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Assessment of the risk of infectious aerosols leaking to the environment from BSL-3 laboratory HEPA air filtration systems using model bacterial aerosols

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To assess the risk of infectious bacterial aerosols leaking to the environment, the filtration efficiency of a biosafety level 3 (BSL-3) laboratory high-efficiency particulate (HEPA) filter was investigated using the aerosolized bacteria Serratia marcescens. The aerosol size was measured using an Andersen sampler. Eight first stage HEPA filters (numbered 1–8) were distributed in contaminated labs and exhausts from each of the first stage HEPA filters were aggregated and filtered through one second stage HEPA filter before being released to the environment. In total, 8 first-stage and 1 second-stage HEPA filters from the BSL-3 air purification system were analyzed. No S. marcescens was detected in first-stage filters 1, 2, 4, 5, 7 and 8 and the second-stage HEPA filter. The filtration efficiencies against aerosolized S. marcescens were >99.9999%. First stage filter numbers 3 and 6 had filtration efficiencies of 99.9825% and 99.9906%, respectively. When filter number 3 was replaced by a new filter and the bracket for filter number 6 was sealed, no aerosolized S. marcescens was detected in the filtered air. Our work suggests that the BSL-3 laboratory HEPA filter air purification system is effective against bacterial aerosols, with little to no bacterial leakage into the environment.

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1. Introduction

Emerging and re-emerging infectious diseases have recently attracted substantial scientific and governmental attention. Multi-drug resistant tuberculosis (MDR-TB), severe acute respiratory syndrome (SARS), H5N1 avian influenza, H1N1 influenza and many other emerging diseases have either proven fatal or caused international alarm. Following the 2002–2003 SARS outbreak, the concept of aerosol transmission has been widely accepted (Tang, Li, Eames, Chan, & Ridgway, 2006). Infectious diseases remain the major causes of morbidity and mortality in China, despite substantial progress in their control and treatment (Wang et al., 2008). China is significantly impacted by emerging infectious diseases because of its population size and density. The public health system must be prepared to handle the challenges presented by new infectious diseases, while at the same time controlling existing infectious diseases. One of the most critical roadblocks to developing biocontainment countermeasures is a lack of specialized research resources, in particular, specialized biocontainment laboratories. To address infectious microbial threats, the government has committed resources to implement new research strategies, including the development of biosafety level 3 (BSL-3) laboratories. BSL-3 laboratories are designed to work with risk group 3 microorganisms in large volumes or high concentrations of risk group 2 microorganisms that pose a high risk of aerosol transmission (WHO, 2004). The general public often opposes high level biosafety laboratories because of the potential for leakage of infectious microorganisms to the environment (Kaiser, 2004; Nisbet, 2007). High-efficiency particulate (HEPA) air filtration systems are used to reduce the airborne release of microbial aerosols. HEPA filters are usually tested using physical particles as challenge aerosols (ASTM, 2009), even though their use is frequently to reduce exposure to biological particles. Attempts to evaluate the performance of respiratory protective equipment against biological particles have typically been performed using airborne bacteria and viruses (Balazy et al., 2006; Brosseau, McCullough, & Vesey, 1997; Eninger et al., 2008; McCullough, Brosseau, & Vesey, 1997; Qian, Willeke, Grinspun, Donnelly, & Coffey, 1998). Studies on respiratory protective equipment against airborne biological agents have recently been reviewed by Rengasamy, Zhuang, and BerryAnn (2004). There is, however, a lack of direct measurements on the efficiency of BSL-3 laboratory HEPA filtration systems against aerosolized biological particles. The BSL-3 laboratory in this study was constructed for group 3 bacteria such as Mycobacterium tuberculosis. To ensure there is no environmental leak risk, the performance of the BSL-3...
laboratory HEPA air filtration system was studied using microbiological aerosol methodology. The bacterial aerosol was generated in the BSL-3 lab containment area and then sampled before and after the testing filters when the BSL-3 lab was operated under normal working conditions. This methodology could be applied anywhere to determine the efficacy of BSL-3 laboratory HEPA filtration systems for reducing airborne contamination by airborne bacteria and evaluate bioaerosol leakage risks for the environment. This is the first attempt to evaluate the HEPA filtration system of BSL-3 lab against airborne bacteria aerosol under normal working conditions.

2. Materials and methods

2.1. Test organism

*Serratia marcescens* (0.7 μm × 1.0 μm) (8039, stored at the Beijing Institute of Microbiology and Epidemiology) is one of the smallest bacteria (Fig. 1) and produces a distinct red colony on nutrient agar plates. It was used as a substitute for high risk bacteria. After incubation, the red colonies of *S. marcescens* could be discriminated from other microorganisms (Fig. 2). *S. marcescens* was aseptically inoculated in a flask using 150 mL of nutrient broth (10 g peptone, 3 g beef extract, 5 g NaCl and 1 L deionized water at a pH of 7.3 ± 0.2) (HuanKai Microbial Sci. & Tech Co., Ltd, Guangdong, China) and then incubated for 24 h at 30 °C with shaking (160 rpm). The culture was centrifuged at 9000 g for 10 min (Sigma 3–18K, Gottingen, Germany) and the sediment was washed three times and then suspended using phosphate buffered saline (PBS). The actual concentration of *S. marcescens* in this preparation was determined by preparing decimal dilutions of the sample and injecting 100 μL of each dilution into normal nutrient agar (10 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar and 1 L deionized water at a pH of 7.3 ± 0.2). After incubation at 30 °C for 24–48 h, the colonies were counted and the concentrations expressed as colony-forming units (cfu) per mL. Fresh preparations were made for each series of tests.

2.2. Personal protective equipment

When performing the bacterial aerosol research, workers were equipped with bioaerosol protection clothing (Shandong Tores-Tex stock Co., Ltd., China), N99 respirators (Firmshield Biotechnology Co., Ltd., China) that demonstrated excellent protection against biological aerosols (Wen et al., 2010), protective goggles (3M Company), protective gloves (Beijing Reagent Latex Products Co., Ltd., China), and protective shoes (Shanghai JWFu Industry Co., Ltd., China).

2.3. Selection of *S. marcescens* aerosolization media

To select the most appropriate *S. marcescens* aerosolization medium, nutrient broth (NB), PBS, and normal saline (NS, 0.85% NaCl water solution) were selected as the nebulization media. A DV40 nebulizer (Qindao Zhongrui Intelligent Instruments Co., Ltd., China) was filled with 30 mL *S. marcescens* in different media. The operating principle of the DV40 nebulizer is similar to that of the collision nebulizer where compressed air shears liquid into droplets. The larger size fraction of droplets was removed and the smaller fraction was ejected by the nebulizer. *S. marcescens* was aerosolized by applying clean compressed air using a GAST pump (GAST Manufacturing Inc., MI, USA) to the nebulizer at the flow rate 101/min and the aerosolization time was 5 min. Before and after aerosolization the concentration of *S. marcescens* was determined using the serial dilution culture method described in Section 2.1. Each media was tested three times and the best aerosolization media was selected. The results from different aerosolization media were analyzed using SPSS 11.0 and p < 0.05 was considered statistically significant. The media that showed the highest survival of *S. marcescens* was chosen as the aerosolization media.

2.4. Survival of *S. marcescens* under different nebulization times

PBS was selected as the nebulization media for the DV40 nebulizer and the nebulizer conditions were the same as described in Section 2.3. The nebulization time was 0, 5, 10, 15, and 30 min. During each time period the concentration of *S. marcescens* suspension was determined using the serial dilution culture method as described in Section 2.1. These tests were performed three separate times and the survival of *S. marcescens* in PBS media under different nebulization times was determined.

2.5. Selection of AGI-30 sampler collection media

An AGI-30 sampler (Qindao Zhongrui Intelligent Instruments Co., Ltd., China) was used to collect the bacterial aerosols before the HEPA filters in the BSL-3 lab. NB, PBS, and NS were selected as potential collection media for the AGI-30 sampler. *S. marcescens* and different media were added to the AGI-30 sampler and after being impinged for 10 min at a flow rate of 12.5L/min by the AGI-30 sampler, the concentration of the *S. marcescens* suspension was

**Fig. 1.** Picture of *S. marcescens* by transmission electron microscopy.

**Fig. 2.** Colony of *S. marcescens* after incubation.
detected using the serial dilution culture method described in Section 2.1. Each collection media was used three times and the best collection media was determined. The results from different collection media were analyzed using SPSS 11.0 and p < 0.05 was considered statistically significant. The media with the highest survival of S. marcescens after impingement was chosen as the collection media.

2.6. Survival of S. marcescens under different impingement times

PBS was selected as the S. marcescens aerosol collection media used by the AGI-30 sampler. The sampler was filled with 20 mL of S. marcescens PBS suspension and the AGI-30 sampler was operated at a flow rate of 12.5 L/min in a biological safety cabinet. The impingement times were set to 0, 5, 10, and 15 min. After each time period the concentration of S. marcescens was determined using the serial dilution culture method described in Section 2.1. These tests were performed three times and the survival of S. marcescens in PBS media under different impingement times was determined.

2.7. Quantity of aerosolized S. marcescens

The DV40 nebulizer was filled with 30 mL of S. marcescens PBS suspension. S. marcescens was aerosolized by applying compressed air using a GAST pump to the nebulizer at 10 L/min. The aerosolization time was 10 min and the volume of S. marcescens PBS suspension was measured. This was performed three times and the quantity of aerosolized S. marcescens under the respective conditions was determined.

2.8. Size of S. marcescens aerosol particles

A six stage Andersen cascade impactor (Kangjie Instruments, China) was used to determine the size of S. marcescens aerosol particles. The sampler was operated at 28.3 L/min for 1 min in an aerosol chamber after S. marcescens aerosol was generated for 5 min. The plates were removed from the sampler and incubated at 30 °C for 24–48 h and the colonies on the plate were counted. The number of colonies on agar plates from each impactor stage were revised (Andersen, 1958) and the size of particles collected were determined from the presence of growth on each of the plates used (Wen et al., 2009).

2.9. BSL-3 laboratory air filtration system

The BSL-3 laboratory HEPA filtration systems had two stage HEPA filters. The first stage HEPA filters were inside the BSL-3 laboratory and were distributed in each contaminated lab as showed in Fig. 3. Labs 1–3 were potentially contaminated areas and lab 4 was a preparation lab. Under normal operation, the negative pressures of the contaminated labs, preparation lab, and clean areas were approximately −50, −25, and −10 Pa, respectively, and the BSL-3 lab was maintained at 25 °C and 50% relative humidity. The HEPA filters (numbers 1–8) were the first stage of airborne contamination control filters in the BSL-3 laboratory. Exhaust air through the first stage HEPA filters was aggregated together and filtered through one second stage HEPA filter before being exhausted outside. The two stage HEPA filter system was designed to avoid the escape of microbial aerosols into the environment. The HEPA filters were tested by smog testing before installation and after installation the HEPA filter system was tested using model bacterial aerosol.

2.10. HEPA filter testing method against bacterial aerosol

Prior to testing the BSL-3 lab was in normal operation as described in Section 2.9 for at least 1 h. The test rig is shown schematically in Fig. 4. Before testing, the air in the BSL-3 lab and exhaust from the HEPA filters were collected by the Andersen samplers as control samples. The sampler flow was 28.3 L/min and the collection time was 10 min. If no S. marcescens was found on the control agar plates then the testing began. The DV40 nebulizer was filled with 30 mL of S. marcescens suspension. The test bacterial aerosol was aerosolized by applying clean compressed air using a GAST pump to the nebulizer at 10 L/min. The DV40 nebulizer position was 80 cm before the filter and the height was 100 cm from the lab floor. The aerosolized microbes were generated before the first stage HEPA filter in the BSL-3 lab for 10 min. At the upstream and downstream sides of the first stage HEPA filter and the second stage HEPA filter, microbiological air samplers were used to sample the air to detect the model microorganism aerosol concentration. Before the first HEPA filter, an AGI-30 sampler was used at a central position 10 cm before the filter, with a collection flow of 12.5 L/min and time of 10 min. In a previous study, a Merck Mas-100 sampler (Merck, Germany) had a collection efficiency that was higher than the AGI-30 air sampler when the concentration of the microorganisms was low (Yang et al., 2009). Therefore, a Merck Mas-100 air sampler was used to collect air after the first and second stage HEPA filters at a central position 30 cm behind the filter and a flow...
rate of 100 L/min. The sampling time was set at 10 min because the prolonged nature of the tests may have caused excessive drying of the agar and loss of microbial viability. Each filter was tested three times. After testing the equipment and the air of the BSL-3 lab were disinfected using aerosolized 1.5% hydrogen peroxide.

The collected samples were incubated at 30 °C for 24–48 h and the red colonies of S. marcescens on the collecting agar plates were counted and their concentrations converted to cfu/m³. By taking pre- and post-HEPA filter air samples with a sampling device, this method allowed the simultaneous measurement of bacterial aerosol concentrations before and after filtration. The percentage efficiency of the HEPA filter system was calculated using Eq. (1), where A was the concentration of bacterial aerosol challenging the HEPA filter system and B was the concentration after filtration:

\[
\text{Filtration efficiency} (\%) = \frac{A - B}{A} \times 100. \tag{1}
\]

The Merck Mas-100 sampler flow rate was 100 L/min and the sampling time was 10 min, so the testing limit was 0.001 cfu/L (1 cfu/m³). The AGI-30 sampler testing limit was 0.008 cfu/L (8 cfu/m³).

3. Results

3.1. Selection of S. marcescens aerosolization media

The survival of S. marcescens in different aerosolization media was: 90.1 ± 1.31% in NB, 96.8 ± 1.83% in PBS and 89.9 ± 2.23% in NS. PBS was identified as the best aerosolization media for the DV40 nebulizer used in this study and was selected to be the aerosolization media.

3.2. Survival of S. marcescens under different nebulization times

The survival of S. marcescens under different nebulization times is shown in Fig. 5. The survival of S. marcescens after nebulization for 0, 5, 10, 15, and 30 min was 100%, 97.3 ± 0.06%, 95.6 ± 0.85%, 92.5 ± 1.26% and 88.9 ± 0.98%, respectively. In our testing method, S. marcescens was nebulized for 20 min and the survival after that time was approximately 90%.

3.3. Selection of AGI-30 sampler collection media

Survival in the AGI-30 sampler after impingement for 10 min was: 91.1 ± 0.67% in NB, 96.4 ± 0.87% in PBS and 90.7 ± 3.74% in NS. PBS was identified to be the best AGI-30 sampler collection media and was chosen to be the collection media for the study.

3.4. Survival of S. marcescens under different impingement times

The survival results under different impingement times are shown in Fig. 6. The survival of S. marcescens after impingement for 5, 10, and 15 min were 97.6 ± 0.21%, 95.6 ± 0.06% and 90.5 ± 1.15%, respectively. An impingement time of 10 min was used for the study.

3.5. Quantity of aerosolized S. marcescens

The DV40 nebulizer produced 250 ± 10 μL of S. marcescens suspension per minute when the flow rate was 10 L/min. In our testing method, the nebulizer generated S. marcescens aerosol for 20 min and the quantity of aerosolized S. marcescens was approximately 5 mL.

3.6. Particle size of S. marcescens aerosol

The particle size distribution of nebulized S. marcescens aerosol was: 0.6–1.0 μm (19%), 1.1–2.0 μm (46%), 2.1–3.3 μm (24%), 3.4–4.7 μm (8%), 4.8–7.0 μm (2%), and >7.0 μm (1%). The count median diameter (CMD) of S. marcescens aerosol was 1.77 μm.

3.7. Efficiency of first stage HEPA filters

A total of 8 first stage HEPA filters were tested. The filtration efficiencies of the first stage HEPA filters against S. marcescens aerosol are provided in Table 1. No S. marcescens aerosol was detected behind the 1, 2, 4, 5, 7, and 8 HEPA filters and the filtration efficiencies were all >99.9999%. The filtration efficiency of HEPA filters 3 and 6 were 99.9825 ± 0.0092% and 99.9906 ± 0.0024%, respectively. After replacing filter 3 and airproofing the brackets of filter

| Table 1 | Filtration efficiency of the first stage HEPA filter of BSL-3 filtration system. |
|---------|--------------------------------------------------------------------------------|
| Number of HEPA filter | Challenge concentration of bacterial aerosol (cfu/L) | Concentration of bacterial aerosol after the first HEPA filters (cfu/L) | Filtration efficiency of the first HEPA filter (%) |
| 1 | 4099 ± 580 | <0.002 | >99.9999 |
| 2 | 5207 ± 841 | <0.002 | >99.9999 |
| 3* | 3651 ± 695 | 0.6 ± 0.2 | 99.9825 ± 0.0092 |
| 4 | 5324 ± 716 | <0.002 | >99.9999 |
| 5 | 2376 ± 541 | <0.002 | >99.9999 |
| 6* | 3171 ± 434 | 0.3 ± 0.1 | 99.9906 ± 0.0024 |
| 7 | 3600 ± 538 | <0.002 | >99.9999 |
| 8 | 2280 ± 245 | <0.002 | >99.9999 |

* After replaced number 3 filter and airproofed number 6 filter fixed bracket no S. marcescens was detected in the filtration air, the filtration efficiency were >99.9999%.
6, no S. marcescens was detected in the filtered air and the filtration efficiencies were >99.9999%.

3.8. Efficiency of second stage HEPA filter

S. marcescens aerosol was not detected after filtering through the second stage HEPA filter during each test and the filtration efficiency was >99.9999%.

4. Discussion

There is constant concern about the health and safety of individuals and the environment being exposed to pathogenic microorganisms. Along with safe microbiological techniques, primary barriers (safety equipment and personal protective equipment) minimize the occupational exposure of laboratory workers and limit the transmission of microorganisms from these workers to others. Secondary barriers (facility safeguards) provide supplementary microbiological containment, serving mainly to prevent the escape of infectious agents into the environment when a failure in the primary barriers occurs (Kimman, Smit, & Klein, 2008). Air filtration systems with HEPA filters are one of the most important secondary barriers to prevent the escape of infectious agents from a BSL-3 lab to the environment. The adverse health effects of biological particles, particularly their pathogenicity, do not depend on the mass of the inhaled particles, but on the number of particles inhaled. Traditional vaccines may not provide adequate protection to humans when infection occurs via inhalation (Ivinis et al., 1995; Reed & Martinez, 2006). For example, inhalation of one Coxiella burnetii bacterium can induce Q fever (Tigertt, Benenson, & Gochenour, 1961). Studies evaluating protection efficiencies of BSL-3 lab HEPA filtration system using biological aerosols are scarce, highlighting a significant gap in current knowledge.

The HEPA filtration system of a specially designed BSL-3 laboratory for the autopsies of patients with SARS was evaluated using a physical particle simulant test (Li et al., 2005). Air filter sets were tested for their efficiency in protecting laboratory animals against potential airborne infections (Mrozek et al., 1994). There have been no reports evaluating the performance of air filtration systems in BSL-3 labs using the deliberate release of model microorganisms in an actual working laboratory setting. Because of the unique aerosol properties of bioaerosols, special equipment used for protection against the aerosol transmission of diseases was required to undergo bioaerosol protection testing (Rengasamy et al., 2004). Because of aerosol safety issues involved with the generation of high microbial aerosol concentrations, the method uses non-pathogenic microorganisms. In this study, a bacterial model (S. marcescens) was used to test the efficiency of an air purification system in a BSL-3 lab. S. marcescens has been used previously as model bacterial aerosol because of its high aerosol stability and low pathogenicity (Furuhashi & Miyamae, 1981; Heidelberg et al., 1997; Ko & Burge, 2007; Ko, First, & Burge, 2000; Lai, Burge, & First, 2004; Patel & Nou, 2008). The BSL-3 lab studied was used to do bacterial research, so S. marcescens was an excellent candidate as a bacterial model organism. In the future, a viral model aerosol should also be used to investigate the risk associated with performing viral research.

The aerosolization and collection processes can inactive or kill some microorganisms. Microorganisms are subjected to numerous stresses during the aerosol generation and collection processes within a device (Liu et al., 2012). Maintaining their integrity and activity depends on the nature of the nebulizers, samplers, and media. In this study, S. marcescens aerosol generation and collection methods were studied. The aerosol particles size was also measured. In this study, the CMD of S. marcescens aerosol was 1.77 μm. Because a dryer was not used, water aerosolized by the DV40 nebulizer was not evaporated, increasing the number of larger particles that can carry single bacteria or bacterial agglomerates. S. marcescens aerosol was deliberately released into potentially contaminated areas when the BSL-3 lab was in normal operation. This test method not only evaluated the HEPA filters, but also the installation parameters of the filters, such as airproofing of the fixed filter bracket, airproofing glue, the fixed screw, and whether the HEPA filter was damaged during the installation. In our study, filters 3 and 6 showed filtration efficiencies against S. marcescens of 99.9825% and 99.9906%. Only HEPA filter 3 was leaking from the filter itself, as determined by aerosol testing after it was removed from the filtration system. It may have been damaged during the transport and the installation processes. Filter 6 was determined to be intact by aerosol testing and it was concluded that S. marcescens aerosol was leaked through other routes. After the fixed filter bracket was airproofed using airproofing glue, there was no S. marcescens aerosol detected, so indicating that the aerosol was leaking from the fixed bracket. There was no S. marcescens aerosol detected in the second stage HEPA filters.

The evaluation of the effectiveness of biosafety measures has not been extensively studied and does not result in a general consensus. Kimman et al. (2008) recommended developing evidence-based practices and criteria to evaluate effectiveness wherever this is possible and feasible. This may optimize, and perhaps, simplify future biosafety measures and stimulate compliance with applicable regulations. In this paper we studied a testing method for evaluating the efficiency of air filtration systems in a BSL-3 lab using the deliberate release of bacterial aerosol in a working laboratory setting. The method included a substitute bacteria, bacterial aerosol nebulizer, samplers, aerosolized bacteria, and collection media. In the BSL-3 lab, the S. marcescens aerosol concentration was >10^3 cfu/L and mimicked a seriously contaminated status and no S. marcescens was detected in the exhaust air. The air filtration system of this BSL-3 lab had excellent filtration efficiency against bacterial aerosol.

5. Conclusions

The BSL-3 laboratory HEPA filter air purification system was found to be effective against bacterial aerosol with several logs of reduction and no bacterial aerosol leakage to the environment. The developed aerosol test method, including the optimal aerosol generation time, nebulization, and collection liquid, can be used to evaluate the HEPA filter air purification system of a BSL-3 lab filtration efficiency against bacterial aerosol in a working laboratory setting. The results obtained from this study can provide guidance in assessing the environmental biosafety of a BSL-3 laboratory.

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