied to an academic environment. This design encourages competency assessments of biosafety and laboratory biosecurity capability of all individuals working in biological laboratories, while focusing on the need for these disciplines to support and integrate with the scientific process.

Definitions

Competency—the ability to successfully complete a skill composed of various tasks, to a required degree given a defined set of conditions

**Biosafety and Laboratory Biosecurity Tasks**—defined set of actions that comprise safe-working procedures and practices in a biological research or biomedical laboratory

Materials and Methods

Instructional Systems Design (ISD) is a quality training methodology used to design task-based training for adult students (Hodell, 2000). This methodology is often called ADDIE, an acronym that stands for the five steps in the training program: Analysis, Design, Development, Implementation, and Evaluation. ISD is a linear method-
ology stressing analysis of training goals and individual needs prior to design and development of a training program. Using ISD, skills are defined by tasks necessary to complete a skill and tasks are broken down into subtasks. Each subtask is a defined action with a performance metric that can be evaluated. In this article, we describe the adaptation of ISD to a biosafety training program for an international clinical laboratory. We further expand on this model to identify core documents that can be analyzed using the ISD methodology for quantitative evaluation of competency and proficiency in laboratory biosecurity.

Results

Training Philosophy

In the international development project described, the training effort included all aspects of program development and implementation. The following training philosophy was developed to accommodate the project scope:

- All persons in a facility, containing or working with biohazards, have biosafety tasks associated with their work.
- All work done in a facility that conducts biological research or infectious disease surveillance has a safety component.

Training Analysis

Based on this philosophy, the goal of the training program was to develop individualized training focused on the tasks necessary to complete a job. As each procedure varies slightly among individuals and a variety of procedures may be used among laboratories, the training design must be flexible to allow for facility-specific adaptation. To do this, we designed a step-wise methodology using templates to allow for adaptation of the task lists. The training analysis of tasks was done using international guidelines and published biosafety documents as reference material. These tasks were further revised to accommodate local procedures and practices, resulting in a final facility-specific task list. Documents used in the template analysis for biosafety in a clinical laboratory included Biosafety in the Microbiological and Biomedical Laboratory (U.S. Department of Health and Human Services, 2009), Laboratory Biosafety Manual (WHO, 2004), and International Health Regulations (IHR) (WHO, 2005).

In the template analysis, biosafety tasks were linked to generalized job descriptions of personnel in an international clinical laboratory. Table 1 provides an example of a task template. Appendix 1 can be found on ABSA’s Training Tools web page—it contains a complete training analysis for this specific biosafety training program (ABSA, 2011). The tasks listed in the analysis are derived from the facility’s standard operating procedures (SOPs) and represent all administrative and technical work completed in the facility. For evaluation of competency, tasks are defined by a list of subtasks that enable completion of the task. Subtasks are individual, measurable actions. Because the subtasks are defined actions that can be evaluated using a specified evaluation method, they can be used to standardize competency training across trainers and laboratories. Table 2 provides an example of the method used to break down a skill into tasks and further details the task using enabling subtasks with corresponding evaluation metrics. This example demonstrates the technique of defining the biosafety component of a task by detailing the enabling subtasks necessary to complete a required skill. In this example, the skill chosen is pipetting. Although pipetting is not considered a biosafety task, one of the enabling subtasks associated with pipetting, the proper disposal of the pipette tips, is common biosafety practice. Defining the subtasks necessary to complete a skill contextualizes the biosafety element as an overall part of the working procedures and practices in the laboratory (Table 2).

The template tasks detailed in the training analysis reflect the training needs of the laboratories participating in the study and are not a complete list for all laboratories. In this study a cumulative number of 374 subtasks were identified. From this list, subtasks were grouped by

Table 1

<table>
<thead>
<tr>
<th>Suggested job titles are listed. Add a job title that is appropriate for your facility.</th>
<th>Add tasks as suggested by the job description.</th>
<th>Add or remove subtasks as appropriate. Use active verbs to describe each subtask.</th>
<th>Insert appropriate evaluation method. Suggested methods are inserted into the cells.</th>
<th>Insert appropriate degree for successful completion of the subtask.</th>
<th>List biosafety document(s) describing the associated theory or procedure.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Training Analysis</strong></td>
<td><strong>Job</strong></td>
<td><strong>Task</strong></td>
<td><strong>Subtask</strong></td>
<td><strong>Evaluation Method</strong></td>
<td><strong>Metric</strong></td>
</tr>
</tbody>
</table>

job title or proficiency level matching common laboratory and managerial jobs. Figure 1 depicts the distribution of subtasks by common laboratory jobs and correlates these to the cumulative number of subtasks learned at a particular scientific skill level. In the laboratory, biosafety proficiency is achieved over time as individuals in the laboratory progress from technician to scientist to laboratory manager. The biosafety professional, although not part of the laboratory staff, must be proficient in all biosafety tasks associated with work in the laboratory and also be knowledgeable about administrative and facility operation tasks not associated with the laboratory. This analysis demonstrates that biosafety-specific laboratory tasks are learned in a cumulative manner over time in the laboratory, suggesting a need for the Biosafety Officer to have laboratory experience. In Figure 1, the number of biosafety subtasks required in each job remains approximately the same (approximately 75-100 tasks/level), but

### Table 2

**Subtask List.** Skills are described by task and enabling subtasks. Each subtask is standardized by detailing an evaluation method and a performance metric or degree.

<table>
<thead>
<tr>
<th>Skill</th>
<th>Task</th>
<th>Subtask</th>
<th>Performance Metric</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Performs a series of 1:5 dilutions</td>
<td>Selects the correct pipettor for the desired volume</td>
<td>Selects a 200 µl pipettor</td>
<td>Demonstration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Accurately measures 120 µl of liquid</td>
<td>± 2 µl accuracy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dispenses liquid in a manner that prevents production of aerosols</td>
<td>Avoids forceful evacuation of liquid from pipette tips by angling tips into the side of the well</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disposes of tips in a designated sharps container</td>
<td>Selects puncture-resistant container with biohazard symbol for sharps disposal</td>
</tr>
</tbody>
</table>

### Figure 1

Required subtasks were stratified by job title and compared to the cumulative number of subtasks for necessary biosafety proficiency in the laboratory. All personnel in a clinical facility have biosafety tasks that define competency in biosafety in a specific job. The number of tasks particular to a job is roughly equivalent among the four selected jobs (black bars). This fact contrasts with the cumulative number of biosafety tasks mastered at each scientific job (Technician, Scientist, and Laboratory Manager) or biosafety proficiency level (white bars). Biosafety Officers, while not part of the laboratory staff, must master all laboratory tasks to be proficient in their job. Proficiency is cumulative and is demonstrated by competency in all tasks.
the cumulative number of subtasks increases from Technician to Scientist to Laboratory Manager, suggesting an increase in overall ability in laboratory procedure-based biosafety over time. Since each subtask is a unique evaluation point, progression from Technician to Scientist to Laboratory Manager reflects an increase in competency in laboratory procedure-based biosafety as measured by the cumulative number of biosafety tasks completed. The Biosafety Officer must be either theoretically or practically trained in all laboratory biosafety tasks (Figure 1). Although knowledge of biosafety practices may substitute for procedural ability when working as a Biosafety Officer, the authors felt that the “hands on” nature of a competency training program requires the biosafety professional to have laboratory experience as a prerequisite for selection into the Biosafety Instructor course.

In the Biosafety Instructor course, Biosafety Officers are trained to integrate technical procedures, biosafety, and quality tasks into a facility-specific task list. To refine the task list to reflect the needs of the facility, the general task analysis is aligned with SOPs, the facility biosafety manual, and other facility-specific biosafety documents and practices. Once complete, the facility task list is defined by working procedures in the facility that have been aligned with biosafety guidelines and regulations and further divided into proficiency levels that reflect the jobs listed in the organizational chart of the facility. The final result is facility-specific, individualized training that can be used to measure and compare individual competency in biosafety or laboratory biosecurity.

Training Design and Development

Grouping of the subtasks into training type details biosafety responsibilities by generalized job descriptions, allowing for analysis of idealized training in a clinical laboratory (Figure 2). Three categories were chosen to describe the type of training needed for the job: basic biosafety skills; biosafety management skills; and Biosafety Instructor skills. Subtasks were grouped by training type and job. In this analysis, Technicians and Scientists working in the laboratory require basic, procedural biosafety skills while Laboratory Managers and Biosafety Officers require skills in biosafety management and biosafety instruction. For our training purposes, the jobs of the Biosafety Officer and the Laboratory Manager, although very different, were found to have similar biosafety responsibilities and training needs (Figure 2). Although this analysis varies according to actual positions and responsibilities in a specific facility, modeling of the system reveals the key position of a Skilled Laboratory or the Laboratory Manager in overall training program design and suggests the need for expanding the number and types of biosafety trainers to include a laboratory-specific trainer with expertise in integration of biosafety techniques into laboratory procedures and practices.

Because of the individualized nature of the competency training, the Biosafety Instructor course was designed to establish two types of biosafety trainers in a facility: Biosafety Instructors and Laboratory Trainers. Biosafety Instructors analyze and design all training for the facility, conduct classroom training, and train mid- and upper-level management, administrators and facility engineers. This position is traditionally filled by the Biosafety Officer. Laboratory Trainers are skilled in the working practices of an individual laboratory. The Laboratory Trainer conducts practical training in the laboratory, using the competency-training checklists developed in collaboration with the Biosafety Instructor, providing

Figure 2

Biosafety responsibilities in a clinical diagnostic laboratory were categorized according to the jobs performed at the facility. Responsibilities included basic biosafety skills, biosafety management skills, and biosafety instructor skills. Stratification of responsibilities by skills demonstrates the similarities in biosafety responsibilities between the biosafety officer and laboratory manager and highlights the biosafety instructor responsibilities shared by these two positions.
continuity to the training program. In the Biosafety Instructor program, the Laboratory Trainer position can be filled by the Laboratory Manager or a skilled scientist with working knowledge of all procedures in the laboratory. The National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (NIH, 2011) cites the need for the Principle Investigator (PI) to assume training responsibilities in laboratories conducting research with recombinant DNA. This training analysis suggests that the role of the PI as a trainer pertains not only to laboratories working with recombinant DNA but also to all biological and biomedical laboratories. The Biosafety Officer Training design reaffirms the Laboratory Manager’s or PI’s responsibilities as a biosafety trainer and recognizes the need to train Laboratory Managers as trainers.

Training Evaluation
In this training design, the Laboratory Trainer collaborates with the Biosafety Instructor, adapts the task and subtasks to the needs of the laboratory, conducts competency training, and tracks individual competency over time (Table 3). In the example, competency is easily quantified as compliance over time (Table 3). In a like manner, competency can be calculated as the percent of subtasks successfully demonstrated in the completion of a task.

Application of ISD ADDIE Methodology to Laboratory Biosecurity
Wilhelm (2010) has defined laboratory biosecurity as “...convergence of science, public health, laboratory management, biosafety and security...minimizing the exposure to existing threats and risks to people, BSATs (Biological Select Agents and Toxins), Especially Dangerous Pathogens (EDPs), VBM (Valuable Biological Materials), information and infrastructure.” Addressed from a procedural level within a facility, biosafety is a subset of laboratory biosecurity as both disciplines involve working procedures and practices, but laboratory biosecurity encompasses a greater number of disciplines. Laboratory biosecurity may be addressed by the standardization of working procedures and practices detailed in published biosafety guidelines, laboratory biosecurity guidelines, quality documents, outbreak response, personal reliability, research, and technical papers. Table 4 provides a suggested list of documents to be used for ISD training analysis of a laboratory biosecurity program.

Discussion
This training program suggests a graded, step-wise method for quantitative assessment of competency in a clinical or BSL-2 research laboratory. In the training program, competency in biosafety is determined by the successful integration of biosafety tasks into working procedures and practices. Using the ISD methodology, assessment of competency is quantitative and can be measured as the number of tasks completed over the total number of tasks or by completion of a task to a specified degree.

One of the unique features of the training program is that it identifies two types of biosafety trainers, the Biosafety Instructor and the Laboratory Trainer. The classroom-to-workbench nature of the training also establishes a need for the expansion of the number of Laboratory Trainers in a facility. The Biosafety Instructor and Laboratory Trainer have distinct background experience, training requirements, and roles in the biosafety training program. Laboratory Trainers, defined by the training analysis as a necessary component of a competency training program, are established in every laboratory. Biosafety Instructors are trained to design and develop institute-specific materials and deliver traditional classroom training. This training design supports a “culture of biosafety” in the facility and encourages consistent, coordinated compliance with biosafety procedures and practices throughout the facility.

A wealth of standardized procedural documents in biosafety and biosecurity makes the development of ISD training templates possible. Although the development of a general subtask list represents a significant amount of labor, the amount of time invested in template development is finite because there are a finite number of standardized materials from which to work. Likewise, there is a significant, up-front time investment at the facility level by the Biosafety Instructor as the materials are detailed to be facility-specific. The resulting training program, however, reflects the cultural and economic reality in the facility, making this type of training program

Table 3
Quantification of Competency Over Time. Competency evaluations over time can be used to give a percent compliance with trained working procedures in the laboratory. Tasks are detailed by subtask with corresponding evaluation methods in the Task Lists. In the example below, in 9 out of 12 months the task of waste disposal was successfully completed for a score of 9/12 or 75%.

<table>
<thead>
<tr>
<th>Task</th>
<th>Monthly Compliance Checklist</th>
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<tbody>
<tr>
<td>Waste Handling</td>
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accessibility for small clinical laboratories and ideal for academic and international audiences.

Clinical and research laboratories following BSL-2 criteria represent the largest number of laboratories and the largest population of laboratory workers working with biohazards. To date, emphasis on competency training has been largely placed on high-containment laboratories following BSL-3 and BSL-4 criteria. The described, graded, step-wise methodology of ISD provides a mechanism to initiate competency training in biosafety and laboratory biosecurity in academic, pharmaceutical, and clinical laboratories, creating awareness and establishing a culture of laboratory biosafety and laboratory biosecurity in the academic and research base that “feeds” high-containment laboratories. The techniques described actively involve scientists in the biosecurity program and give the Laboratory Manager responsibility and oversight for the design and implementation of biosafety and biosecurity in the laboratory. Program standards are then used to track program success. This suggests a more “science and user-friendly” way to address the issue of laboratory biosafety and biosecurity, especially in an international forum.

This article details a methodology that can be extended to include all aspects of procedural biosafety and biosecurity in the laboratory. The training analysis of laboratory tasks contains biosafety, biosecurity, and quality tasks demonstrating the multidisciplinary nature of laboratory work. Biosafety competency training is possible using ISD methodology, providing a quantitative method to measure individual success in an infectious disease surveillance training program.

This training program has been successfully used in Eastern Europe in the Ukraine, and in Central Asia in the Republic of Kazakhstan and the Republic of Georgia. In these countries, biosafety specialists are usually facility scientists, and biosafety is much more procedurally-based than in the United States. As such, the program

<table>
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<tr>
<th>Table 4</th>
<th>Suggested Documents for Laboratory Biosecurity Competency Training</th>
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<tbody>
<tr>
<td><strong>Document Type and Reference</strong></td>
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<tr>
<td><strong>Facility-Specific Procedural Documents</strong></td>
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<tr>
<td>Standard Operating Procedures</td>
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<tr>
<td>Biosafety Manual</td>
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<tr>
<td>Equipment Manuals</td>
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<tr>
<td><strong>Biosafety</strong></td>
<td></td>
</tr>
<tr>
<td><em>Biosafety in Microbiological and Biomedical Laboratories</em>, 5th ed.</td>
<td></td>
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<tr>
<td>WHO Laboratory Biosafety Manual, 3rd ed.</td>
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<tr>
<td>Rutala, W. A. et al., <em>Guidelines for disinfection and sterilization in healthcare facilities</em> (Rutala et al., 2008)</td>
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<tr>
<td>NIH Guidelines (NIH, 2011)</td>
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<tr>
<td>CWA 15793 (European Committee for Standardization Workshop, 2008)</td>
<td></td>
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<tr>
<td><strong>Quality</strong></td>
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<tr>
<td>Clinical Laboratory Standards Institute, <em>Quality Manual</em>, 3rd ed. <em>(Clinical Laboratory Standards Institute, 2004; 2006)</em></td>
<td></td>
</tr>
<tr>
<td>CWA 15793 (European Committee for Standardization Workshop, 2008)</td>
<td></td>
</tr>
<tr>
<td><strong>Biosecurity/Personal Reliability</strong></td>
<td></td>
</tr>
<tr>
<td>DoDi Number 5210.89 <em>(U.S. Department of Defense, 2006)</em></td>
<td></td>
</tr>
<tr>
<td>Epidemic and Pandemic Alert Response <em>(WHO, 2006)</em></td>
<td></td>
</tr>
<tr>
<td>Select Agent Regulation 7 CFR 331 and 9 CFR 121 <em>(USDA, 2005)</em></td>
<td></td>
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<tr>
<td>Select Agent Regulation 42 CFR 73 <em>(U.S. Department of Health and Human Services, 2005)</em></td>
<td></td>
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<tr>
<td><strong>Incident Response</strong></td>
<td></td>
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<tr>
<td>IHR, 2nd ed. <em>(WHO, 2005)</em></td>
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was easily integrated into the existing system. Most, but not all, differences in local regulations were addressed by the interactive nature of the “template” materials; however, some legal changes will be required to completely integrate U.S. and international risk-based biosafety guidelines into Eastern European and Central Asian facilities. Awareness training for Laboratory Managers and establishing Biosafety Instructors within the institute increased the chances of program acceptance as participants take ownership of the training modules they develop for their respective institutes. In the countries where this program has been trained, local Biosafety Instructors are now responsible for defining their institute’s biosafety and biosafety training programs.

Acknowledgments

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References


Case Studies for Dual Use Research

The Federation of American Scientists has a compelling educational program “Case Studies on Dual Use in Biological Research” available at www.fas.org/biosecurity/education/dualuse/index.html.

Module titles are: 1) introductory information; 2) synthesizing polio virus; 3) aerosol drug delivery research; 4) unexpected results in virus research; 5) experiments in antibiotic resistance; 6) genetic control with RNA interference; 7) reconstruction of the 1918 influenza virus; 8) public reaction to science research; and 9) biosecurity resources. These training modules are available in English, French, and Chinese.

For extensive and up-to-date information on biosecurity visit www.fas.org/.
The molecular biology and biotechnology fields are growing by leaps and bounds. Molecular Biosafety aims to shed light on how these cutting-edge techniques impact safety. Please e-mail your insights and questions to Margy Lambert at margylambert@gmail.com or Co-Editor Barbara Johnson at barbara_johnson@verizon.net or Co-Editor Karen B. Byers at karen_byers@dfci.harvard.edu.

**An Update on Alternatives to Antibiotics—Old and New Strategies**

The ability of bacterial pathogens to rapidly evolve enables these pathogens to find new ways to evade host defenses and to become resistant to antibiotic treatments. High levels of antibiotics used in humans and animals have also significantly amplified the emergence of antibiotic-resistant strains. There is great public health concern that humans are losing the antibiotic war against bacterial pathogens with steeply increasing numbers of humans and animals infected with resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA) and gram-negative bacteria resistant to β-lactam antibiotics (Bush, 2010; Culos et al., 2011), more bacterial strains showing multi-drug resistance to last resort antibiotics such as vancomycin, slow progress in development of new antibiotics, and increasing numbers of resistant strains being found in the broader community rather than being localized to healthcare environments (Carson & Riley, 2003; Gould, 2008; Parisien et al., 2008).

Concerns regarding the efficacy of existing antibiotic treatments have resulted in a fresh look at alternatives to antibiotics, both novel treatments and lesser-known existing methods that have gotten little attention previously due to the focus on antibiotics. Some of these antimicrobial agents show substantial promise in becoming viable alternatives to antibiotics.

**Old Antimicrobial Treatments Drawing Renewed Attention**

**Natural/Traditional Antibacterial Treatments**

Probiotics such as *Lactobacillus spp.* and *Saccharomyces boulardii* have been used successfully for prevention of bacterial infections and to treat symptoms of bacterial infections affecting the GI tract (e.g., *Clostridium difficile*-associated diarrhea). Probiotics are the “good” microorganisms normally found in the gut while prebiotics are compounds that promote growth of the “good” microorganisms (Quigley, 2010). Besides protection from “bad” microorganisms via a competition mechanism, a primary mechanism for the beneficial effect of probiotics is their interaction with the gut-associated immune system, boosting the host’s immune response (Oelschlaeger, 2010).

A number of traditional medicinal plant extracts and phytomedicines have again gained attention in the fight against bacterial infections, particularly in use against some GI tract pathogens and against certain skin infections. Willow herb has been used traditionally in some cultures to treat infected wounds. Recent research indicates that willow herb extract can significantly inhibit growth of gram-negative and gram-positive bacteria including *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* (Bartfay et al., 2011). The whole herb extract was able to inhibit growth of bacteria in culture more effectively than vancomycin or tetracycline. Phytomedicines that have been used effectively in the treatment of infections include artesunate for malaria, tea tree oil for skin infections, honey for wound infections, mastic gum and honey for *Helicobacter pylori* gastric ulcers, and cranberry juice for urinary tract infections (Carson & Riley, 2003; Tanih et al., 2009).

**Vaccines**

The primary strategy used against pathogenic viruses involves vaccines while the primary strategy used against bacteria has historically been antibiotics. Vaccines against certain bacterial pathogens have been developed and used over many years. However, vaccine development is costly, there have been cases of safety concerns and a number of vaccine trials have demonstrated mixed results in terms of efficacy achieved against bacterial targets. Some vaccine research efforts, such as those targeted against Lyme disease (Poland, 2011), have been abandoned for the foreseeable future. Although vaccines will continue to be an important option to prevent some bacterial infections, particularly ones with very serious potential outcomes, it seems unlikely that vaccines will become broadly available as an alternative to antibiotics.
**Bacteriophages (Phages)**

One antimicrobial treatment for humans and animals garnering renewed interest is bacteriophage (or phage) therapy. Bacteriophages are viruses that invade and kill bacteria, but do not infect eukaryotes. Phages have many different uses in biotechnology in addition to their use as alternatives to antibiotics. These include: 1) delivery vehicles for vaccines and gene therapy; 2) detection of pathogenic bacteria; 3) screening libraries of proteins, peptides, and antibodies (Clark & March, 2006); 4) biopreservation of food (Garcia et al., 2008); and 5) surface decontamination (Viazis, 2011).

Phage therapy is an effective treatment against many types of bacterial pathogens and has been shown to be relatively safe with few side effects. Some countries, such as the former Soviet Union and Poland, have used phage therapy effectively for many years. Potential drawbacks to phage therapy include a narrow host range (a drawback for targeting multiple pathogenic strains while being a benefit for maintaining normal microbiota), phage resistance, and phage-mediated transfer of genetic material to hosts (Joerger, 2003). Gene transfer via bacteriophages is a type of horizontal gene transfer that is one of the primary routes to increased bacterial pathogenicity (Lambert, 2010a). The main caution, therefore, in use of phage therapy is that phages used in treatment must be carefully monitored to ensure that phages do not contain toxin genes or virulence genes that could be inserted into bacterial hosts, potentially increasing toxicity or pathogenicity (Parisien et al., 2008). Overall, phage therapy shows great promise as an antimicrobial treatment due to its high efficacy, high safety, high specificity, and relatively low cost.

**New Antimicrobial Strategies**

**Bacterial Cell Wall Hydrolases**

Bacterial cell wall hydrolases (BCWH) are enzymes that attack bacterial cell walls, lysing the walls and killing the bacteria, and are divided into three groups: lysozymes, autolysins, and virolysins (Parisien et al., 2008). Of these, the phage-encoded virolysins show the most promise as alternatives to antibiotics since they are highly specific, having the capability to lyse bacteria on a generally species-specific basis. Phage therapy (an older antimicrobial treatment) and phage-encoded virolysins (a relatively new research avenue for antimicrobial treatment) are two of the most promising families of alternative antibacterial agents being investigated for treatment and prophylaxis of bacterial infections in veterinary and human medicine (Courchesne et al., 2009).

**Antivirulence Drugs Targeting Secretion Systems**

Some antivirulence drugs have recently been developed that target bacterial secretion systems such as the Type III secretion systems used by many gram-negative pathogens (Baron, 2010). These drugs that disarm rather than kill bacterial pathogens provide a viable alternative or adjunct to classical antibiotics and merit further research.

**Strategies Targeting Host Immune Systems**

Techniques that target the host’s immune system have been developed and used effectively to combat bacterial pathogens, including use of non-pathogen-specific immunomodulatory therapy to boost the host’s immunity (Anisimov & Amoaka, 2006), antimicrobial peptides (AMPs) produced by bacteria, and endogenous AMPs that are components of the host’s innate immunity systems. Non-specific immunomodulatory therapies, such as use of probiotics, provide a useful strategy for prevention of bacterial infections and for adjunct treatments of such infections. AMPs are ubiquitous, gene-encoded families of natural proteins produced by eukaryotic and prokaryotic organisms or encoded by phages. The vast diversity in AMPs generates a high potential for clinical applications.

**Bacteriocins: AMPs of Bacterial Origins**

Bacteriocins are types of AMPs of bacterial origin that are lethal to bacteria other than the producing strain (Joerger, 2003). Nisin is a bacteriocin produced by Lactococcus lactis that has been approved as a preservative. Bacillus AMPs show unique promise as antimicrobial agents because they have a broad inhibition spectrum against gram-negative bacteria, yeasts, and fungi in addition to gram-positive bacterial species, including a number that are pathogenic to humans and/or animals. Bacteriocins of Bacillus origin are also being used for food preservation and in environmental applications such as bioprotection against pre-harvest and post-harvest decay of vegetables (Abriouel et al., 2011).

**Endogenous AMPs: AMPs of Eukaryotic Origins**

Besides using AMPs of bacterial origin, another extremely promising alternative to antibiotics is the use of endogenous AMPs produced by eukaryotic hosts as part of their immune response. AMPs of eukaryotic origin such as defensins and cathelicidins supply comprehensive protection against infection with a broad spectrum of activity against bacterial, fungal, and viral pathogens (Andres & Dimarco, 2007; Sang & Blecha, 2008). In vertebrates, AMPs form a first line of defense against pathogens and are a key element of innate immunity. A common feature of most AMPs is that they kill bacteria by disrupting the bacterial cell membrane. This mechanism is considered a key advantage in the use of AMPs as antimicrobial treatments since this route of action is judged to be less likely to induce resistance. Some AMPs have intracellular targets and those AMPs that have both extracellular and intracellular targets, whether natural or synthetic, are especially appealing as poten-
tial antimicrobial agents since their attacks on pathogens are multi-pronged (Nguyen et al., 2011; Rozgony et al., 2009).

Extensive research in this area over the last 10 years has revealed some potential drawbacks in the clinical applications of AMPs as antimicrobial drugs. These include bioavailability (limited tissue distribution), stability, potential immunogenicity, potential eukaryotic toxicity, and high production costs (Liu et al., 2010; Rotem & Mor, 2009; Tew et al., 2010). Many laboratories are developing synthetic AMPs and peptidomimetics in an attempt to overcome these limitations of natural AMPs while maximizing the antimicrobial effects. Innate immunity, like intrinsic immunity, is very complex (Lambert, 2010b). Therapeutic interventions based on either of these host immune mechanisms, therefore, merit close scrutiny regarding potential negative impacts on the host’s immune defenses. Despite these challenges, natural and synthetic AMPs that mimic the host’s innate immunity defenses remain a top target for development as antimicrobial agents.

**Conclusion**

Increased concerns about reliance on antibiotics as the primary (or only) treatment targeting many bacterial infections have led to a reassessment of other existing treatments as well as a search for novel alternatives. The need for alternatives to antibiotics is heightened because of surging numbers of antibiotic-resistant strains, including increasing numbers of multi-drug resistant strains in non-hospital settings.

Natural treatments such as probiotics remain important for prevention and for treatment of symptoms of bacterial infection. Some alternatives such as vaccines offer valuable strategies against specific bacterial pathogens, but are unlikely to have broader applicability. Additional research is called for in some areas such as with certain natural plant extracts (e.g., willow herb extract) and with antivirulence drugs targeting bacterial secretion systems. The most promising approaches for developing alternatives to antibiotics are phages,ophage-encoded virolysins, bacteriocins (AMPS of bacterial origin), and AMPs of eukaryotic origin or synthetic versions that mimic the innate immunity mechanism of eukaryotic AMPs.

Phages, one of the most promising alternatives to antibiotics, have been around for quite a while. When it comes to bacterial pathogens, bacteriophages and eukaryotes have a common enemy. It makes sense for humans to use this commonality when pursuing treatment options for human and animal diseases. In addition, a newer phage-based strategy (phage-encoded virolysins) shows considerable potential as a therapeutic option.

Antimicrobial peptides, whether of bacterial or eukaryotic origin, are part of organisms’ natural defenses against bacteria. Similar to the common enemy argument about phages, it makes sense for humans to use bacteriocins (bacterial AMPs) to combat bacterial pathogens. *Bacillus* AMPs, in particular, deserve close attention as antimicrobial agents due to their broad inhibition spectrum. It also is logical to pursue research on the diverse families of eukaryotic AMPs that are part of innate immune defenses. Research on bacterial AMPs and endogenous host AMPs has yielded very promising results in the search for alternatives to antibiotics.

Therapeutic use of some synthetic versions of AMPs is being actively explored with the aim of optimizing efficacy while minimizing the limitations of endogenous AMPs (Rozgony et al., 2009; Tew et al., 2010). Because of the complexity of the vertebrate immune system, however, use of synthetic AMPs as antimicrobial agents will need to be carefully tested to ensure that such treatments do not result in negative unforeseen impacts on the host’s immune response.

**References**


Capsule

Felix K. Gmuender
Basler & Hofmann Singapore Pte Ltd., Singapore

What’s new, what’s hot, what’s timely? If you don’t have time to search the Internet for the latest developments that might impact your work environment, you just might find some of this information in this “Capsule” column. Please e-mail any comments or suggestions to felix.gmuender@bh.com.sg or to Co-Editor Barbara Johnson at barbara_johnson@verizon.net or Co-Editor Karen B. Byers at karen_byers@dfci.harvard.edu.

Viral Infections in Workers in Hospital and Research Laboratory Settings, Maintenance of Influenza Virus Infectivity on the Surfaces of Personal Protective Equipment, and Transocular Entry of Seasonal Influenza-Attenuated Virus Aerosols and the Efficacy of PPE

Pedrosa & Cardoso (2011) present one of the most comprehensive reviews of means of occupational laboratory and hospital infections with viruses. Based on strict selection criteria, the authors selected 66 out of 141 papers published between 1935 and 2006 for detailed analysis. Thirty-six papers with a total of 211 cases deal with laboratory-acquired (or associated) infections (LAI). The time period was divided into three 24-year periods. For the first period from 1935-1958, 28 LAI were analyzed; for the second period from 1959-1982, 68 cases were analyzed; and for the final period from 1983-2006, 157 LAI were analyzed. The author of this Capsule noticed that only 6 cases of LAI came from the time period after 2000. Notwithstanding, the general trend for LAIs is up, not down, despite increasing efforts to improve biosafety at the bench and biosafety management. Pedrosa & Cardoso (2011) comment that “the volume of virological research and the number of patients with viral diseases in hospitals have both grown over time.”

Many of the case studies reviewed by Pedrosa & Cardoso (2011) deal with viruses that infect via the mucosa, in particular the eye. While most of these occurred in hospital settings, the lessons learned are highly relevant for biomedical and microbiological laboratories. In many BSL-2 laboratories, staff are not wearing eye protection, probably unaware of the mucosal route of transmission and/or claiming that they would conduct work that creates droplets and aerosols in a biosafety cabinet. They forget that in BSL-2 laboratories, many tasks are carried out on the open bench, outside of primary containment systems, and that while the BSC very efficiently captures aerosols, higher velocity large droplets may escape the BSC. Furthermore, BSCs do not prevent contact infections via mucocutaneous membranes or infections via ingestion. Flasks, vials, and tubes are frequently manipulated outside primary containment systems, including their transport from the incubator to the BSC. Spill and leak accidents, and splashes to the face and eye do occur. In many biological and microbiological laboratories, workers wearing gloves walk around the lab, and even outside of one lab into another lab or support area, and touch common items. Lack of a glove policy can spread contamination into laboratory areas perceived by laboratory staff as “clean.” Thus, we should keep an eye (sic!) on contact infections (i.e., How many times per day do we touch the face near or at the eye?). Using safety glasses in the laboratory will prevent the potential risk of transmission in addition to protecting against splashes. An adequate glove policy, hand washing, and strict adherence to the rules would eliminate or at least reduce the likelihood of fomite and finger transmission.

This review article (Pedrosa & Cardoso, 2011) helps us to better reason with staff who are reluctant to wear eye protection at all times in BSL-2 laboratories and provides evidence-based examples to laboratory managers that enforcing an appropriate glove and hand-hygiene policy is important in reducing the risk of LAI.

The LAI quoted in this review occurred predominantly when workers were processing risk groups 3 and 4 viral agents, which are known to be transmitted through aerosols. In a laboratory situation, the infectious dose for the aerosol route may be generated during certain procedures (nebulizers, resuspension of centrifuge pellets, etc.), when a spill occurs outside primary containment equipment, or when primary containment systems fail. For example, the authors quote the following accident, published in 1995:

“A second laboratory infection with Sabia virus [Ed.: Today this is a risk group 4 virus] and aerosol inhalation as the mode of infection occurred in a biosafety level 3 (BSL-3) facility. Despite that, the researcher only wore a disposable gown, two pairs of gloves, and a surgical mask (droplet protection only), which constitute BSL-2 personal protective equipment. The researcher had no positive pressure high efficiency particulate air (HEPA) filtered respirator, and after performing a centrifugation of 200 ml of viral suspension in a bottle with Vero cell culture (high
titer viral suspension with high volume), viral suspension was found in the bottom of the rotor and the outside of the bottle was wet when the lid of the rotor was opened by the virologist." (Pedrosa & Cardoso, 2011)

With hindsight we can identify the chain of events that caused the above-mentioned LAI and what should have been done to prevent it and break the links in the chain of infection. The biosafety community benefits when information from such accidents is published so lessons are learned and knowledge about the root causes and appropriate precautionary safety measures can be adopted worldwide. Accidents still do happen, but fortunately we continue to learn from them. The above-quoted case was totally preventable. Most exposures occur because lab workers are not aware of the risks when, for example, a sealed biosafety rotor or cup is not used, rotors are opened inappropriately, or inappropriate PPE such as surgical masks are worn. One single safety measure might have prevented this LAI. With the tools of microbiological risk assessment and management as described in current biosafety guidance documents, the risks in the biosafety laboratory can be minimized to a negligible or acceptable level.

To the uninitiated reader of this review article the conclusion might be that the risk situation in a biosafety laboratory has not changed since the last century, because numbers of LAI are going up. However, to quote the authors: “The measures and protocols of biosafety generated to minimize the number of these accidental infections involving specific modes of infection were less effective than commonly supposed.” The analyzed case studies show that non-compliance with biosafety protocols for aerosol containment and personal protective equipment results in LAI; thus, the point could be made that the problem is in the adoption and adherence to biosafety protocols.

For those who are new to the field of biosafety, the article helps to better understand the risks and shows that biohazards do not pose a phantom risk. The two authors have provided a meticulous and valuable review.


**Maintenance of Influenza Virus Infectivity on the Surfaces of Personal Protective Equipment and Clothing Used in Healthcare Settings**

In healthcare settings (and biosafety laboratories as well), the tenacity and viability of infectious agents on surfaces are of high relevance. In healthcare settings, surfaces typically get contaminated by secretions and droplets spread by patients and in laboratories by droplets and aerosol particles large enough to settle quickly. Personal protective equipment (PPE), including clothing and respirators, can prevent direct contact and inhalation, but at the same time the PPE may become fomites that can cross-infect other surfaces and eventually even people. Inappropriate usage and disposal of PPE may result in an inadvertent direct exposure or contamination of surfaces. Sakaguchi et al. (2011) studied whether and for how long influenza A viruses (H1N1) maintain infectivity on PPE worn in healthcare institutions; these include rubber gloves, N95 respirators, surgical masks, and Tyvek® suits. 1280 hemagglutination (HA) units of the ATCC VR-95 influenza A strain were applied to the surfaces and left for 1, 8, and 24 hours. Samples were collected to determine the 50% tissue culture infective dose TCID\(_{50}\)/ml. Sakaguchi et al. (2011) report that the HA titer did not decrease in any of the materials even after 24 hours, which served as a control (virus particles did not detach). The infectivity (TCID\(_{50}\)/ml) was maintained on all materials for 8 hours and on rubber gloves for 24 hours. The authors conclude that periodic replacement and correct removal of PPE are necessary to prevent cross infections.


**Transocular Entry of Seasonal Influenza—Attenuated Virus Aerosols and the Efficacy of N95 Respirators, Surgical Masks, and Eye Protection in Humans**

Bischoff et al. (2011) investigated to what extent the airborne transmission route for a live attenuated influenza vaccine strain can be barred by N95 respirators, surgical masks, and/or eye protection (non-vented goggles). Twenty-eight test subjects were assigned to 6 groups: (1) no protection; (2) eye protection only; (3) surgical mask without eye protection; (4) surgical mask with eye protection; (5) fit-tested N95 respirator without eye protection; and (6) fit-tested N95 respirator with eye protection. The test subjects were exposed to monodispersed viral aerosols with a particle size of 4.9 μm. All 4 participants in the control group (no protection) exhibited successful transmission. Successful transmission was confirmed in: 3 of 4 subjects with eye protection only; all of 5 subjects wearing surgical masks only; all of 5 subjects wearing surgical masks and goggles; 2 of 5 subjects wearing a fit-tested N95 respirator; and 1 of 5 subjects wearing a fit-tested respirator and goggles. Despite the small sample size, the authors concluded that the eyes play a relevant entry route for influenza viruses.

Animal Bytes examines biosafety challenges posed when conducting work with animals and provides solutions that promote both safe and responsible research. Good safety and animal husbandry are essential for good science. Learn about best practices when working with animals and applied safety information that can be used every day. Please e-mail your comments, questions, and insights to barbara_johnson@verizon.net or Co-Editor Karen B. Byers at karen_byers@dfci.harvard.edu.

**Animal Care and Use**

An institution’s decision to conduct programs involving animals for teaching, research, or testing comes with significant responsibility for the welfare and safety of the animals, workers, and the community. In the United States, two federal agencies regulate the care and use of animals: the United States Department of Agriculture (USDA) and the Public Health Service (PHS). An institution may fall under one or both of these entities’ jurisdictions as a function of funding sources or animal species. Regardless of which mandate is applicable, all institutions should adhere to the *Guide for the Care and Use of Laboratory Animals* (ILAR, 2010) to ensure best practices and to maintain regulatory compliance. The USDA enforces the Animal Welfare Act and Regulations (2010) while the Office for Laboratory Animal Welfare (OLAW) in the PHS enforces the Health Research Extension Act of 1985, Public Law 99-158 (OLAW, 2002a) through the Public Health Service Policy (PHS Policy, 2002). In addition, many institutions seek program accreditation through the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Although voluntary, many organizations view AAALAC accreditation as demonstration they meet or exceed the USDA and PHS requirements on animal care and use. Institutions planning to conduct research with animals must develop an animal care and use program that accounts for all activities that involve animals including policies and procedures, veterinary and animal care, personnel training, animal facility design and management, occupational safety and health, and the constitution and function of the Institutional Animal Care and Use Committee (IACUC) (ILAR, 2010). This column focuses on the basics of creating an IACUC.

**IACUC: Background, Charter, and Membership**

According to U.S. federal law, the IACUC must be established by institutions that use laboratory animals for research, testing, or instructional purposes (IACUC, 2011). The IACUC is a self-regulating entity that is appointed by the highest operating official (i.e., CEO) of the organization and reports to the Institutional Official (IO). Although the CEO may delegate responsibilities to an appointed IO, that individual, as a representative of the institution, at a minimum must be authorized to legally commit on behalf of the institution that the federal animal welfare requirements will be met. This generally also requires that the IO have the authority to commit institutional resources in terms of funding and administration to ensure the animal care and use program is effective and the institution maintains compliance with USDA Animal Welfare Regulations (AWR) and PHS Policy requirements. Guidance on membership requirements for constituting a functional and recognized IACUC depends on whether one adheres to Animal Welfare Act (AWA, 2009), Animal Welfare Regulations (AWR) (CFR, 2009) or PHS Policy requirements. For example, the PHS Policy requires the IACUC to have five members and USDA regulations require three members (OLAW, 2002b). A charter should be developed to clearly define the IACUC and identify its purpose, membership, roles, functions, meeting frequency, and other responsibilities. The charter is a guidance document and should be reviewed annually with changes made as needed, with the goal to continuously improve the animal care and use program.

This column adopts guidance provided by ILAR (2010) that states the required IACUC members must include at a minimum:

- A veterinarian with experience in laboratory animal science or the species of research animals at the institute
- A practicing scientist with experience in research involving animals
- An individual from within or outside the organization (i.e., ethicist, lawyer, etc.) whose primary focus is non-scientific
- A community member representing the community’s interest in animal care and use who does not use laboratory animals and has no institute affiliation or an immediate family member with institute affiliation.

Most institutions appoint more than one scientist, non-scientist, and community member to ensure the IACUC is constituted with appropriate members and achieves a quorum of greater than 50% of voting members. These appointments may be made as full voting members or alternate members. Alternate members vote only if the primary voting member for whom they are serving as an alternate is not in attendance. To avoid conflict of interest, members are excused by the Chair or recuse themselves from discussions pertaining to topics...
where they are personally involved in the activity or when their participation would provide them an unfair competitive advantage (i.e., against a competing laboratory). Having alternate or additional voting members becomes advantageous when voting members must excuse themselves or for other reasons cannot attend meetings or facility inspections.

The Chair, as all committee members, is appointed by the CEO and should be familiar with animal research or regulatory requirements and the parliamentary process. That person should have leadership skills to ensure participation of all members and be able to enlist the support of the IO. It is inadvisable for the attending veterinarian (AV) to serve as IACUC Chair or IO to maintain the proper balance of checks and balances between the responsibilities of the IACUC and AV.

To coordinate with other institutional regulatory committees and functions (i.e., the Institutional Biosafety Committee (IBC), Institutional Review Board (IRB), occupational health and safety), representatives such as the biosafety professional, safety officer, and medical officer may sit on the IACUC, often as ex-officio members. This serves to streamline and facilitate the review process across the committees, facilitate communications among committees, and ensure all regulatory compliance is addressed. In some institutions software programs for filing IACUC, IBC, and IRB proposals are electronically linked and information cross-populates the forms to reduce the researcher’s administrative burden, ensure the same information is entered on all forms, and rapidly notify the IACUC members from the IBC and IRB of a protocol requiring their attention. While such a program bears financial expense in design, implementation, and system maintenance, it may pay for itself many times over in processing efficiency, notification, and tracking. A site-specific assessment and consideration of institutional needs drive the decision on how to best address achieving the necessary communication and cross-references across institutional research and regulatory compliance functions.

IACUC: Roles and Training

The role of the IACUC is to oversee, review, and evaluate the animal care and use program. Regardless of whether an institute falls under USDA or PHS requirements, the key components have similar intent for both agencies and include:

- Conducting a review of the institution’s animal care and use program at least every 6 months
- Conducting a physical inspection of the animal facilities, including satellite sites, where animals may be housed, cared for, or worked with at least every 6 months
- Reviewing a protocol with notification to the PI of the approval, modification, or denial of the protocol; and review of significant changes made to existing protocols (i.e., species, number of animals, endpoints, materials and methods, etc.)
- Reviewing concerns regarding animal care and use
- Suspending previously approved animal work that is not in compliance with regulations or consistent with the conditions of approval
- Reporting findings and recommendations regarding the status of the program, facilities, and personnel training

All voting members must receive training to fulfill their IACUC duties. A number of training programs, resources, and opportunities are available and can be found through consultation with the IACUC Chair or AV, or by visiting several online resources such as the HHS Office of Research Integrity (ORI, 2011), OLAW (OLAW, 2011), the American Association for Laboratory Animal Science Learning Library (AALAS, 2005), AAALAC education and outreach (AAALAC, 2011), Collaborative Institutional Training Initiative (CITI, 2011), Public Responsibility in Medicine and Research (PRIMR, 2011), and other reputable resources.

As stated earlier, the decision to conduct programs involving animals comes with great scientific and social responsibility. As the regulating body of the institution, the IACUC is charged with ensuring all work is conducted responsibly, ethically, safely, and humanely. The information in this column is intended to serve as a primary resource in the development of an informed, educated, and committed IACUC.

References


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