

unique challenge since the revision summaries of rapidly and dramatically changing guidance documents are often scant or non-existent. The change matrix we have developed is intended to be a resource for biological safety officers, investigators, and others with a vested interest in biosafety to assist in the identification of the issues most pertinent to their facility or institution. Individual statements or statement pairs have been presorted based on a number of criteria therefore allowing the individual user the freedom to filter the data based on their specific needs. For instance, an individual primarily interested in facility changes affecting laboratory exhaust requirements may sort through the changes based on those specific criteria.

The change matrix we have created presents a systematic and streamlined methodology for identifying changes in the Laboratory Biosafety Level Criteria sections between the fourth and fifth editions of BMBL. An overview of this process is represented graphically in Figure 7. We believe this methodology can also be applied to future revisions as well as to other elaborate guidance documents where a precise awareness of changing guidance is crucial to safety and regulatory compliance. In addition, the organization of the matrix makes it possible for the

individual user to add additional criteria (e.g., level of resource investment) and search for them against existing criteria.

## Authors' Note

The information contained herein does not necessarily represent the position of the federal government.

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## Inactivation of *Francisella tularensis* Schu S4 in a Biological Safety Cabinet Using Hydrogen Peroxide Fumigation

James V. Rogers and Young W. Choi

Battelle Memorial Institute, Columbus, Ohio

### Abstract

This study evaluated the inactivation of *Francisella tularensis* Schu S4 on various materials (acrylic, glass, polyamide, polyethylene, polypropylene, silicone rubber, and stainless steel) using hydrogen peroxide fumigation of a Class III Biological Safety Cabinet (BSC III). A suspension of *F. tularensis* Schu S4 ( $7 \times 10^7$  CFU) was dried on seven different types of test surfaces and exposed to vaporous hydrogen peroxide (VHP) fumigation for a contact time of two hours. Qualitative growth assessment showed that VHP exposure inactivated *F. tularensis* on all replicates of the seven test materials up to four days post-exposure. The effectiveness of VHP fumigation on the growth of biological indicators (*Bacillus subtilis* or *Geobacillus stearothermophilus*) and spore strips (*Bacillus atrophaeus*) was evaluated in parallel as a qualitative assessment of decontamination. At one and four days post-exposure, decontaminated biological indicators and spore strips exhibited no growth,

while the non-decontaminated samples displayed growth. This study provides information for using VHP fumigation as an alternative approach for the decontamination of virulent *F. tularensis* when the current accepted method of 10% household bleach followed by 70% alcohol may not be practical for decontamination of a BSC III.

### Introduction

The gram-negative coccobacillus, *Francisella tularensis* (formerly known as *Pasteurella tularensis*), is the etiologic agent of the zoonotic disease tularemia. *Francisella tularensis* is classified as a Category A select agent due to its infectivity and capacity to cause illness and death, thus heightening the concern of using this microorganism as a potential biological weapon (Dennis et al., 2001; Peterson & Schriefer, 2005). Such concerns have prompted decades of research investigating and developing vaccines and medical countermeasures against *F. tularensis* infection (Ellis et al., 2002).

In the environment, various factors such as temperature, relative humidity, sunlight, mode of dispersion (e.g., aerosols), and environmental material matrices (soil, water, etc.) influence the duration that *F. tularensis* can remain viable, which can last for weeks or months (Anda et al., 2001; Feldman et al., 2001; Abd et al., 2003; Feldman, 2003). The persistence of *F. tularensis* necessitates effective decontamination technologies and applications since only a few viable organisms are sufficient to cause potential infection (Saslaw & Carlisle, 1969). This is especially crucial in laboratories conducting research under Biosafety Level 3 (BSL-3) containment where *F. tularensis* is potentially applied or dispersed in various forms such as liquids or aerosols. Although *F. tularensis* is susceptible to heat inactivation (Ehrlich & Miller, 1973; Anda et al., 2001), there are often situations in which potentially contaminated items such as surfaces and sensitive equipment cannot be treated by autoclaving, heat, or liquid decontaminants. Therefore, fumigating agents such as VHP are often more suitable for decontaminating these materials within equipment in a BSL-3 containment facility.

Currently, the decontamination approach for treating the intentional release of *F. tularensis* on contaminated inanimate surfaces is achieved by spraying the suspected contaminated area with a 10% household bleach solution (one part household bleach and nine parts water) for at least a 10-minute contact time, followed by further cleaning with a 70% alcohol solution (Dennis et al., 2001). As a fumigant, hydrogen peroxide has been shown to decontaminate a wide range of microorganisms; however, efficacy against *F. tularensis* has not been demonstrated. Therefore, this study was conducted to evaluate the decontamination of *F. tularensis* using VHP fumigation of a BSC III.

## Materials and Methods

### Test Organisms

The virulent strain of *F. tularensis* Schu S4 was propagated on Chocolate II agar with hemoglobin and IsoVitaleX™ (CII+H+I agar; BD Diagnostic Systems, Sparks, Maryland). Isolated colonies were transferred to individual CII+H+I agar plates and grown for 72-96 hours at 37°C until lawns were formed. Once the lawns were inspected for homogeneity, they were scraped into tubes with Mueller-Hinton broth (BD Diagnostic Systems) supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% IsoVitaleX™ (BD Diagnostic Systems), at pH 7.0 (Baker et al., 1985), with 10% sucrose for long-term storage at ≤ -70°C (Conlan & North, 1992). Bacterial viability and titer for this trial were verified once the stock was thawed using dilution plating on CII+H+I agar. The stock was diluted in Dulbecco's phosphate-buffered saline (Sigma, St. Louis, Missouri) prior to inoculation onto the material coupons.

### Test Materials

Seven materials commonly found in laboratories and BSCs were used as test surfaces: acrylic, glass, polyamide, polyethylene, polypropylene, silicone rubber, and stainless steel. Samples of each test material were cut from a larger piece of the representative materials to form 2.0 cm x 5.0 cm coupons with the exception of glass (microscope slides; VWR International, Inc., West Chester, Pennsylvania) and stainless steel (1.9 cm x 7.5 cm coupons). A visual inspection of the physical integrity and appearance of the test material coupons was performed before and after decontamination to detect any damage to the test materials. All coupons were sterilized by autoclaving at 121°C for 20 minutes prior to testing.

### Decontamination Procedure

All testing was performed under BSL-3 conditions. Each coupon was laid flat on a wire rack with support fencing (Rogers et al., 2005) in a BSC III, and contaminated with a suspension of approximately  $7.0 \times 10^7$  CFU of *F. tularensis* Schu S4. This inoculation consisted of placing two rows of five droplets (10 µL/droplet) using a multi-channel micropipette (Figure 1). The droplets were allowed to dry for one hour prior to decontamination. For each test material, three inoculated coupons and one blank (not inoculated) were used for decontamination. In parallel, two sets of inoculated coupons (N=3/material) and blanks (N=1/material) were used as controls. The first set was used to control for any potential decrease in microorganism viability during the one-hour drying time. The second set of control coupons were maintained in a separate, isolated BSC III to control for any potential decrease in microorganism viability during the entire three-hour decontamination run.

Biological indicators (BI) containing *Bacillus subtilis* (ATCC 19659) and *Geobacillus stearothermophilus* (ATCC 12980) spores on stainless steel disks sealed in Tyvek pouches (Apex Laboratories, Inc., Apex, North Carolina) and spore strips (SS) containing *B. atrophaeus* (ATCC 9372) spores on filter paper strips sealed in glassine envelopes (Raven Biological Laboratories, Omaha, NE) were also used to evaluate decontamination as previously described (Rogers et al., 2005; Rogers et al., 2007). These BI and SS contained approximately  $1 \times 10^6$  spores per indicator. Three of each BI and SS were subjected to hydrogen peroxide fumigation and three of each BI and SS not subjected to decontamination were used as positive controls.

The BSC III used for the decontamination run was approximately 128 ft<sup>3</sup> (approximately 3,625 L). An Amsco VHP® Generator Series 1000 (STERIS Corporation, Mentor, Ohio) was connected to the BSC Class III cabinet and used for the hydrogen peroxide fumigation. The decontamination process consisted of dehumidification (four minutes), conditioning (20 minutes), sterilization (120 minutes), and aeration (30 minutes) phases, which were controlled by the hydrogen peroxide generator. The

injection rates during the conditioning and sterilization phases were set at 3.0 g/L and 3.1 g/L, respectively. Prior to initiating the decontamination run, the temperature and relative humidity inside the BSC III were measured using a NIST-traceable thermometer/hygrometer (VWR International, Inc.).

### Sample Processing

Following the one-hour drying time, one set of control coupons (inoculated and blank coupons for each material type) were placed into 50 mL conical tubes containing 10 mL of Mueller-Hinton broth containing 0.025% iron pyrophosphate, 0.1% glucose, and 2% IsoVitaleX™. At the end of the decontamination run, the second set of control and decontaminated coupons inoculated with *F. tularensis* (and corresponding blanks) were placed into 50 mL conical tubes containing 10 mL of Mueller-Hinton broth supplemented with 0.025% iron pyrophosphate, 0.1% glucose, and 2% IsoVitaleX™. The BI and SS were placed into 50 mL conical tubes containing 10 mL of tryptic soy broth (Remel, Lexena, Kansas). All tubes containing coupons were gently agitated and incubated at 37°C for *F. tularensis*, *B. subtilis*, and *B. atrophaeus*, and at 55-60°C for *G. stearothermophilus*. The cultures were visually inspected for growth at one and four days of incubation. This one- to four-day time frame was chosen based on previous qualitative growth studies evaluating the efficacy of antimicrobial agents against *F. tularensis* (Baker et al., 1985; Brown et al., 2004).

## Results

The decontamination run lasted approximately three hours from start to finish; no physical damage was observed for the hydrogen peroxide-exposed coupons upon visual inspection. The starting temperature and relative humidity were approximately 20°C and 40%, respectively. For all coupon materials, the one-hour drying controls and unexposed decontamination controls exhibited positive growth at one and four days (Table 1). VHP fumigation of inoculated coupons in the 3,625 L BSC Class III resulted in the complete inactivation of *F. tularensis* as demonstrated by the lack of viable growth in liquid cultures at one and four days (Table 1). All control and decontamination blanks were negative for growth (Table 1).

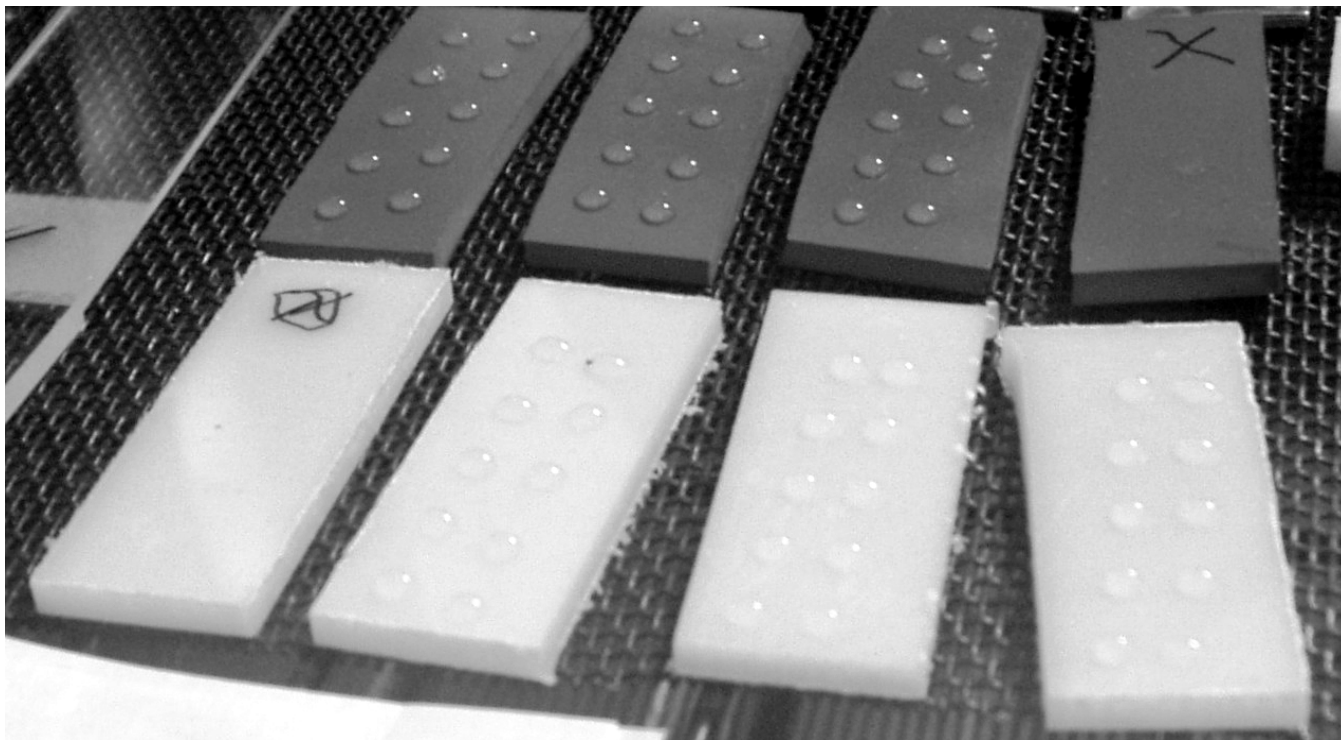
For all BI and SS exposed to VHP, no growth was observed as determined by the lack of visibly cloudy liquid cultures at one and four days post-exposure (Table 2). All of the unexposed BI and SS exhibited growth as determined by the presence of visibly cloudy liquid cultures at both one and four days (Table 2).

## Discussion

As a fumigant, hydrogen peroxide exhibits decontamination efficacy against a wide range of microorganisms (Klapes & Vesley, 1990; Heckert et al., 1997; French et al., 2004; Johnston et al., 2005; Rogers et al., 2005). Hydrogen peroxide is considered less toxic than other

### Figure 1

Representative inoculation of the test coupons with a suspension (10 µL drops) of *Francisella tularensis* Schu S4.



fumigants such as chlorine dioxide, ethylene oxide, methyl bromide, and formaldehyde, and is neutralized by catalytic breakdown into water and oxygen. Moreover, hydrogen peroxide fumigation does not appear to damage materials or sensitive equipment (French et al., 2004; Rogers et al., 2005). Therefore, hydrogen peroxide fumigation has been used for decontaminating laboratories and laboratory equipment (Klapes & Vesley, 1990; Kahner et al., 2005; Hall et al., 2007), medically-implanted wires (Fichet et al., 2004) and medical equipment

(Andersen et al., 2006), hospital rooms (French et al., 2004), biological safety cabinets (Hillman, 2004), and animal holding rooms (Krause et al., 2001).

The present study demonstrates that *F. tularensis* Schu S4 is inactivated on various material surfaces when exposed to VHP fumigation in a BSC III. This fumigation did not promote observable physical damage to the test material surfaces, which is similar to previous observations (Rogers et al., 2005). In parallel, BI containing *B. subtilis* and *G. stearothermophilus* spores and *B. atrophaeus*

**Table 1**

Decontamination efficacy of *Francisella tularensis* Schu S4 following hydrogen peroxide fumigation on seven different test materials (N=3/material).

Treatment/Test Material	No. Tested (No. Positive)	
	Day 1	Day 4
<b>Hydrogen Peroxide Fumigation<sup>a</sup></b>		
Acrylic	3 (0)	3 (0)
Glass	3 (0)	3 (0)
Polyamide	3 (0)	3 (0)
Polyethylene	3 (0)	3 (0)
Polypropylene	3 (0)	3 (0)
Silicone Rubber	3 (0)	3 (0)
Stainless Steel	3 (0)	3 (0)
<b>No Fumigation (1 hr drying controls)<sup>a</sup></b>		
Acrylic	3 (3)	3 (3)
Glass	3 (3)	3 (3)
Polyamide	3 (3)	3 (3)
Polyethylene	3 (3)	3 (3)
Polypropylene	3 (3)	3 (3)
Silicone Rubber	3 (3)	3 (3)
Stainless Steel	3 (3)	3 (3)
<b>No Fumigation (decontamination controls)<sup>a</sup></b>		
Acrylic	3 (3)	3 (3)
Glass	3 (3)	3 (3)
Polyamide	3 (3)	3 (3)
Polyethylene	3 (3)	3 (3)
Polypropylene	3 (3)	3 (3)
Silicone Rubber	3 (3)	3 (3)
Stainless Steel	3 (3)	3 (3)

<sup>a</sup>All blanks (not inoculated) for each material were negative for growth.

**Table 2**

Decontamination efficacy of biological indicators and spore strips following hydrogen peroxide fumigation.

Treatment/Test Material	No. Tested (No. Positive)	
	Day 1	Day 4
<b>Hydrogen Peroxide Fumigation</b>		
<i>B. subtilis</i> ATCC 19659 Biological Indicator	3 (0)	3 (0)
<i>G. stearothermophilus</i> ATCC 12980 Biological Indicator	3 (0)	3 (0)
<i>B. atrophaeus</i> ATCC 9372 Spore Strip	3 (0)	3 (0)
<b>No Fumigation (decontamination controls)</b>		
<i>B. subtilis</i> ATCC 19659 Biological Indicator	3 (3)	3 (3)
<i>G. stearothermophilus</i> ATCC 12980 Biological Indicator	3 (3)	3 (3)
<i>B. atrophaeus</i> ATCC 9372 Spore Strip	3 (3)	3 (3)

SS were also completely inactivated by VHP fumigation. The growth assessments of various BI and SS are often used to qualitatively evaluate decontamination performance (Heckert et al., 1997; Sigwarth & Stark, 2003; French et al., 2004; Hillman, 2004; Johnston et al., 2005; Rogers et al., 2005; Rogers et al., 2007). In the present study, this growth assessment demonstrated that both BI and SS exposed to VHP fumigation were completely inactivated as demonstrated by no observed growth in the liquid cultures at one and four days. These results suggest the VHP fumigation of the BSC III inactivated bacterial spores to the level of at least 6-logs, supporting the use of BI or SS during hydrogen peroxide fumigation of *F. tularensis* contaminated materials to qualify decontamination runs.

In this study, the approach used to evaluate the effectiveness of VHP fumigation against *F. tularensis* was a qualitative growth/no growth assessment. Such evaluations do not provide quantitative values, such as a log reduction that can be useful in assessing the degree of decontamination, or enabling statistical comparisons. However, this qualitative assessment was performed for two reasons. First, *F. tularensis* Schu S4 can persist in the environment for extended periods of time (Anda et al., 2001; Feldman et al., 2001; Abd et al., 2003; Feldman, 2003), and the median intracutaneous and respiratory infectious doses are less than 10 cells (Saslaw & Carlisle, 1969), making it important to completely inactivate *F. tularensis* by decontamination. The persistence on material surfaces was demonstrated previously where approximately  $1 \times 10^7$  *F. tularensis* cells deposited on metal surfaces remained viable for days, depending on the relative humidity, with the fastest decline of 4-logs observed at one and a half days under 100% relative humidity (Wilkinson, 1966). Consistent with previous observations, our results showed that viable *F. tularensis* remained on the control coupons after the one-hour drying time as well as during the three-hour decontamination run. Related to persistence, the aging of aerosols at 24°C under 85% relative humidity affects the infectivity of *F. tularensis* Schu S4 in humans and non-human primates where low inhaled bacterial concentrations (150-750 viable cells) aged for 60 minutes exhibited 75% infectivity of exposed humans, while no humans were infected when the bacteria were aged 120 minutes or longer (Sawyer et al., 1966). However, *F. tularensis* at concentrations of 7,500-50,000 viable cells were infectious when aged for up to 180 minutes (Sawyer et al., 1966). Therefore, based on the worst case scenario of a 4-log decrease within one and a half days (Wilkinson et al., 1966), it is possible that with a starting inoculum of approximately  $1 \times 10^8$  CFU *F. tularensis* in the present study, there may be at least 10,000 viable CFU remaining on the contaminated surfaces, which could potentially serve as a biological hazard requiring decontamination. However, this is only an assumption based on published inactivation rates, and fur-

ther studies would be needed to quantitatively determine any loss in titer of *F. tularensis* Schu S4 on the test materials after drying for one hour.

Secondly, qualitative growth/no growth assessment can potentially provide a better estimate of complete inactivation compared to test material extraction and dilution plating. The extraction and plating procedures have been used previously to provide quantitative log-reduction values for decontaminating *B. anthracis* and surrogate spores using fumigants (Rogers et al., 2005; Rogers et al., 2007). This extraction and dilution plating approach typically yields less than 100% recovery of microorganisms from test material coupons, which is dependent on material porosity and complexity. Thus, the data derived from this type of analysis often provides a conservative estimate of microorganism decontamination at least to the level of recoverable, detectable microorganisms in the unexposed controls. However, this approach in log reduction determinations may be suitable for discerning effective decontamination of organisms such as *B. anthracis* where the estimated LD<sub>50</sub> for pulmonary anthrax in humans is at least 8,000 spores (Inglesby et al., 2002). This is much higher than the reported median infectious and lethal dose concentrations of *F. tularensis* in humans (Saslaw & Carlisle, 1969). Complete inactivation (i.e., no growth) of *F. tularensis* may be necessary for effective decontamination remediation since such a requirement is the current criterion for remediation of a *B. anthracis*-contaminated site (Canter, 2005).

This study provides information for using hydrogen peroxide fumigation as an alternative approach to the current accepted method of 10% household bleach followed by 70% alcohol for decontamination of virulent *F. tularensis*. In this study, there were additional variables not evaluated that include other test material types such as porous surfaces, effects of relative humidity and temperature, and various methods of applying *F. tularensis* to test materials. Further studies are needed to address these parameters with respect to hydrogen peroxide decontamination of virulent *F. tularensis*.

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