Efficacy of Dry Mist of Hydrogen Peroxide (DMHP) against *Mycobacterium tuberculosis* and use of DMHP for Routine Decontamination of Biosafety Level 3 Laboratories[▽]

M. Grare,[1] M. Dailloux,[1] L. Simon,[2] P. Dimajo,[3] and C. Laurain[1]*

Laboratoire de Bactériologie,[1] Laboratoire d'Hygiène Hospitalière,[2] and Département Environnement Hospitalier,[3] Centre Hospitalier et Universitaire de Nancy, Nancy, France

***Mycobacterium tuberculosis* is a major cause of morbidity and mortality worldwide and is becoming a greater concern due to the development of multidrug-resistant strains. *M. tuberculosis* can contaminate rooms, medical equipment, and research laboratories and has the propensity to be highly resistant to decontamination. The aim of this study was to determine the efficacy of room disinfection with a dry mist of hydrogen peroxide (DMHP) in a biosafety level 3 laboratory in the event of contamination with *M. tuberculosis*. The biological indicators (BIs) were comprised of presterilized cotton tissues on which amounts of about 10^7 CFU/ml of *M. tuberculosis* H37Ra were dried. The device (Sterinis; Gloster Sante Europe) provided a DMHP of 5% hydrogen peroxide during 25 min. Three experiments were performed. The viable bacteria were reduced by values of more than 5 $\log_{10}$, and no colony grew from any BI. In conclusion, DMHP shows promise as an effective and safe alternative to the currently used formaldehyde.**

Tuberculosis is a major public health problem, particularly with the development of multidrug-resistant strains (22). It was declared to be a global emergency by the World Health Organization (WHO) in 1993. *Mycobacterium tuberculosis* bacteria are most likely transmitted by aerosols and so can easily contaminate rooms in health care facilities or research laboratories. Moreover, these bacteria present high intrinsic resistance to disinfection and fumigation processes and possess effective defense mechanisms against oxidative stress (4, 5). Their resistance to disinfectants has been considered intermediate between those of other vegetative bacteria and spores. The components of the cell wall that are responsible for this high level of resistance are unknown, but both mycolic acids and arabinogalactan appear to be involved (3, 20).

Chemical liquids and vapors are mainly used as decontaminating agents. Traditional room fumigation has been conducted by using formaldehyde or ethylene oxide gas. These methods have been given up due to their toxicity and carcinogenicity. In June 2004, the International Agency for Research on Cancer (IARC) classified formaldehyde as carcinogenic for humans (6, 12). It has been recommended in France since September 2006 that this substance not be used for room decontamination, and its production was definitively stopped in January 2007. Thus, research laboratories and hospitals must find other solutions for decontamination; H_2O_2 provides an alternative to formaldehyde fumigation because of its biological efficacy against various microorganisms (7, 8, 11, 13, 17) and its safety (hydrogen peroxide readily decomposes to form water and oxygen).

Hydrogen peroxide has already been recommended for the disinfection of a large variety of materials, including dental instruments (18) and bronchoscopes (2). The use of H_2O_2 vapor (VHP) for the biodecontamination of biological safety cabinets (BSCs), rooms in health care facilities (8), ambulances (1), pharmaceutical facilities, and laboratories (10, 14, 15, 16) has been increasing. A few studies have focused on its use for disinfection of biosafety level 3 laboratories (BSL3s), especially for efficacy against *Mycobacterium tuberculosis* or other, nontuberculous *Mycobacteria* (NTM) species (1, 2, 9, 10, 14).

In this study, a patented, programmable device (Sterinis; Gloster Sante Europe) that provides a dry mist of hydrogen peroxide (DMHP) as a disinfectant was tested for air and surface decontamination of a BSL3. The objective was to determine the efficacy of DMHP diffusion in eliminating contaminations with laboratory strain *M. tuberculosis* H37Ra, an avirulent strain.

MATERIALS AND METHODS

Preparation of biological indicators (BIs). Assays were conducted by following standards AFNOR 72-281 and AFNOR 72-190 of the Association Française de Normalisation (for surface disinfection with aerosols), adapted for slowly growing mycobacteria. *M. tuberculosis* H37Ra (ATCC 25177) was subcultured in Löwenstein Jensen medium (Bio-Rad). After 15 days of subculture, a bacterial suspension was prepared in sterilized distilled water and adjusted at a 1 McFarland standard, and colony counts were performed in duplicate on Löwenstein Jensen medium for each experiment. Amounts of 100 µl of this suspension were applied to the center of 11 presterilized cotton tissues (4.5 × 3 cm) which were air dried in glass tubes.

BSL3 room experiments. The trials were conducted in an 80-m^3 BSL3 which contained two class II BSCs (Securiplus M5142; Astec Microflow, England), freezers, and incubators. Three cycles were conducted. In each cycle, BIs were removed from the glass tubes and were then placed at the following nine locations (lettered A to I) in the laboratory: two in operational BSCs (front panel open 20 cm; mean flow speed, 0.45 m^3/s), two in two different incubators (closed, one ventilated and the other not ventilated), and five on the ceiling (in the middle and in the corners of the laboratory, with one behind the DMHP apparatus) (Fig. 1). BIs were placed at various distances from the apparatus (1 to 9.5 m). Two BIs were used as controls and maintained outside the BSL3 throughout the duration

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Hôpital Brabois, CHU, 5 Rue du Morvan, 54111 Vandoeuvre-les-Nancy, France. Phone: (33) 3-83-15-34-72. Fax: (33) 3-83-85-43-76. E-mail: c.laurain@chu-nancy.fr.
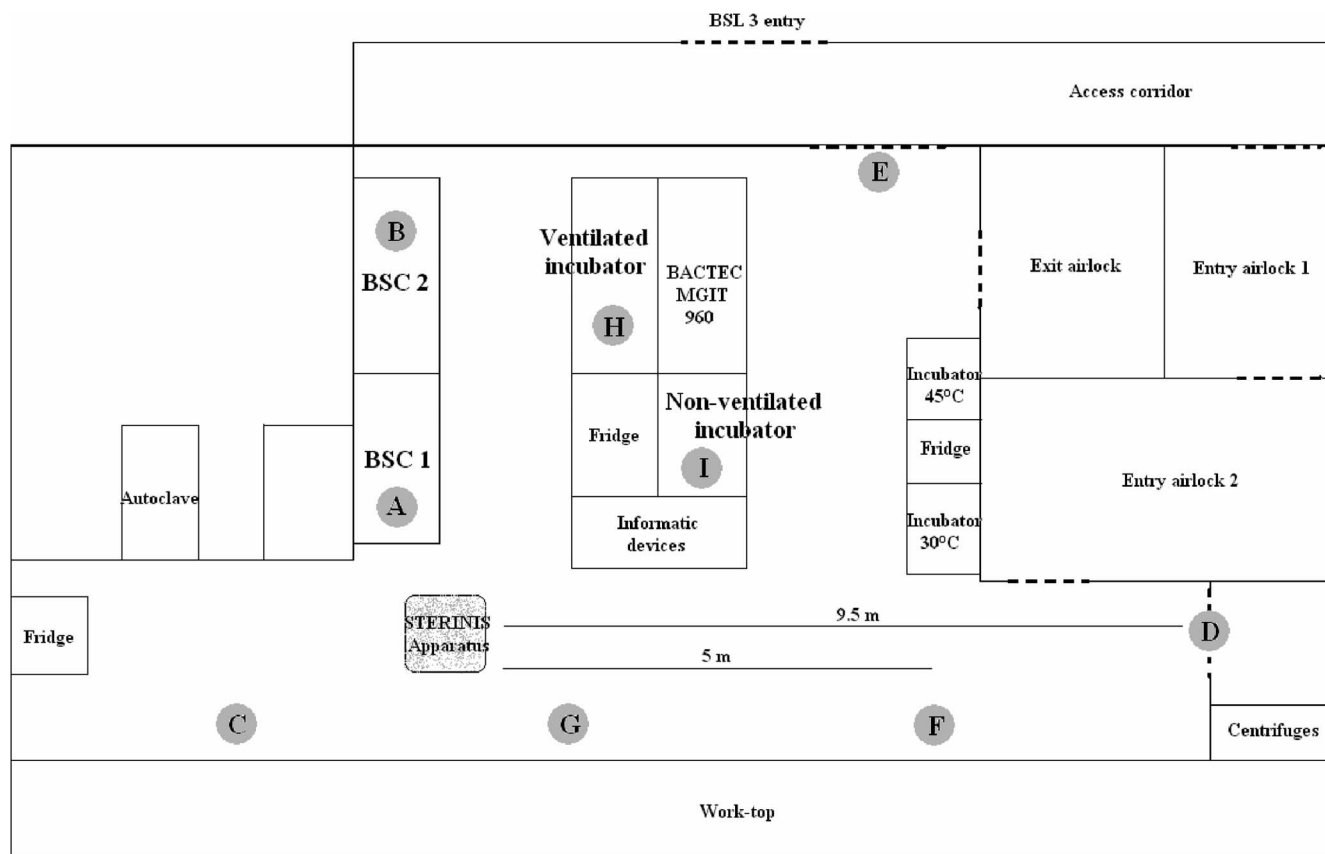
FIG. 1. Schematic plan of the BSL3 with the location of each BI. Fridge, refrigerator.

of sample exposure to DMHP in order to validate our methods for culturing and enumerating bacteria on the BIs (the bacterial inoculum, ultrasound detachment, filtration on 0.45-μm filter, and washing steps) and to be sure that *Mycobacteria* cells remain viable after these treatments. The system providing heating, ventilation, and air conditioning in the room was disabled to prevent unwanted dispersion or dilution of DMHP during the exposure period. This system was reinstated after a 2-h exposure to DMHP. The room temperature was within the range of 20 to 23°C, with relative humidity at 45 to 50%, measured just before starting diffusion. DMHP (Sterusil) disinfection was performed by means of a Sterinis apparatus (Gloster Sante Europe, Toulouse, France) using Sterusil. Sterusil is a disinfecting solution containing 5% hydrogen peroxide, stabilized orthophosphoric acid at less than 50 ppm, silver cations at less than 50 ppm, gum arabic at 1 ppm, and 95% osmosed water. This solution is not considered carcinogenic by IARC. Toxicity assays have been already made in rats, and the results showed innocuousness. The apparatus produced 6 ml/m³ H_2O_2 during 25 min.

Culture and enumeration of BIs. The nine BIs that were exposed to DMHP and removed from the room 1 night after the aeration phase and the two BIs not exposed to DMHP (controls) were transferred into plastic tubes containing 10 ml sterilized distilled water. They were subjected to ultrasound during 3 min (2 W; frequency, 20 KHz) (Vibracell; Sonics Materials). Within the 10 ml recovered, dilution at 10^{-2} was realized in sterilized distilled water. Then, 2 ml of each suspension (pure and 10^{-2} diluted) was filtered on 0.45-μm filters (Millipore Corporation, Bedford, MA). The filters were washed three times with 50 ml sterilized distilled water and then placed onto Middlebrook 7H11 plates supplemented with OADC (lot no. 5188292; Becton Dickinson, Sparks, MD). All enumerations were made in duplicate. CFU were counted after incubation at 37°C for 4 weeks. After ultrasound detachment, all BIs were cultured in Middlebrook 7H9 broth (reference no. 271310, lot no. 1298006; Becton Dickinson, Sparks, MD) in order to ensure that this technique efficiently collected all *Mycobacteria* and that no viable *Mycobacteria* remained on the support.

RESULTS

The results of the three DMHP diffusion exposure runs are presented in Table 1.

We made colony counts on Lowenstein Jensen medium for all initial suspensions in order to evaluate the quantity of *M. tuberculosis* H37Ra applied to the BIs. We considered these first values to be a theoretical initial concentration. We obtained values between 10^6 and 10^8 CFU/ml for the initial inoculum for the three assays. This variation underlines the difficulty of achieving a homogeneous suspension with *Mycobacteria*, despite taking all the precautions that could be taken and using McFarland standard measurement (all suspensions were adjusted at 1 McFarland standard before dilution). The numerations obtained for BIs not exposed to DMHP were considered real concentrations, taking into account the efficacy of *Mycobacteria* cell detachment by ultrasound and the potentially decreased *Mycobacteria* cell viability from 24 h of drying on BIs. The bacterial numbers in nonexposed BIs ranged from 5×10^5 to 5×10^6 CFU/ml (depending on the initial inoculum). For all experiments, we observed about a 1-$\log_{10}$ decrease, but in all cases, we obtained at least 5×10^5 CFU/ml from controls, which allowed us to note a 5-$\log_{10}$ decrease for the other BIs.

During run 1, a decrease of more than 5 $\log_{10}$ from the initial inoculum amount was observed for the samples exposed to

TABLE 1. Total bacterial counts per ml after DMHP[a]

Sample, location	Run 1		Run 2		Run 3	
	Bacterial count (CFU/ml)	Growth of BI subculture	Bacterial count (CFU/ml)	Growth of BI subculture	Bacterial count (CFU/ml)	Growth of BI subculture
Unexposed control 1	5×10^5	Yes	5×10^6	Yes	5×10^5	Yes
Unexposed control 2	5×10^5	Yes	5×10^6	Yes	5×10^6	Yes
A, in operational BSC 1	0	No	0	No	0	No
B, in operational BSC 2	0	No	0	No	0	No
C, behind the VHP apparatus	0	No	0	No	0	No
D, 9.5 m from apparatus	0	No	0	No	0	No
E, on exit door	0	No	50	No	0	No
F, 5 m from apparatus	0	No	0	No	0	No
G, above apparatus	0	No	0	No	0	No
H, in incubator	0	No	50	No	0	No
I, in incubator	0	No	0	No	0	No

[a] The enumerations of the initial suspensions were 10^6, 10^8, and 10^7 CFU/ml for runs 1, 2, and 3, respectively, on Löwenstein Jensen medium.

DMPH after 6 weeks at 37°C (no colony grew on filters on 7H11 plates). No growth was noted for cotton tissues subcultured in 7H9 broth after 6 weeks at 37°C. This confirms that the ultrasound technique is effective for detaching viable *Mycobacteria* from BIs and that DMHP is an efficient decontamination tool.

During run 2, the same observations were made, with two exceptions. Some *Mycobacteria* cells remained on BI E (one colony grew, which corresponds to 50 CFU/ml). This can be explained by the distance between this BI and the apparatus and by the number of obstacles between the two elements. One colony also grew on BI H but with a great reduction in numbers compared to the initial suspension (50 CFU/ml versus 10^7 CFU/ml). This is disturbing because it could signify that DMHP entered the incubator and so could alter cultures stocked in the incubator. However, no growth was noted for cotton tissues subcultured in 7H9 broth after 6 weeks at 37°C.

During run 3, a decrease of more than 5 $\log_{10}$ was observed for the exposed samples after 6 weeks at 37°C, and none of the BIs produced colonies on subcultures.

For three assays, we obtained a decrease of more than 5 $\log_{10}$ from the initial inoculum amount. These results demonstrate that DMHP dispersal within the room was efficient and that the short exposure time (25 min) was sufficient to deactivate the BIs.

DISCUSSION

The challenge for biodecontamination is to find a product able to reach infectious particles everywhere. DMHP diffusion seemed to represent a good alternative to formaldehyde fumigation, particularly for decontamination of a BSL3. According to the AFNOR 72-281 and 72-190 standards, at least a 5-$\log_{10}$ reduction of the initial inoculum is required to decide that a decontaminant is efficient. The results of in vitro studies investigating the resistance of *M. tuberculosis* to oxidative stress demonstrated that low concentrations of H₂O₂ (between 10 and 100 mmol · liter^{-1}) can kill the bacteria (4, 5). Accordingly, the DMHP diffusion system described in this report used a 5% H₂O₂ solution to generate and maintain the dry mist within the room at 6 ml/m³.

In our study, we used a Sterinis apparatus, which achieves a DMHP. As previously discussed, *M. tuberculosis* is highly con-

taminative because of its capacity to form an aerosol and then to disperse widely in the environment, onto all surfaces. Manual disinfection using alcohol wipes and sprays treats only the zones that are easily accessible. The DMHP diffusion process could be a useful tool because its dry mist contains H₂O₂ as electrically charged particles of such a small size (approximately 10 μm in diameter) that they can circulate freely in air as a dry aerosol. This makes the DMHP diffusion a suitable substance for decontaminating a BSL3. In the study whose results are presented here, we showed efficiency of this process against *M. tuberculosis* H37Ra, using a fumigation cycle of only 25 min in an automated mode which does not require human intervention, in three independent trials.

In these conditions, DMHP diffusion efficiently kills *M. tuberculosis* bacteria. This is consistent with the findings of other authors. Kahnert et al. have showed that VHP treatment is an effective means of eliminating room contaminations of *M. tuberculosis* H37Rv (14). Their experiments were quite similar to our study, but they used another VHP system (VHP1001; Steris Ltd., United States). A 5-$\log_{10}$ reduction of the inoculum was obtained after 90 min of exposure (14). More recently, Hall et al. concluded that VHP (Clarus S HPV system; Bioquell, Inc., United States) provides an alternative to traditional decontamination methods for laboratories and other areas contaminated with *M. tuberculosis* bacteria (bacteria were deactivated in all locations following 90 min of VHP exposure) (10). They obtained a 3-$\log_{10}$ reduction in less than 90 min in room experiments (10). They evidently considered this lower decrease sufficient, because they cleaned manually before VHP fumigation. In our BSL3, we preferred to start the diffusion of H₂O₂ immediately, in order to prevent contamination of laboratory staff, and then follow up with a process of decontamination (repeated once).

No surface or material damage could be noticed after DMHP exposure.

One question asked by our study was whether there would be penetration of H₂O₂ mist into incubators. We effectively showed a decrease of CFU on all BIs, including those present in incubators. In the event of a contamination accident in a BSL3, all cultures could not be transferred to other incubators, and *Mycobacteria* cells might be altered if H₂O₂ mist pene-

trated an incubator. More experiments must be conducted in order to confirm this observation.

In conclusion, our study showed that a DMHP process (Sterinis) is effective for decontamination of *M. tuberculosis* bacteria in a BSL3. In order to avoid the use of carcinogenic formaldehyde, DMHP diffusion technology is a promising method of decontamination of rooms and research laboratories, such as BSL3s. However, we have tested only one strain of *M. tuberculosis* (H37Ra), which presents the same characteristics as the virulent strain H37Rv, including susceptibility to antibiotics. This is the principal limitation of our study. Effectively, a large variety of *Mycobacteria* strains could be cultured in a BSL3, including multidrug-resistant strains (19). Some studies have found that some NTM are more resistant to disinfectants than *M. tuberculosis* bacteria (21). It could be interesting to evaluate this process against other *M. tuberculosis* strains or against NTM (*Mycobacterium avium* complex or *Mycobacterium abscessus* strains, for example, which are often isolated from clinical specimens), even if NTM are less dangerous for immune-efficient laboratory staff.

REFERENCES

1. **Andersen, B. M., M. Rasch, K. Hochlin, F.-H. Jensen, P. Wismar, and J.-E. Fredricksen.** 2006. Decontamination of rooms, medical equipment and ambulances using an aerosol of hydrogen peroxide disinfectant. J. Hosp. Infect. **62:**149–155.
2. **Bär, W., G. Marquez de Bär, A. Naumann, and S. Rüsch-Gerdes.** 2001. Contamination of bronchoscopes with *Mycobacterium tuberculosis* and successful sterilization by low-temperature hydrogen peroxide plasma sterilization. Am. J. Infect. Control **29:**306–311.
3. **Best, M., S. A. Sattar, V. S. Springthorpe, and M. E. Kennedy.** 1990. Efficacies of selected disinfectants against *Mycobacterium tuberculosis.* J. Clin. Microbiol. **28:**2234–2239.
4. **Dussurget, O., G. Stewart, O. Neyrolles, P. Pescher, D. Young, and G. Marchal.** 2001. Role of *Mycobacterium tuberculosis* copper-zinc superoxide dismutase. Infect. Immun. **69:**529–533.
5. **Edwards, K. M., M. H. Cynamon, R. K. R. Voladri, C. C. Hager, M. S. DeStefano, K. T. Tham, D. L. Lakey, M. R. Bochan, and D. S. Kernodle.** 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis.* Am. J. Respir. Crit. Care Med. **164:**2213–2219.
6. **Environmental Protection Agency.** 1999. Technology transfer network air toxics website: formaldehyde. http://www.epa.gov/ttn/atw/hlthef/formalde.html.
7. **Fichet, G., E. Comoy, C. Duval, K. Antloga, C. Dehen, A. Charbonnier, G. McDonnell, P. Brown, C. I. Lamezas, and J. P. Deslys.** 2004. Novel methods for disinfection of prion-contaminated medical devices. Lancet **364:**521–526.
8. **French, G. L., J. A. Otter, K. P. Shannon, N. M. T Adams, D. Watling, and M. J. Parks.** 2004. Tackling contamination of the hospital environment by methicillin-resistant *Staphylococcus aureus* (MRSA): a comparison between conventional terminal cleaning and hydrogen peroxide vapour decontamination. J. Hosp. Infect. **57:**31–37.
9. **Griffiths, P. A., J. R. Babb, and A. P. Fraise.** 1999. Mycobactericidal activity of selected disinfectants using a quantitative suspension test. J. Hosp. Infect. **41:**111–121.
10. **Hall, L., J. A. Otter, J. Chewins, and N. L. Wengenack.** 2007. Use of hydrogen peroxide vapor for deactivation of *Mycobacterium tuberculosis* in a biological safety cabinet and a room. J. Clin. Microbiol. **45:**810–815.
11. **Heckert, R. A., M. Best, L. T. Jordan, G. C. Dulac, D. L. Eddington, and W. G. Sterritt.** 1997. Efficacy of vaporized hydrogen peroxide against exotic animal viruses. Appl. Environ. Microbiol. **63:**3916–3918.
12. **International Agency for Research on Cancer.** 2004. IARC classifies formaldehyde as carcinogenic to humans. http://monographs.iarc.fr/ENG/Monographs/vol88/index.php.
13. **Johnston, M. D., S. Lawson, and J. A. Otter.** 2005. Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores. J. Microbiol. Methods **60:**403–411.
14. **Kahnert, A., P. Seiler, M. Stein, B. Aze, G. McDonnell, and S. H. E. Kaufmann.** 2005. Decontamination with vaporized hydrogen peroxide is effective against *Mycobacterium tuberculosis.* Lett. Appl. Microbiol. **40:**448–452.
15. **Klapes, N. A., and D. Vesley.** 1990. Vapor-phase hydrogen peroxide as a surface decontaminant and sterilant. Appl. Environ. Microbiol. **56:**503–506.
16. **Krishnan, J., J. Berry, G. Fey, and S. Wagener.** 2006. Vaporized hydrogen peroxide-based biodecontamination of a high-containment laboratory under negative pressure. Appl. Biosafety **11:**74–80.
17. **Quilez, J., C. Sanchez-Acedo, C. Avendano, E. del Cacho, and F. Lopez-Bernad.** 2005. Efficacy of two peroxygen-based disinfectants for inactivation of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. **71:**2479–2483.
18. **Ricklöff, J., and P. Orelski.** 1989. Resistance of various microorganisms to vapor phase hydrogen peroxide in a prototype dental hand piece/general instrument sterilizer, abstr. Q-59, p. 339. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, DC.
19. **Robert, J., N. Veziris, C. Truffot-Pernot, C. Grigorescu, and V. Jarlier.** 2007. Surveillance of antituberculosis drug resistance in France: recent data. Bull. Epidemiol. Hebd. **11:**90–91.
20. **Russell, A. D.** 1999. Bacterial resistance to disinfectants: present knowledge and future problems. J. Hosp. Infect. **43**(Suppl.)**:**S57–S68.
21. **Wang, G. Q., C. W. Zhang, H. C. Liu, and Z. B. Chen.** 2005. Comparison of susceptibilities of *M. tuberculosis* H37Ra and *M. chelonei* subsp. *abscessus* to disinfectants. Biomed. Environ. Sci. **18:**124–127.
22. **World Health Organization.** 2005. Global tuberculosis control. WHO report 2005, p.1–247. WHO, Geneva, Switzerland.