博士論文（要約）

Study on development of test method to evaluate correctly the air cleaning performance against airborne microbes in indoor environment

（室内環境における浮遊微生物に対する空気清浄性能を正しく評価するための試験法開発の研究）

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Abstract

Recent studies have investigated the efficiency of air-cleaning products in reducing the concentration of pathogenic aerosols in indoor environment. A standard method for evaluating the reduction in airborne viruses using air-cleaning products has been developed using a safe bacteriophage instead of pathogenic viruses. The reduction in airborne viruses is determined by counting the number of viable airborne phages in a culture after the air-cleaning product is used.

Some reports suggest that air-cleaning products are not effective in reducing the number of viable pathogens in aerosols. Therefore, further research is required to accurately evaluate the performance of these products based on a mechanistic understanding of pathogen reduction when they are used. The purpose of this study is to improve current standardized methods and to improve our understanding of the reduction mechanisms of airborne microbes, especially viruses. An evaluation method is proposed that correlates the performance of a safe bacteriophage against real pathogens.

This study reached the following conclusions:

(1) Currently available standardized tests are not capable of identifying mechanisms to physically decrease or inactivate pathogenic aerosols when reducing viable microbial levels. Thus, a polymerase chain reaction (PCR) was used to quantify the number of phi-X174-specific particles in the aerosol. A behavior analysis using this quantitative PCR method and a culture assay identified a humidity range within which viable airborne phages were inactivated. This observation may help to identify mechanisms that

differentiate between the physical decrease and inactivation of viable airborne phages.

(2) The physical decrease in the viable airborne phage is primarily caused by an air filter. To compare the filter efficiency of the filter for the phage with that of a real pathogen, such as the influenza virus, a safe method was developed. It involved quantitating an inactivated influenza virus aerosol with an antigen-capture enzyme-linked immunosorbent assay (ELISA). Using this ELISA, the filter efficiencies for aerosols of three types of spherical microbes (phi-X174 phages:28nm, an inactivated influenza virus:120nm, and *Staphylococcus aureus*:0.7 μ m) were tested. These results indicated that the filter efficiency was lowest for the inactivated influenza virus aerosol and highest for the phi-X174 phage. This suggests that the standard test for evaluating the reduction of airborne viruses using phi-X174 phage may overestimate the performance of filters compared to the filter efficiency for real pathogens such as the influenza virus. It is recommended that the standard test be updated to use inactivated influenza virus aerosols to improve the accuracy of performance evaluations of air-cleaning products.

(3) To verify whether the phage is surrogate virus when inactivating airborne viruses captured with a filter, the inactivation efficiencies of some pathogens were compared with that of the phage by heat treatment, UV irradiation, or ethanol treatment. It was found that the phage was more resistant to inactivation than the real pathogen. The inactivation efficiencies of each method were also found to vary with different pathogen strains. This suggests that it is important to carefully evaluate the inactivation efficiency of each method, so as to select an appropriate virus strain as the inactivated

pathogen for the standard inactivation efficiency test. It needs to be sufficiently resistant to the inactivation treatment.

(4) To improve the dry dispersion technique of airborne fungal spores in the current standard test, a new device was developed. An aerial ultrasonic oscillator is used to disperse the dry fungal spores in the current standard test; however, the aerial ultrasonic technique requires the use of skilled operators for accurate evaluations. A new device using glass beads for dry dispersion was successfully trialed in this study as a more versatile apparatus for use with the compact test chamber. Therefore, this newly developed technique may present an improved method for generating dry airborne fungal spores to use in the current standard performance test for air-cleaning products.

This study will help to accurately evaluate the performance of air-cleaning products against microbial aerosols and will contribute to a cleaner and safer indoor environment.

Contents

Note: This is an abridged version of my doctorate thesis. Chapter 3, 5, 6, and 7 are under unavoidable circumstances that web-based publication of doctorate thesis is impossible. The manuscripts related to chapter 3, 5 were submitted to a journal "Biocontrol Science" and will be expected to be published within two year. The papers related to chapter 6, 7 were not able to be agreed with web-based publication of doctorate thesis by all co-authors because some co-authors passed away. Therefore, chapter 3, 5, 6, and 7 are abridged with article information.

Chapter 1

Introduction

Respiratory diseases are serious problems worldwide. For instance, cases of the highly pathogenic avian influenza (Claas et al., 1998), severe acute respiratory syndrome (Ksiazek et al., 2003), Middle East respiratory syndrome (Bermingham et al., 2012), and human avian influenza A (H7N9) infection (Liu et al., 2013) have been reported recently. Pathogen aerosols have a high risk of spreading infections in an indoor environment. Infection control against pathogen aerosols is important especially in hospitals, elderly care facilities, and general houses where cancer patients under treatment live who are susceptible to infectious diseases because their immune system have reduced (Ganjoho of NCC, 2015). Thus, many air-cleaning technologies have been developed (Nishikawa et al., 2003; Ogata et al., 2008; Asano et al., 2010; Tanino et al., 2015), and then, some standard test methods have been established to evaluate the reduction in airborne microbes caused by an air cleaner in Japan. For evaluating the performance against airborne fungi, the Society of Indoor Environment Japan (SIEJ) published a standard method (SIEJ No. 20110001, 2011). For evaluating the performance against airborne viruses, the Japan Electrical Manufacturers' Association (JEMA) published a standard test method using a safe bacteriophage, a virus that infects only bacteria not human, instead of pathogenic viruses; the reduction in airborne viruses is determined by counting the number of viable airborne phages by culture, after operating

the air cleaner (JEM1467, 2015).

Recent studies have investigated the performance of air-cleaning products advertising virus inactivation against pathogen aerosols, influenza virus (Mimura et al., 2010; Fujita et al., 2011; Nishimura, 2011; Ogata et al., 2016; Nishimura, 2016). Some epidemiological studies reported that the effectiveness of reducing influenza infection was observed with the products operating (Mimura et al., 2010; Fujita et al., 2011), and a paper reported that the products reduced the number of microbes aerosols effectively using *Staphylococcus aureus* and bacteriophages which is not pathogen. However, other studies reported that the products did not reduce the number of viable influenza virus in the aerosols effectively as a fundamental function, and thus speculated that the effectiveness against influenza infection might be caused by another factors in the epidemiological studies (Nishimura, 2011; Nishimura, 2016). Therefore, further studies are required to evaluate correctly the performance of air-cleaning products based on understanding the reduction mechanism.

The reduction in the number of viable microbes might be due to "physical decrease" or "inactivation"; physical decrease refers to the removal of microbe particles from the air through filter adsorption, while inactivation indicated that microbe particles exist in the air, but have lost their infectivity. I speculated that understanding the mechanism behind the reduction was important for proper usage of air-cleaning products. In the case of physical decrease, it is necessary to consider the secondary contamination of the destination of the viable microbes. In the case of

inactivation, mutant or revertants of the microbes must be considered. This distinction is especially important when developing new devices for cleaning the air to elucidate the mechanism of decreasing the number of viable airborne microbes. However, current standardized tests are not capable of distinguishing between these two possibilities while evaluating removal of airborne microbes by air cleaners because they involve only culture assays of airborne microbes.

The purpose of this study is to improve the current standard method, and to correctly understand the mechanism of reducing the amount of airborne microbes, especially viruses, and to propose an evaluation method that correlates with the performance against real pathogens.

In chapter 2, an aerosol test system using both airborne bacteria and airborne phage in a small chamber (1 m^3) was prepared and was preliminarily characterized (Okuda et al., 2008), in order to improve the current test method and to propose an advanced analysis for distinguishing between inactivation and physical decrease in airborne phage number in chapter 3.

In chapter 3, using phi-X174 phage aerosols in the small chamber, a behavior analysis to distinguish between physical decrease and inactivation of viable phages was performed. In order to distinguish between these two, it is necessary to quantify the total number of phage particles, regardless of the infectivity titer. I established a suitable polymerase chain reaction (PCR) system for quantifying phi-X174-specific particles in the aerosol. This chapter showed one example that a behavior analysis using this

quantitative PCR system and culture assay could successfully distinguish between physical decrease and inactivation of viable phages at particular levels of humidity.

Next, I addressed whether there are any points on physical reduction to be improved in the current standard method using phages. Physical decrease is caused by an air filter as a major means. I validated whether collection efficiency of a filter against phage is as same as that against real pathogen such as influenza virus. To compare collection efficiency of a filter against phage with that against influenza virus, a novel method that can be performed safely and that involves quantitating influenza virus aerosols is necessary.

In chapter 4, I designed a novel method for safely measuring virus aerosols for the performance evaluation of a filter; a method of safely measuring inactivated Influenza virus aerosol using antigen-capture enzyme-linked immunosorbent assay (ELISA) (Shimasaki et al., 2016b).

In chapter 5, using this ELISA, the filter efficiency against each aerosol of the different diameter of three spherical microbes (phage, inactivated influenza virus, and *Staphylococcus aureus*) was tested, indicating differences in the penetration ratio. This chapter could propose an improvement on physical reduction in the current standard method.

Next, I addressed whether there are any points on inactivation to be improved in the current standard method using phages. Inactivation is caused by heat treatment, UV irradiation, and ethanol treatment as major

means. In chapter 6, inactivation efficiencies of these means against phage with that against real pathogen were compared (Shimasaki et al., 2009). This chapter could suggest an important point for validating inactivation.

In chapter 7, finally, a new technique was suggested for the current standard method against airborne fungi. To evaluate the removal of airborne fungi, a technique for generating airborne fungal spores in the dry state in a test chamber (dry dispersion) is required. Although an aerial ultrasonic oscillator was used as the device for dry dispersion in the current standard test, the aerial ultrasonic technique is not easy to perform by non-skilled operators. Therefore, this chapter suggested a new device using glass beads for dry dispersion as a more versatile apparatus (Shimasaki et al., 2015).

I hope that this study will help to accurately evaluate the air cleaning performance of microbial aerosols and will contribute to the sanitary engineering of the indoor environment mainly for hospitals, elderly care facilities, and general houses where cancer patients under treatment live.

Chapter 2

Characterization and Performance Evaluation of an Aerosol Test System in a Small Chamber Using Airborne Bacteria and Airborne Phage

2.1 Purpose of this chapter

It is necessary to understand the behavior of pathogen-bearing aerosols, i.e., to identify whether the number of pathogens decrease physically or whether the pathogens are inactivated, which reduces the number of viable pathogens in the aerosol. This distinction is especially important when developing new devices for cleaning the air to elucidate the mechanism of decreasing the number of viable airborne microbes. If the number of viable airborne microbes decreases physically but the microbes are not inactivated, the surviving microbes can congregate and become a new infection source. Therefore, preventive measures are required for such pathogens.

However, the current standardized test (JEM1467, 2015) is not capable of distinguishing between these two possibilities while evaluating removal of airborne microbe by air cleaners because they involve only culture assays of airborne microbe.

To distinguish between inactivation and physical decrease in airborne pathogen number in the laboratory, it is necessary to quantify the total number of microbial particles regardless of the infectivity titer. The number

of airborne microorganisms, especially bacteria, can be monitored using a particle counter, and the size of single microbial particle can also be measured.

To improve the current test method against airborne virus (JEM1467, 2015), I preliminarily characterized an aerosol test system using airborne bacteria in a small chamber (1 m^3). Then, using airborne bacteria in the small chamber, I evaluated the performance of ultraviolet (UV) irradiation or high efficiency particulate air (HEPA) fan filter unit (FFU) operation, the antimicrobial mechanisms of which are well-known. Moreover, using airborne phage in the chamber, I identified caveats in this system pertaining to application of viral aerosols.

2.2 Materials and Methods

Preparation of test bacteria

S. aureus (NBRC 12732) was cultured on tryptic soy agar (TSA; Difco, Becton, Dickinson, and Company, NJ, USA) for 24 h at 37°C, as described previously (Shimasaki et al., 2016a). *S. aureus* colonies on the agar plate were suspended in distilled water (DW). The suspension was roughly filtered with a piece of sterilized absorbent cotton to remove extra lumps of bacteria. The filtered suspension was diluted with DW at a concentration of ca. 10^9 colony-forming units (CFU)/mL.

Culture assay

The number of viable *S. aureus* was determined using a culture assay, as described previously (Shimasaki et al., 2016a). Briefly, the assay fluid was serially diluted 10-fold with PBS and 0.1 mL of each diluted assay fluid,

along with the undiluted sample, was spread onto two TSA plates. Additionally, 1 mL and 10 mL aliquots of the assay fluid were filtered through membrane filters (0.45 μm pore size, Millipore, MA, USA) and the membrane filters were placed on the TSA plates. After cultivation for 48 h at 35°C, the bacterial colonies on the plates were counted.

Preparation of test phage

The phi-X174 phage (ATCC 13706-B1 or NBRC 103405) and *Escherichia coli* (ATCC 13706 or NBRC 13898) were prepared, as described in a previous study (Shimasaki et al., 2016a), with some modifications. The host *E. coli* was grown in a nutrient broth (NB; Difco™, Becton, Dickinson and Company, NJ, USA) with 0.5% NaCl (0.5% NaCl-NB) and 2 mM $CaCl_2$ at 36 °C overnight, and the cell density was adjusted to 10^8–10^9 colony-forming units (CFU)/mL. The phi-X174 phage at a density of 10^5–10^6 plaque-forming units (PFU)/mL was mixed with an equal volume of host suspension, and 2 volumes of 0.5% NaCl-NB containing 0.5% agar (Difco™, Becton, Dickinson and Company) were added. The mixture was poured onto a plate of nutrient agar (NA; Difco™, Becton, Dickinson and Company). After cultivation for 18 h at 36 °C, the upper soft-agar layer containing phi-X174 phages was collected and transferred into a stomacher bag. The bag was stomached for 2 min to crush the soft-agar and then warmed at 36 °C for 1 h. The lysate in the bag was transferred into centrifuge tubes and centrifuged at 2,380 ×*g* for 10 min. The supernatant was collected and centrifuged twice. The final supernatant was filtered using a membrane filter (0.22 μm pore size; Stericup, MILLIPORE, Darmstadt, Germany) and stored below −80 °C until it is used for the test. The stock phi-X174 phage

was at a concentration of approximately 1×10^{10} PFU/mL.

Phage plaque assay

The phage titer was determined by a soft-agar-overlaid plaque assay, as previously described (Shimasaki et al., 2016a). Briefly, the assay fluid was 10-fold serially diluted with PBS, and 0.2 mL of each serially diluted assay fluid sample, along with the undiluted sample, was mixed with 0.2 mL of host *E. coli* (ca. 10^9 CFU/mL) and added to tubes with 4 mL of 0.5% NaCl-NB containing 0.5% agar. After gentle mixing, the suspension was poured into three plates of NA. After cultivation for 18 h at 36 °C, the plaques on the plates were counted.

Generation and sampling of microbial aerosol in the test chamber

The experimental setup of the test chamber is shown in Fig. 2-1. The test chamber (1 m^3 in volume, constructed from boards of polyvinyl chloride) was fitted with a nebulizer (custom-made glass one or NE-C16, Omron healthcare Co., Ltd., Kyoto, Japan) for generating the aerosol and a midget impinger (based on JIS K 3800:2009) for sampling. Two stirring fans (SERVO CN55B5, NIDEC SERVO Co., Gunma, Japan) were operated in the chamber during the experiment such that the wind speed was 1.5 m/s. Five test pieces (polyvinyl chloride, 5×5 cm^2 each) were placed at the center of the bottom of the test chamber for harvesting the adhering bacteria. During the experiment, a light-scattering particle counter (MODEL 3886, Kanomax Japan, Osaka, Japan) was used to monitor the concentration of particles in the test chamber at a flow rate of 2.83 L air/min, based on our previous report (Shimasaki et al., 2015). The number of particles in each size range was calculated by subtraction, i.e., number of 0.3–0.5 μm particles =

number of ≧ 0.3 μm particles − number of ≧ 0.5 μm particles. Before generating the aerosols, particles with diameter ≧ 1 μm were removed from the test chamber by running the fan filter unit.

S. aureus aerosols were generated for 1 or 10 min (approximately 0.15 mL/min) using the nebulizer with the test suspension at a dispersion rate of 5 L/min. Subsequently, the aerosols inside the chamber were stirred for 2 min with fans and then sampled for 4 min at 5 L/min (20 L in total) with the midget impinger containing 20 mL of phosphate buffered saline (PBS, ELMEX Ltd., Tokyo, Japan). Aerosols were sampled every 20 min for 60 min for each experiment, followed by the culture assay. To investigate the amount of test bacteria adhering to the surface of the test chamber after aerosolization, the bacterial aerosol was incubated in the chamber for 40 min, followed by collection of the test pieces from the chamber and transfer to a stomacher bag with 10 mL of soybean casein digest broth with lecithin and polysorbate (Eiken Chemical Co., Ltd., Tokyo, Japan). The bag was shaken for 1 min to detach *S. aureus* from the test pieces and the wash solution was assayed. Phage aerosols were also generated and collected according to the same procedure. All experiments were conducted at 20-25°C. The relative humidity (RH) in the aerosol test chamber was set at 40-69%.

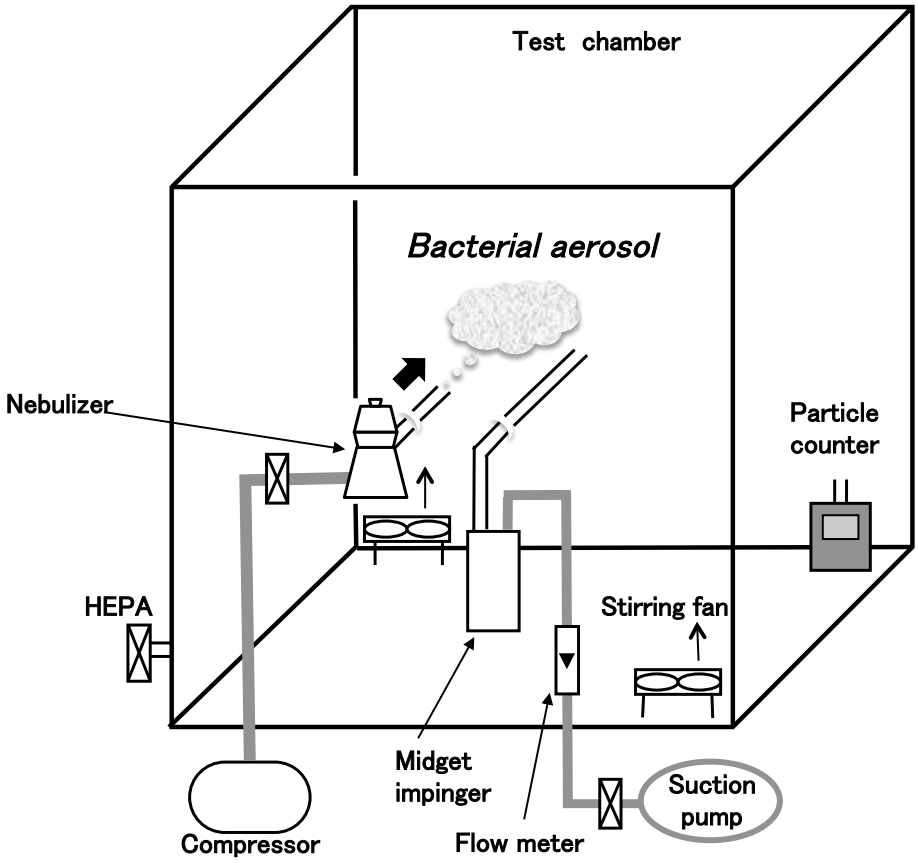

FIG. 2-1. Experimental setup of the test chamber.

The test chamber (1 m^3 in volume) was fitted with a nebulizer for generating the bacterial or viral aerosol and a midget impinger for sampling. Two stirring fans were operated at a wind speed of 1.5 m/s in the chamber during the experiment. During the experiment, a light-scattering particle counter was used to monitor the concentration of particles in the test.

UV irradiation

UV lamp (GL15, HITACHI, Tokyo, Japan) was set at the center of the back wall in the test chamber. The UV intensity at a distance of 50 cm from the lamp was 0.1 mW/cm^2 with a wavelength of 254 nm, as measured by an UV meter (Model UVC-254, LUTRON ELECTRONIC ENTERPRISE Co., Ltd., Taipei, Taiwan).

HEPA FFU operation

HEPA FFU (MAC-11FR, AIRTECH JAPAN Ltd., Tokyo, Japan) was set at the center of the back wall in the test chamber. The HEPA FFU was operated in weak mode, at a circulating airflow rate of 0.6 m^3/min.

2.3 Characterization of the bacterial aerosol

First, I compared the particle size distribution of the aerosols containing *S. aureus* suspension with DW and DW alone in the test chamber (Fig. 2-2). The *S. aureus* suspension was nebulized into the chamber for 1 min (approximately 10^8 CFU/0.15 mL). The main sample peak obtained for the *S. aureus* suspension with DW showed particles with diameter of 0.5–1 μm similar to the size of bacterial single cells at concentration above 2×10^7 (count/1,000L air). The size distribution of *S. aureus* aerosol was considered to show a lognormal distribution, as described in a handbook (Heber, 1995) and a previous paper (Yanagi, 2008), with a mean diameter ranging from 0.5 to 1 μm. In contrast, the main peak obtained with DW aerosols showed particles with diameter of 0.3–0.5 μm at concentration $\leq 5 \times 10^5$ (count/1,000L air), which is considered as background.

Next, I investigated the natural decay behavior of each particle diameter in the aerosol using a light-scattering particle counter when approximately 10^8 CFU was nebulized into the test chamber (Fig. 2-3). The number of particles in the aerosol for each size decreased to approximately half the $\log_{10}$ original value naturally in one hour. At 60 min, the percentage in total number of all particles of each particle diameter were 66% for 0.5–1 μm, 22% for 1–3 μm, 12% for 0.3–0.5 μm, 1% for 3–5 μm, and 0% for over 5μm, indicating that a main peak corresponding to particle diameter of 0.5–1 μm was maintained.

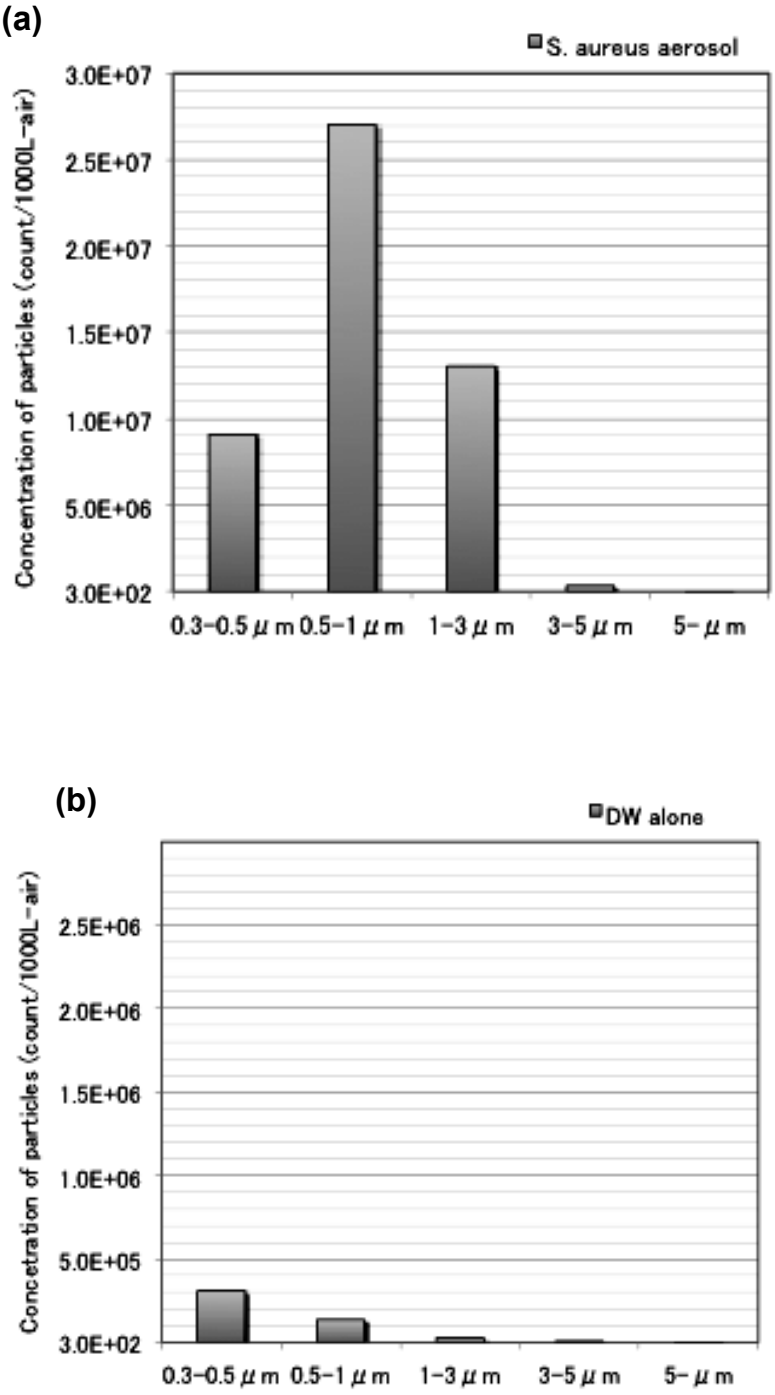

FIG. 2-2. Comparison of particle size distribution

I compared the particle size distribution between *S. aureus* suspension with DW (a) nebulized into the test chamber (approximately 10^8 CFU) and DW alone (b). The vertical axis shows converted values per 1,000 L of air.

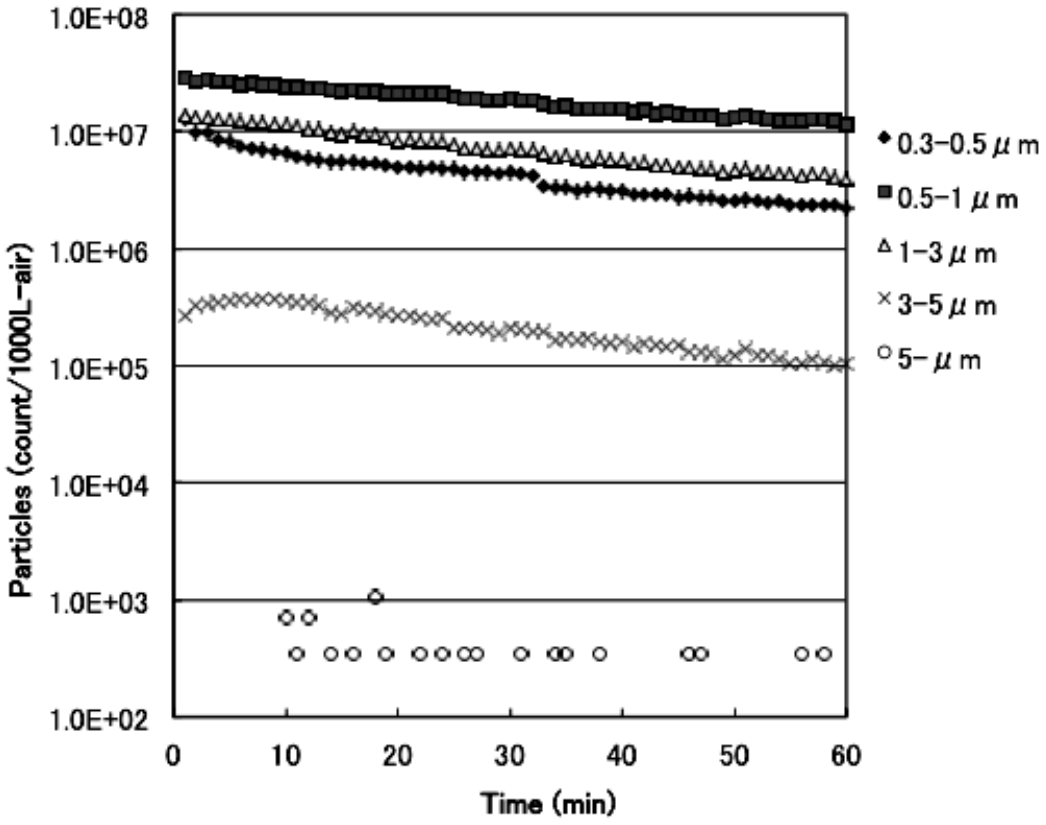

FIG. 2-3. Natural decay behavior and number ratio of each particle diameter in the aerosol of *S. aureus*.

The natural decay behavior of each particle diameter in the aerosol was measured using the light-scattering particle counter when *S. aureus* of approximately 10^8 CFU was nebulized into the test chamber. The numbers ratio of each particle diameter at 60 min were 66% for 0.5–1 μm, 22% for 1–3 μm, 12% for 0.3–0.5 μm, 1% for 3–5 μm, and 0% for over 5μm. The vertical axis shows converted values per 1,000 L of air. (Okuda et al., 2008)

Next, I investigated the natural decay behavior of different amounts of *S. aureus* aerosols (ca. 10^7, 10^8, 10^9) using *S. aureus* suspension 10-fold diluted with DW nebulized into the test chamber for 10 min (Fig. 2-4). The natural decay behavior of bacterial aerosols was measured by sampling airborne bacteria with midget impingers. The slopes of exponential regression were -0.011 for 10^9 CFU/m^3, -0.014 for 10^8 CFU/m^3, -0.039 for 10^7 CFU/m^3. This suggested that higher bacterial load increased the persistence time of the aerosols in air. For attenuation to remain within one $\log_{10}$ value in one hour, more than 10^8 *S. aureus* had to be nebulized into the chamber for the aerosol test.

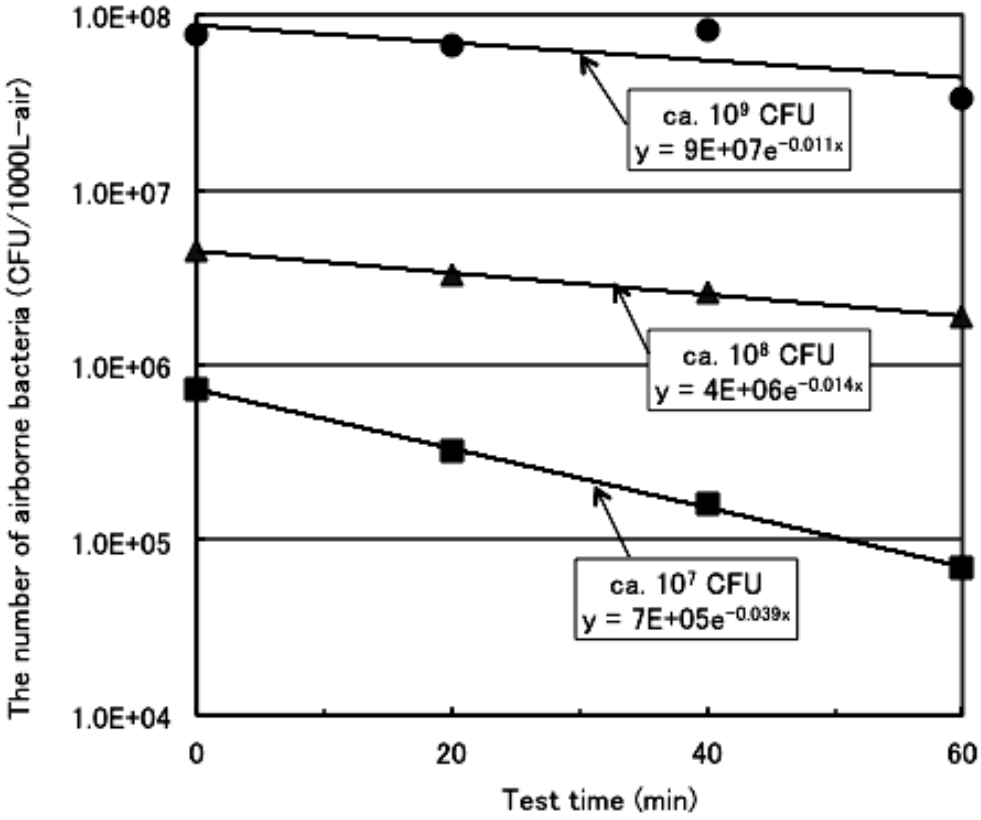

FIG. 2-4. Natural decay behavior of bacterial aerosol upon changing the bacterial amount nebulized into the test chamber.

The natural decay behavior of bacterial aerosol measured by sampling airborne bacteria at 5 L/min for 4 min (total 20 L-air/sampling) with midget impingers varied when the bacterial amount nebulized into the test chamber (1 m^3) was approximately 10^7, 10^8, 10^9 CFU of *S. aureus*. The vertical axis shows converted values per 1,000 L of air. (Okuda et al., 2008)

Next, I analyzed the sizes of the particles associated with the amount of viable bacteria (Fig. 2-5a, 2-5b). Fig. 2-5a and Fig. 2-5b show the correlation between particle concentration and the amount of viable *S. aureus* in the chamber (1,000L). These data were analyzed by simple linear regression. Results show that particles with 0.5–1 μm, 1–3 μm, and 3–5 μm diameter were highly associated with viable *S. aureus* (R^2 = 0.94038 for 0.5–1 μm particles, R^2 = 0.97513 for 1–3 μm particles, R^2 = 0.93318 for 3–5 μm particles) (Fig. 2-5a). In contrast, particles with diameter of 0.3–0.5 μm, 0.5–1 μm, and ≧5 μm were not significantly associated with viable *S. aureus* (R^2 = 0.64528 for 0.3–0.5 μm particles, R^2 = 0.00906 for ≧5 μm particles) (Fig. 2-5b).

Finally, I investigated the amount and uniformity of bacterial adhesion on the inner wall surface of the chamber, and whether these are affected by the amount of the aerosol (Fig. 2-6). Bacteria (ca. 10^9 CFU) was nebulized into the test chamber (1 m^3) with five test pieces placed on each inner wall surface, and the amount of adhered test bacteria on the test pieces was analyzed 40 min after the completion of nebulization. The experiments were repeated thrice. The amount of test bacteria adhering to each test piece was detected to be 2.0 × 10^8 CFU/m^2 in the average. There was no significant difference in the average adhesion amount. When bacterial amount of 3.4 × 10^9 CFU was nebulized, the total adhesion amount of six sides of the inner wall was 1.5 × 10^9 CFU/6-m^2. Thus, approximately 50% of the nebulized bacterial amount was estimated to be adhered to all inner wall of the chamber.

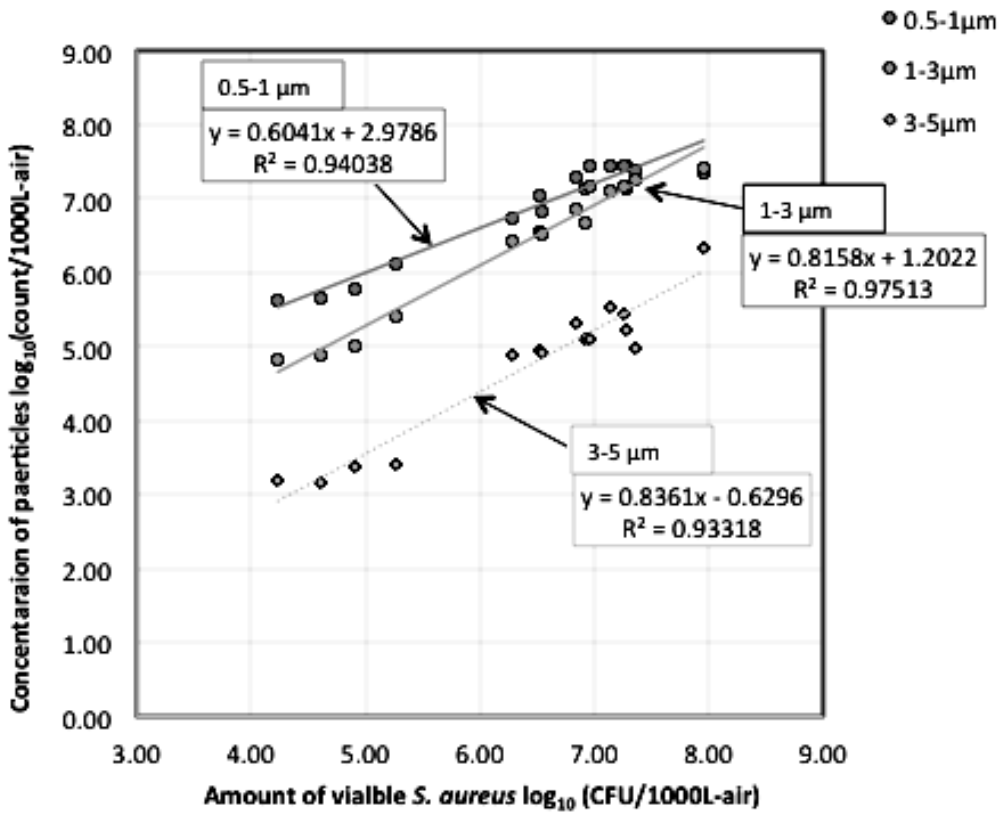

FIG. 2-5a. Correlation between particle concentration and the amount of viable bacteria.

For particles with 0.5–1 µm, 1–3 µm, and 3–5 µm diameter, the particle concentration was highly associated with the amount of viable test bacteria (*S. aureus*) detected ($R^2 \geqq 0.9$). The vertical axis shows converted values per 1,000 L of air.

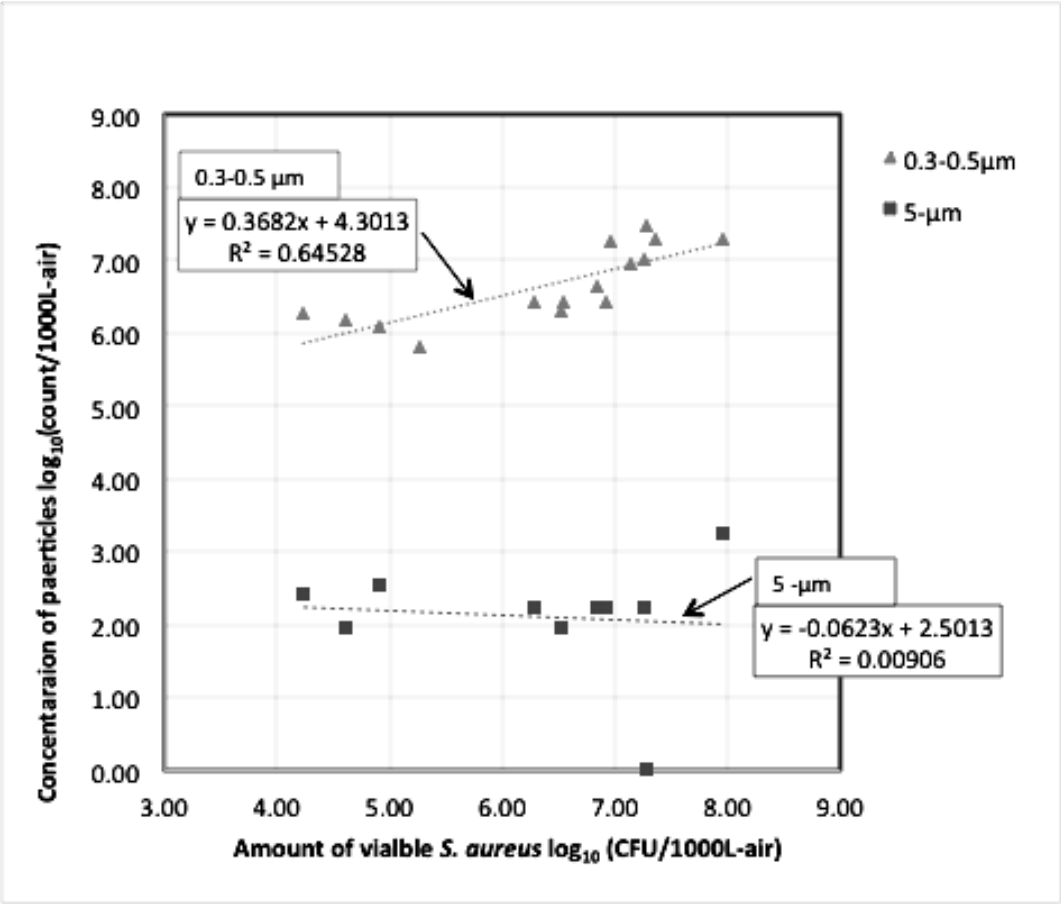

FIG. 2-5b. Correlation between particle concentration and the amount of viable bacteria.

For particles with 0.3–0.5 µm and ≧ 5 µm diameter, the particle concentration was not highly associated with the amount of viable bacteria (*S. aureus*) detected ($R^2 < 0.9$).

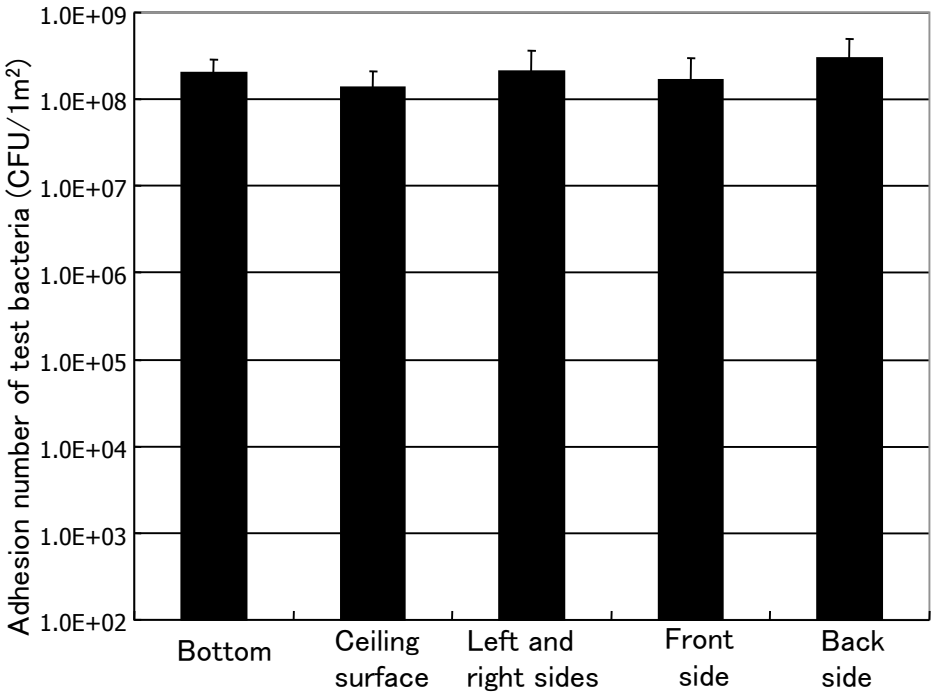

FIG. 2-6. Bacterial adhesion amount on the inner wall surface of the chamber.

Five test pieces (5 × 5 cm^2/test piece) were placed on each inner wall surface of the test chamber. When approximately 10^9 CFU was nebulized into the test chamber (1 m^3), the amount of adhered test bacteria on the test pieces in 40 min after completion of nebulization was assayed. The experiments were repeated thrice. The averages of the amount of adhered bacteria for each particle size is shown. Error bars denote standard deviation. There was no significant difference in adhesion amount. The vertical axis shows converted values per 1m^2 of the chamber surface. (Okuda et al., 2008)

2.4 Evaluating the performance of UV irradiation and HEPA FFU operation against bacterial aerosol

I evaluated the decrease in detection of *S. aureus* aerosols by UV irradiation and HEPA FFU operation using the aerosol test system (Fig. 2-7a). Aerosols of *S. aureus* were nebulized independently into the test chamber (ca. 10^9 CFU/m^3) and the test device (UV lamp or HEPA FFU), and the behavior of the viable airborne bacteria was analyzed over time. Both UV irradiation and HEPA FFU operation decreased the number of viable airborne bacteria as seen from the rapid decrease in the slopes of the regression equation compared to that of the natural decay.

In contrast, there was large difference between the amount of bacteria adhering to the bottom of the chamber under conditions of UV irradiation and HEPA FFU operation (Fig. 2-7b). After UV irradiation or HEPA FFU operation, the amount of adhering viable bacteria was assayed by wiping out the bacteria on the bottom surface (in 10 × 10 cm^2) of the chamber. Moreover, the particle behavior was measured using a particle counter (Fig. 2-7c). The results can be explained as follows; in case of UV irradiation, bacterial particles floated during the experimental time and were inactivated in the air. Furthermore, the adherent viable bacteria were inactivated because the UV light can reach the surface of the chamber. In the case of HEPA FFU, viable bacterial particles were filtered during the experimental time, which decreased the particle number; however, the bacteria were not inactivated in the air. Similarly, the adherent viable bacteria were not inactivated, and therefore, the number of viable bacteria

was the same as that observed with the natural decay.

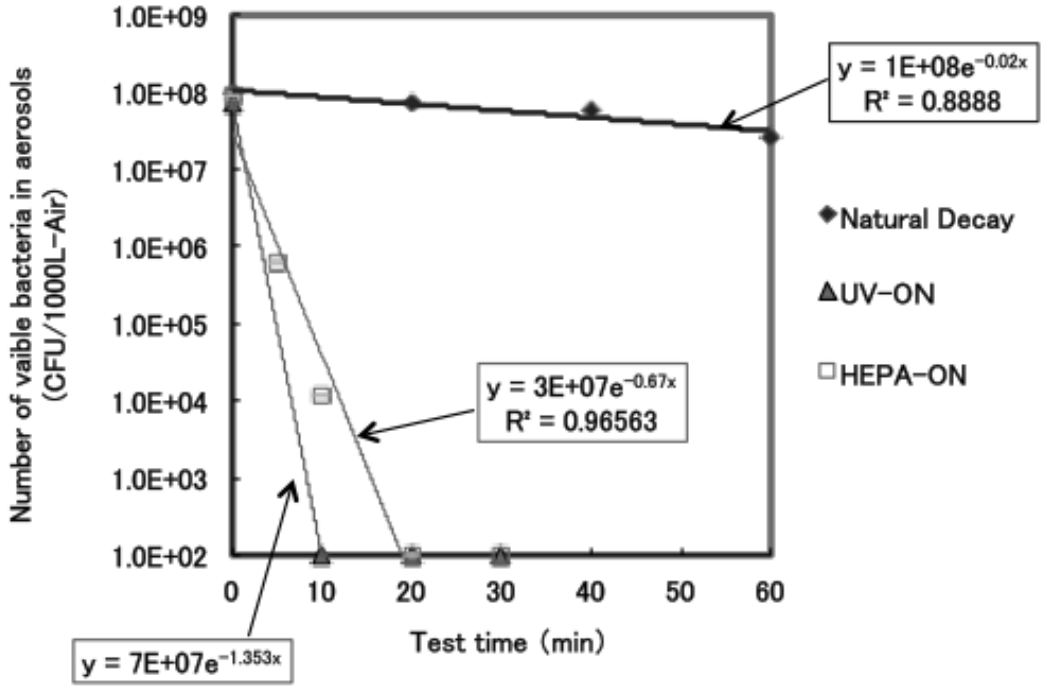

FIG. 2-7a. Analysis of airborne bacteria under different operating device conditions.

The behavior of viable airborne bacteria was analyzed over time when UV irradiation or HEPA FFU was operated. Both UV irradiation and HEPA FFU operation decreased the number of viable airborne bacteria because the slopes of both regression equation were steeper than that of the natural decay. The vertical axis shows converted values per 1,000 L of air.

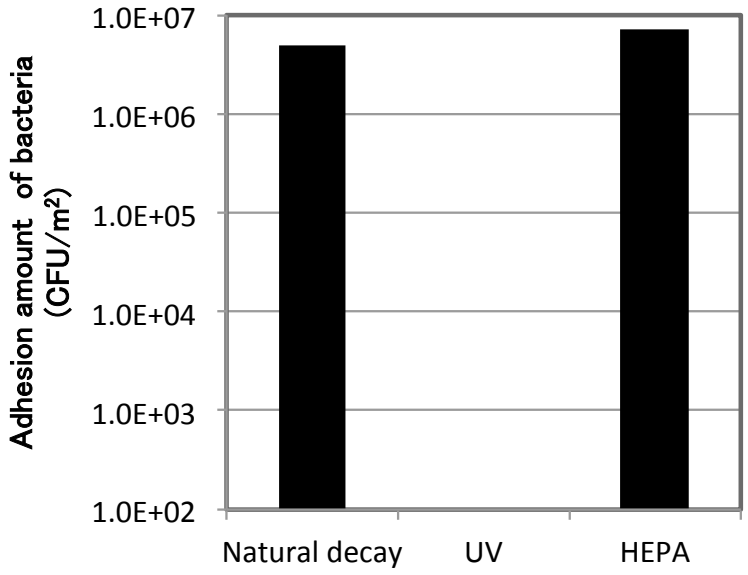

FIG. 2-7b. Analysis of adhered bacteria under different operating device conditions.

After UV irradiation or HEPA FFU operation, the amounts of adhered viable bacteria were assayed by wiping out the bacteria on the bottom surface of the chamber. The values on the vertical axis shows converted values per $1m^2$ of the chamber surface.

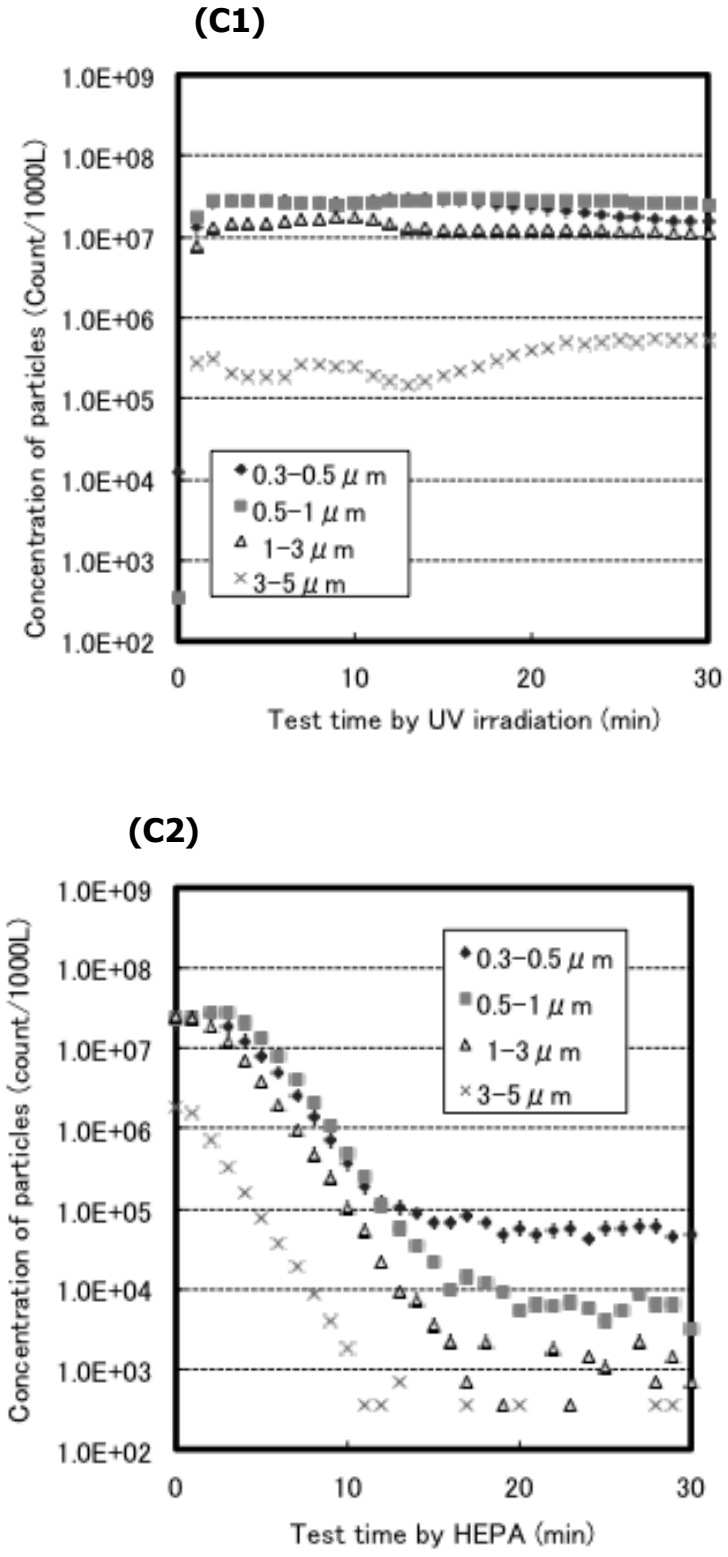

FIG. 2-7c. Behavior of aerosol particles after UV (C1) and HEPA (C2) treatment. The vertical axis shows converted values per 1,000 L of air.

2.5 Characterization of the phage aerosol

I investigated the natural decay behavior of each particle diameter in the phage aerosol using a light-scattering particle counter when approximately 10^8 PFU was nebulized into the test chamber (Fig. 2-8). This condition, approximately 10^8 PFU/m^3, was set aiming that the concentration of phage nebulized into the chamber was almost same as that in a previous study (Nakanishi et al., 2012), where the phage aerosol test was performed in a similar volume chamber of the current standard method (Nakanishi's study: 2.9×10^8 PFU/mL × 4.5 mL = 1.3×10^9 PFU in 10 m^3). The number of particles in the aerosol for each size decreased to approximately half the $\log_{10}$ original value naturally in one hour. At 60 min, the percentage in total number of all particles of each particle diameter were 87% for 0.3–0.5 μm, 12% for 0.5–1 μm, 1% for 1–3 μm, 0% for 3–5 μm, and 0% for over 5μm, indicating that a main peak corresponding to particle diameter of 0.3–0.5 μm was maintained.

Next, I investigated the natural decay behavior of different amounts of phi-X174 phage aerosols (ca. 10^8, 10^9 PFU) nebulized into the test chamber (Fig. 2-9). The natural decay behavior of viral aerosols was measured by sampling airborne phage with midget impingers. The slopes of exponential regression were -0.065 for 10^8 PFU/m^3, -0.02 for 10^9 PFU/m^3. This result suggested that higher viral load increased the persistence time of the aerosols in air when 10^9 PFU/m^3 was nebulized, whereas the number of airborne phage was reduced by appropriately 2 $\log_{10}$ PFU when 10^8 PFU/m^3 was nebulized. It was considered that the number of airborne phage of 10^8 PFU/m^3 was reduced faster than that of airborne *S. aureus* of 10^8 or 10^7

CFU/m^3 due to deposition by diffusion of phage particles with the diameter of 28 nm (Fig. 5-2a) (Takahashi, 1982). However, the ratio of test phage adhering test pieces at the center of the bottom of the test chamber was detected to be 13% when 10^8 PFU/m^3 and 22% when 10^9 PFU/m^3, which were smaller than the adhering ratio of *S. aureus*. When 10^8 PFU/m^3 was nebulized, the behavior of viable phage aerosol was different from those of any particles of each particle diameter monitored by the light-scattering particle counter. To investigate what material made these airborne particles, I compared the particle size distribution of aerosols of phage fluid diluted with DW (approximately 10^8 PFU/0.2 mL), ultrafiltration liquid removed of phage from the phage fluid (<1 PFU/0.2 mL), DW alone in the test chamber (Fig. 2-10). Each liquid was nebulized into the chamber for 1 min (approximately 0.2 mL/m^3). The concentration of each particle diameter in the aerosols of phage fluid was almost same as that of the ultrafiltration liquid without phage, although DW alone aerosols showed as a few particles as background. This indicated that the liquid derived from phage fluid containing NaCl and peptone made these airborne particles, and that the particles measured by a particle counter were not related with phage-specific particles. The results suggested that a detection method for viral-specific particles is required for accurate analysis of phage aerosol.

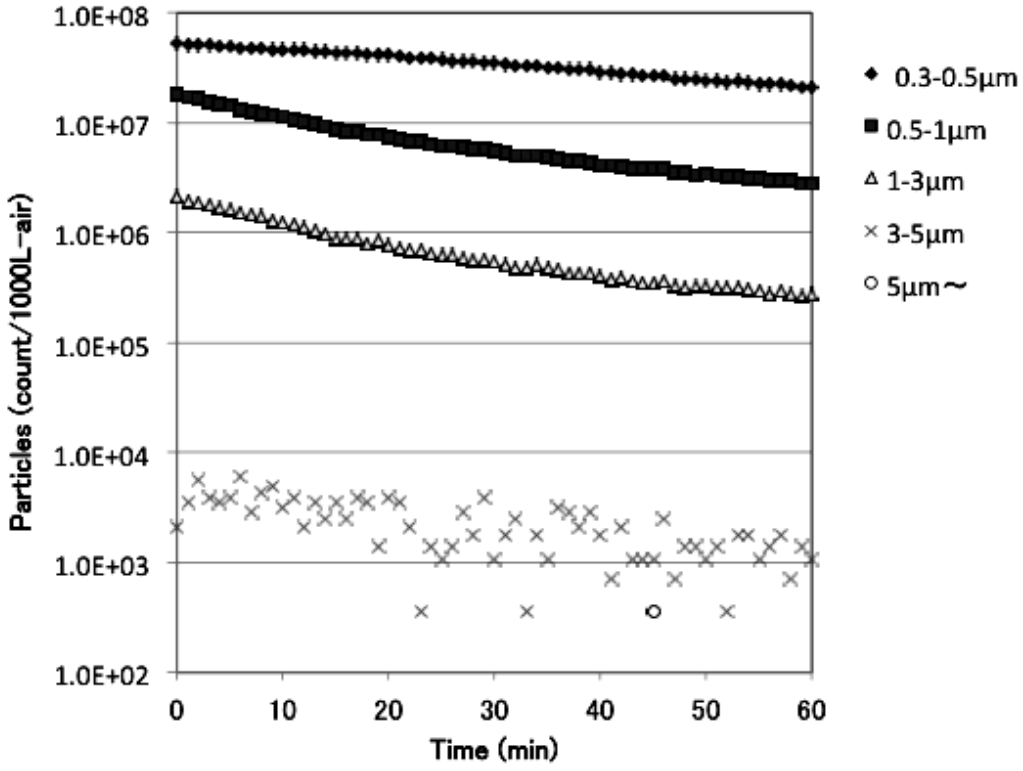

FIG. 2-8. Natural decay behavior and number ratio of each particle diameter in the aerosol of phi-X174 phage.

The natural decay behavior of each particle diameter in the phage aerosol was measured using the light-scattering particle counter when approximately 10^8 PFU was nebulized into the test chamber. The numbers ratio of each particle diameter at 60 min were 87% for 0.3–0.5 μm, 12% for 0.5–1 μm, 1% for 1–3 μm, 0% for 3–5 μm, and 0% for over 5μm. The vertical axis shows converted values per 1,000 L of air.

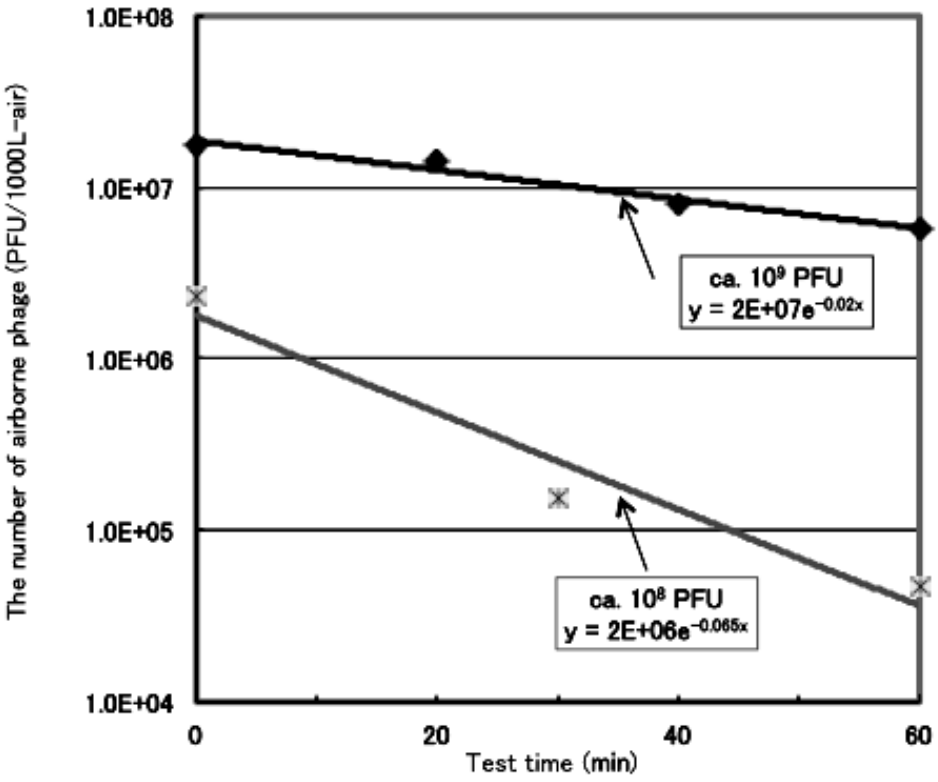

FIG. 2-9. Natural decay behavior of viral aerosol upon changing the viral amount nebulized into the test chamber.

The natural decay behavior of viral aerosol measured by sampling airborne phage varied when the phage amount nebulized into the test chamber (1 m^3) was approximately 10^8, 10^9 PFU of phi-X174 phage. The airborne phage was sampled with a midget impinger at 5 L/min for 6 min when 10^8 PFU or at 5 L/min for 4 min when 10^9 PFU. The vertical axis shows converted values per 1,000 L of air.

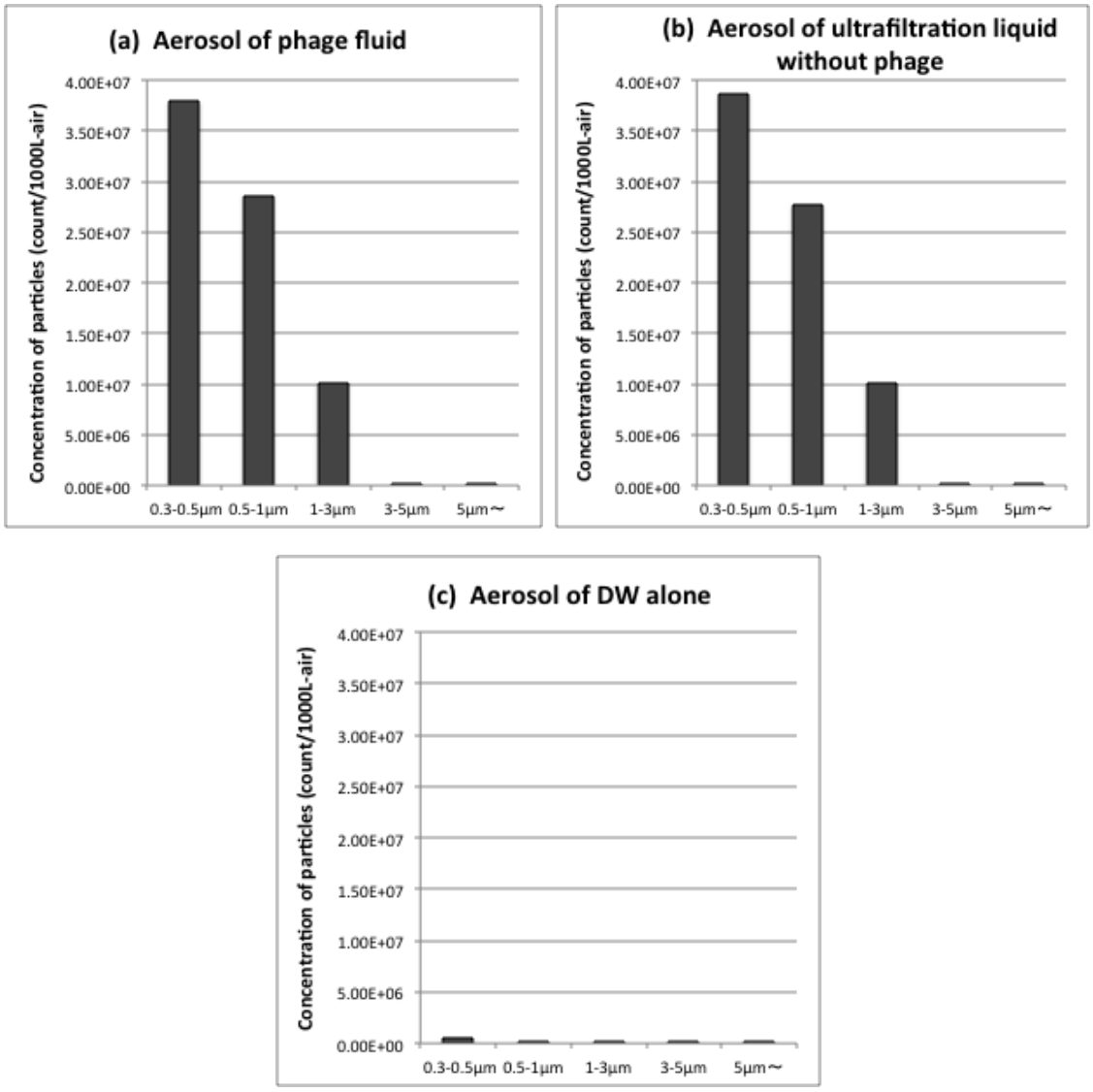

FIG. 2-10. Comparison of particle size distribution

I compared the particle size distribution of aerosols of phage fluid diluted with DW nebulized into the test chamber (approximately 10^8 PFU) (a), ultrafiltration liquid without phage (<1 PFU) , and DW alone (c). The vertical axis shows converted values per 1,000 L of air.

2.6 Discussion and Conclusion

In this chapter, I characterized an aerosol test system using airborne bacteria in a small chamber (1 m^3). This aerosol test system has the following characteristics. DW appeared as a negligible background in the particle size distribution when *S. aureus* suspension in DW was nebulized. A main peak corresponding to particle diameter of 0.5–1 μm was obtained when type in the aerosol decreased to approximately half of the original $\log_{10}$ value naturally in one hour. Higher concentration of bacteria persisted longer in the air as aerosols when 10^7 to 10^9 CFU of bacteria were nebulized in the chamber. The number of particles with diameter of 0.5–1 μm, 1–3 μm, and 3–5 μm showed strong correlation with the viability of *S. aureus*.

Approximately 50% of the nebulized bacterial amount adhered to the inner wall of the chamber when ca. 10^9 CFU was nebulized. I investigated the number of bacteria adhering to the surface of the chamber because adsorption of airborne bacteria would affect the amount of floating bacteria in a small chamber. The test device would have removal effect if the amount of floating airborne bacteria decreases by adhesion. However, results showed that the amount of bacterial aerosol decreased slowly and was not affected by bacterial adhesion (Fig. 2-4) although approximately 50% of the nebulized bacterial amount adhered to the inner wall of the chamber.

I evaluated the performance of the test system using UV irradiation or HEPA FFU, the antimicrobial mechanisms of which are well-known to identify the drawbacks of this system prior to the application of viral aerosols. The results indicated that the system could distinguish between physical decrease and inactivation of viable airborne bacteria using culture

assay and particle counting. Results showed that HEPA FFU removed bacterial aerosol but not bacteria adhering to the surface of the chamber, and that use of both culture assay and particle counting could distinguish between inactivation and physical decrease in the amount of bacterial aerosol. However, a normal particle counter cannot be used when the measurement range is ≤ 0.3 μm, and the particle size of viruses is even smaller, which is a limitation of the method.

I characterized an aerosol test system using airborne phage in the small chamber to improve the current test method for airborne virus (JEM1467, 2015). The number of airborne phage was reduced by appropriately 2 $\log_{10}$ PFU in one hour when 10^8 PFU/m^3 was nebulized, although the ratios of the viable phage adhering the bottom surface of the test chamber was detected were smaller than that of *S. aureus*. It was confirmed that the light-scattering particle counter could not detected the phage particles correctly, since the behavior of viable phage aerosol was different from those of any particles of each particle diameter monitored by the particle counter. Thus, a detection method for viral-specific particles is required in this system for assessing viral aerosols in future studies.

In conclusion, this chapter could clarify the characterization of the aerosol test system using airborne *S. aureus* and airborne phi-X174 phage in a small chamber ($1m^3$). In the next chapter, I would discuss the development of a method for detecting viral-specific particles.

Chapter 3

Advanced Analysis to Distinguish between Physical Decrease and Inactivation of Viable Phages in Aerosol by Quantitating Phage-Specific Particles

The manuscript related to chapter 3 was submitted to a journal “Biocontrol Science”, and will be expected to publish within two years. Until the paper is published, web-based publication of this chapter is impossible. Therefore, chapter 3 is abridged as follows;

A method of polymerase chain reaction (PCR) was developed to quantify the number of phi-X174-specific particles in the aerosol. I selected an appropriate primer-probe set for PCR considering UV resistance. I validated the sensitivity, linearity, and specificity in the presence of other environmental microbes of the quantitative PCR (qPCR) assay for phi-X174 phage. Using this qPCR method and a culture assay, an advanced analysis was performed and identified a humidity range within which viable airborne phages were inactivated in the small chamber system,. This observation may help to identify mechanisms that differentiate between the physical decrease and inactivation of viable airborne phages.

Incidentally, chapter 3 in the full version of my doctorate thesis consists of as follows;

Chapter 4

A Novel Method of Safely Measuring Influenza Virus Aerosol Using Antigen-Capture Enzyme-Linked Immunosorbent Assay

4.1 Purpose of this chapter

As I described in chapter 1, when a surrogate microbe is used for evaluation tests such as JEM1467, it is worthwhile to verify whether it is an appropriate substitute microbe for the real pathogen, based on the mechanism behind the reduction.

In the case of physical decrease caused by an air filter as a major means, the similarities in the physical properties of the particle (such as particle size and surface charge) between the test phage and the pathogenic virus may be critical. I aimed to validate whether collection efficiency of a filter against phage is as same as that against real pathogen virus.

Referred to my previous paper (Shimasaki et al., 2016b), when an aerosol test is performed using a live virus, test-operators might be exposed to risk of infection. In a previous study, to enable safe handling of viral aerosols, an aerosol of live poliovirus was generated in a special large glove box (Shinohara et al., 1995). Similarly, an aerosol of a live influenza virus was generated in a special sealing chamber (Nishimura, 2011), and an aerosol of live Ebola virus was generated in a biosafety level (BSL) 4

laboratory (Piercy et al., 2010). Tests that use infectious viral aerosols must be carried out strictly in special closed chambers or facilities to ensure biosafety. It is difficult to conduct tests involving infectious viral aerosols in a general BSL 2 laboratory. Therefore, a safe method for the virus-aerosol-based test is required.

In this chapter, I designed a new method for safely measuring virus aerosols for the performance evaluation of a nonwoven filter. To safely carry out aerosol tests, an inactivated virus was used. I selected the influenza virus as the test virus because it is the most major airborne pathogen in Japan (http://www.stat.go.jp/data/chouki/24.htm; the data of 2003, accessed on February 15th, 2017). I aimed to build a system that quantifies the total amount of particles of inactivated influenza virus in the aerosol.

Since the inactivated influenza virus cannot be cultured, I developed an antigen-capture enzyme-linked immunosorbent assay (ELISA) that estimates the amount of the M1 protein. The M1 protein was chosen as the target for ELISA because it is expressed in larger quantities than nucleoprotein (data not shown) in influenza virus particles; moreover, the M1 protein can be detected easily (Eierhoff et al., 2009), thereby enabling accurate quantification of viral particles. Furthermore, the amount of M1 protein might be correlated to the intact viral particle amount because the M1 protein is a structural protein that is encapsulated within the intact viral particle (Veit et al., 2011). Surface proteins such as hemagglutinin and neuraminidase were considered unsuitable as markers of intact particles, because they can be detected even if the viral particle is not intact. The capturing-antibody of the ELISA was used to prepare a peptide antibody

capable of targeting the antigen region I intended to capture. Furthermore, to confirm the utility of the method, I evaluated existing protective clothing materials using this new method.

4.2 Materials and Methods

Test influenza virus preparation

Influenza virus A/Puerto Rico/8/34 (PR8) strain, which was provide by Dr. S. Itamura at the National Institute of Infectious Diseases, was inoculated into 10-day-old embryonated chicken eggs, and the allantoic fluid was collected after incubation for 48 h at 34 °C. PR8 viruses were propagated and purified through a 20−60% sucrose gradient prior to pelleting by ultracentrifugation at 82,700 × *g* for 90 min at 4 °C. The virus pellets were then resuspended in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies Japan Ltd., Tokyo, Japan) to a total protein content of approximately 0.6 mg/mL. The total protein content was determined using the RC DC™ Protein Assay (Bio-Rad, CA, USA).

The purified viruses were inactivated using 0.025% ß-propiolactone (BPL; final concentration; Tokyo Chemical Industry Co. Ltd.,Tokyo, Japan) for 18 h at 4 °C, and the activity of the excess BPL in PR8 virus suspension was eliminated by incubating the sample at 37 °C for 90 min. Inactivation of PR8 virus was confirmed by non-activity in the hemagglutination test for allantoic fluid harvested from 6 embryonated chicken eggs into which the inactivated virus was inoculated and incubated for 48 h at 34 °C. The test suspension was diluted to a total protein content of 150 μg/mL with DPBS before use.

Influenza virus plaque assay

The virus titer of intact influenza particles was assayed according to a published manual (Nagata, 1995), with modifications. The influenza virus (100 μL) was inoculated into a confluent monolayer of Madin-Darby canine kidney cells on 6-well plates, and the plates were incubated for 1 h at 37 °C. The cells were then washed with warm Dulbecco's minimal essential medium (DMEM; Life Technologies Japan Ltd., Tokyo, Japan). The cells were overlaid with maintenance medium containing 0.8% agarose and 10 μg/mL trypsin (Sigma-Aldrich, MO, US). The plates were inverted and incubated for 3–4 days at 34 °C. For plaque counting, the cells were fixed with 3.65% formalin for 60 min, and the agarose was removed. The cells were then stained with 0.5% crystal violet (Sigma-Aldrich, MO, US).

Dynamic light scattering (DSL)

The particle size distribution of the BPL-inactivated PR8 virus (inactPR8) suspension was determined by DLS. The suspension was measured for 10 s at room temperature in a Viscotek Model 802 DLS system with Omni Size 3.0 (Viscotek, Texas, USA).

Transmission electron microscopy (TEM)

A droplet of inactPR8 was placed on a 400-mesh copper grid coated with carbon, and then negatively stained with 2% phosphotungstic acid. Images were obtained using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at 80 kV.

Peptide monoclonal antibody

We selected 67-TVPSERGLQRRRFVQ-81 as the M1 protein target peptide sequence on the basis of previous studies (Ye et al., 1989; Bucher et

al., 1989; Okuda et al., 2001). The peptide, containing a C-terminal cysteine for conjugation to keyhole limpet hemocyanin (KLH), was synthesized in Operon Biotechnologies (Tokyo, Japan). Then, the monoclonal antibody was prepared in BEX Co., Ltd. (Tokyo, Japan) as follows. Briefly, BALB/c mice were immunized with this peptide antigen using the Titer Max™ adjuvant. After boosting, the spleens of the mice were excised, and the spleen cells were fused with P3U1 myeloma cells. The fused cells were cultured on 96-well plates and were selected with hypoxiantine-aminopterin-thymidine (HAT) medium. The first screening test by ELISA using viral M1 protein and the culture supernatants of HAT-selected hybridomas resulted in a monoclonal antibody-expressing hybridoma, clone 29D2. This hybridoma was cultured and the monoclonal antibody 29D2 (29D2-mAb) was purified in PBS containing 0.05% NaN_3.

Western blot analysis

Twenty-microliters of egg allantoic fluid (control) or the PR8 virus propagated in egg allantoic fluid were loaded per lane on sodium dodecyl sulfate-polyacrylamide gels (PAGEL-12.5%™; ATTO, Tokyo, Japan) under reducing conditions. The proteins were then transferred to a polyvinylidene fluoride membrane (ATTO, Tokyo, Japan). After blocking with EzBlock Chemi™ reagent (ATTO, Tokyo, Japan), the proteins were detected with 29D2-mAb (1:2,000 dilution) or anti-flu A M1 goat polyclonal antibody (pAb; Abcam, Cambridge, UK; 1:2,000 dilution). After washing, each membrane was reacted with anti-mouse immunoglobulin G (IgG) (H+L) from Goat poly, horseradish peroxidase (HRP)-conjugated (BETHYL Laboratories, TX, USA; 1:10,000 dilution) or anti-goat IgG (H+L) from Donkey poly,

HRP-conjugated (anti-goat IgG-HRP; BETHYL Laboratories, TX, USA; 1:10,000 dilution), and the bands were stained with tetramethylbenzidine (TMB) reagent (ATTO, Tokyo, Japan).

Antigen-capture ELISA

The wells of a microplate (Nunc MaxiSorp™, Thermo Fisher Scientific, MA, USA) were coated with 100 μL of antigen-capturing 29D2-mAb (0.2 mg/mL) in 50 mM carbonate-bicarbonate buffer (pH 9.6; BETHYL Laboratories, TX, USA) and incubated at 4 °C overnight. After washing with Tris-buffered saline (pH 7.5) containing 0.1% Tween20® (TBS-T) 5 times, the microplate was blocked with 300 μL of blocking buffer (EzBlock Chemi™, ATTO, Tokyo, Japan) at room temperature for 1.5–2 h. After washing with TBS-T, the microplate was reacted with serial dilutions (DPBS containing 0.02% gelatin [Sartorius, Gottingen, Germany] and 0.1% Triton® X-100 [Polyoxyethylene(10) Octylphenyl Ether, Wako, Tokyo, Japan]; gelaDPBS-TX) of the PR8 virus or M1 protein standard (recombinant H1N1 [A/Puerto Rico/8/34/Mount Sinai] M1 protein, Sino Biological Inc., Beijing, P.R. China) at 37 °C for 1.5 h. After washing with TBS-T, 100 μL of anti-flu A M1 goat pAb (1:6,000 dilution) was added to the wells, and the plate was incubated at 37 °C for 1 h. After washing with TBS-T, 100 μL of anti-goat IgG-HRP (1:10,000 dilution) was added to the wells, and the plate was incubated at 37 °C for 1 h. The plate was then washed with TBS-T, and 100 μL of TMB-substrate (Thermo Scientific, MA, USA) was added. The reaction was stopped by adding 100 μL of TMB Stop Buffer (Scytek, UT, USA), and the optical density at 450 nm (OD_{450}) was measured using a multi-well plate reader (Spectra max®, Molecular device

Corporation, CA, USA).

Other bacteria and virus preparation

Bacillus subtilis ATCC 6633 was prepared by diluting a commercially available *B. subtilis* spore suspension (ca. 10^9 CFU/mL, Eiken Co., Tokyo, Japan) with PBS (1:5,000,000) before use. *Staphylococcus epidermidis* NBRC 12993 was cultured on Tryptic Soy Agar (TSA; Difco™, Becton, Dickinson and Company, NJ, USA) for 24 h at 34 °C. The colonies on the agar plate were suspended in PBS at a concentration of approximately 10^8 CFU/mL, and the suspension was diluted with PBS (1:1,000,000) before use. To measure the numbers of each bacterium, 1 mL of test bacterial suspension was added to pour plates with TSA medium and cultured for 48 h at 30 °C. The number of the colonies was counted. The *B. subtilis* and *S. epidermidis* test suspensions contained 2.3×10^2 CFU/mL and 1.5×10^2 CFU/mL, respectively.

Human adenovirus 3 (strain GB, ATCC VR-3) was prepared as follows. Human lung carcinoma A549 cells were infected with human adenovirus 3, and were incubated in DMEM containing 0.2% fetal bovine serum for 72–96 h at 37 °C in the presence of 5% CO_2. The infected cells were harvested, and the virus stock was obtained as supernatants after centrifugation at 2,380 × *g* for 10 min following 2 freeze-thaw cycles. The virus stock was concentrated using an ultrafiltration membrane (Kvick Lab cassettes, Kvick Lab 0.11 m^2, 100 KD, PES, GE Healthcare), and was purified by sucrose gradient centrifugation (108,000 × *g* for 3 h at 4 °C). The titer of purified virus was ca. 10^{10} 50% tissue culture infective dose ($TCID_{50}$)/mL. The suspension was diluted with DPBS (1:10,000) before use. The *Human*

adenovirus 3 test suspension contained 10^6 $TCID_{50}$/mL.

Filter performance test against the Influenza virus aerosol

Fig. 4-1 depicts the experimental setup of the test device in a biosafety cabinet. The nebulizer was fitted upstream of the airflow in the test duct (inner diameter, 5 cm; made from acrylic resin), which contained the sample holder. The filter sample was held in place in the sample holder. The distance between the nebulizer nozzle tip and the sample was approximately 28 cm. The inactPR8 test suspension (total protein content of 150 μg/mL and a virus titer of 5×10^8 PFU/mL before inactivation) was placed in the nebulizer. A gelatin filter (12602-47-ALK; Sartorius, Gottingen, Germany) with a dedicated filter case was connected to the test duct, downstream of the airflow. A gelatin filter was used because this filter was reported to have high collection efficiency for microbial aerosols (Sudharsanam et al., 2012).

Air from a compressor was fed into the nebulizer at an airflow rate of 3 L/min to spray the inactivated influenza virus aerosol into the test duct. At the same time, a suction pump was actuated at an airflow rate of 5 L/min for 3 min (15 L in total) to collect the virus aerosol penetrating through the test materials on the gelatin filter. The test was performed more than 3 times at 23 °C and 30% relative humidity.

To determine the amount of virus aerosol penetrating through the test material, the gelatin filter was dissolved in 5 mL of DPBS at 37 °C, and then gelaDPBS-TX (9 volumes) was added to the filter. This dissolved solution was diluted 4-fold serially with gelaDPBS-TX, and 100 μL of each serial dilution or undiluted solution was assayed by the antigen-capture

ELISA.

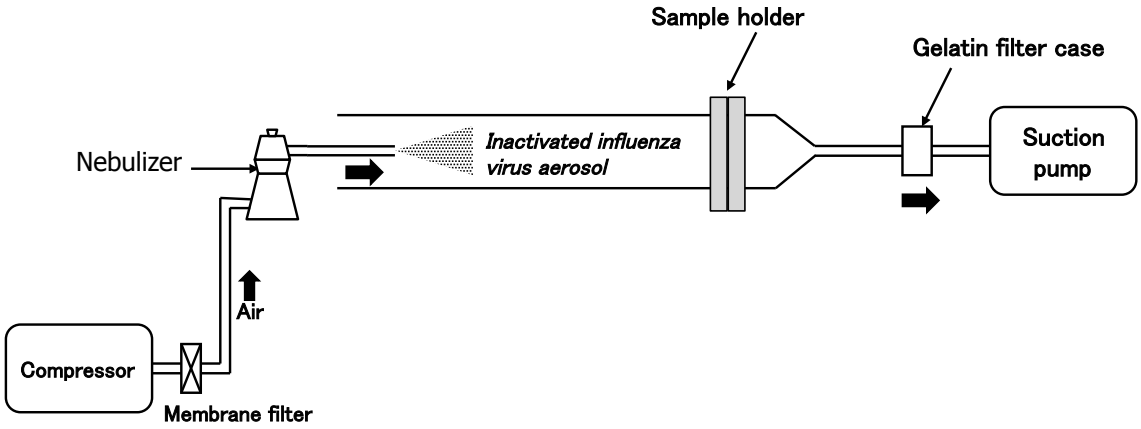

FIG. 1 Experimental setup of the test device.

FIG. 4-1. Experimental setup of the test device.

The nebulizer was fitted upstream of the airflow in the test duct, which contained the sample holder. The filter sample was held in place in the sample holder. The inactPR8 test suspension was placed in the nebulizer. A gelatin filter with a dedicated filter case was connected the test duct, downstream of the airflow. Air from a compressor was fed into the nebulizer at 3 L/min to spray inactivated influenza virus aerosol into the test duct. At the same time, a suction pump was actuated at 5 L/min for 3 min (15 L in total) to collect the virus aerosol penetrating through the test materials on the gelatin filter. The test was performed more than three times at 23 ℃ and 30% relative humidity.

4.3 Characterization of the inactPR8 virus

To confirm the size of the BPL-inactivated virus particles in the test suspension and to detect any aggregation of the virus particles caused by BPL, I investigated the characteristics of the inactPR8 virus by DLS (Fig. 4-2). The particle size distribution of the inactPR8 test suspension was 59.8 nm expressed in terms of the hydrodynamic radius (Rh). TEM of inactPR8 revealed that the virus particles were intact and almost spherical in shape, and that the diameter of a virus particle was 120–130 nm (data not shown). Using the Rh value, the diameter of the particles in the inactPR8 test suspension was estimated to be approximately 120 nm, which is equivalent to that observed by TEM. Moreover, one sharp peak for particle distribution was observed. This result indicated that the inactPR8 virus particles were monodisperse.

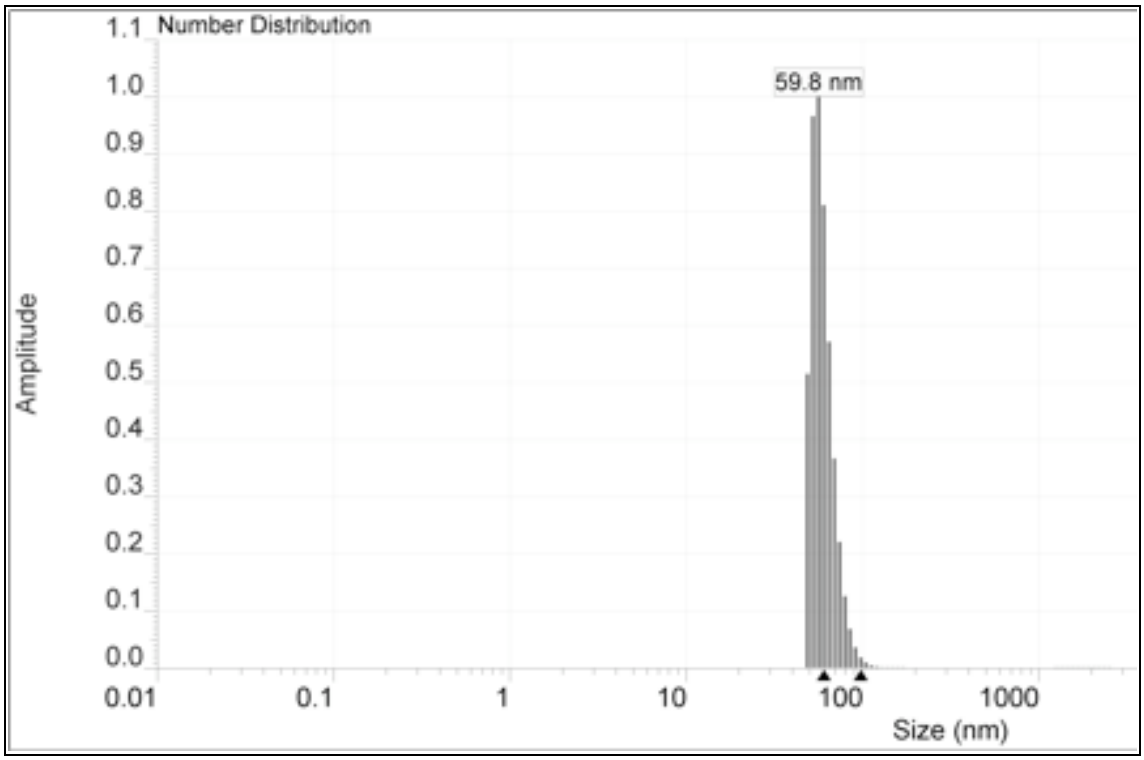

FIG. 4-2. Dynamic light scattering (DLS) analysis of the test virus suspension (inactPR8).

The particle size distribution of the inactPR8 virus in the test suspension was measured by DLS. The size on the horizontal axis indicates the hydrodynamic radius (Rh). The diameter of the inactPR8 particles was estimated using the Rh value. One sharp peak for particle distribution was observed.

4.4 Qualitative and quantitative response of the antigen-capture ELISA

Detection of the M1 protein in the PR8 virus

The antibodies used in the antigen-capture ELISA, 29D2-mAb and anti-flu A M1 goat pAb, successfully detected the M1 protein of PR8 in the western-blot analysis (data not shown). These antibodies did not react with egg allantoic fluid; they reacted only with the 29-kDa M1 protein of the PR8 virus propagated in egg allantoic fluid. Both 29D2-mAb and anti-flu A M1 goat pAb demonstrated specificity toward the M1 protein of the PR8 virus.

Next, I measured the OD_{450} of the M1 protein of inactPR8 using Triton® X-100 in the ELISA solution three times (Table 4-1). The total protein content of inactPR8 was 60 ng/mL and that of the recombinant M1 (rM1) protein standard was 40 ng/mL, respectively. The OD_{450} values of inactPR8 and rM1 were approximately 0.9 with Triton® X-100. However, inactPR8 was not detected in the absence of Triton® X-100 as the OD_{450} was under the cut-off value (2 standard deviations above the mean absorbance value obtained in the control well [no sample]). In contrast, the rM1 protein was detectable to approximately 0.9 at OD_{450} even in the absence of Triton® X-100, because it was not covered with the envelope of the viral particle. This result demonstrates that the M1 protein of inactPR8 expressed within the intact viral particle can only be detected by the ELISA after dissolving the envelope of intact viral particles with Triton® X-100.

Table 4-1. The OD_{450} of the M1 protein of inactPR8, detected using Triton® X-100 in the enzyme-linked immunosorbent assay (ELISA) dilute solution.

	OD_{450} in ELISA [Ave. ± S.D.]	
Protein sample	Triton X-100®(+)	Triton X-100®(-)
inactPR8	0.903 ± 0.052	not detected
rM1	0.846 ± 0.026	0.912 ± 0.035
		(n=3)

The optical density at 450 nm (OD_{450}) of the M1 protein was measured with and without Triton® X-100 in the ELISA solution. The total protein content of inactivated PR8 and rM1 used here were 60 and 40 ng/mL, respectively. The values in the table are the average (Ave.) and the standard deviation (S.D.) of three measurements.

Triton® X-100 (+), with Triton® X-100; Triton® X-100 (-), without Triton® X-100; not detected, the OD_{450} was under the cut-off value.

Standard curve

Fig. 4-3 shows a representative standard curve obtained for the M1 protein in the antigen-capture ELISA using rM1 at a protein concentration of 80 ng/mL in 2-fold serial dilutions. The standard curve demonstrated that the OD_{450} values of different concentrations of the M1 protein followed a linear fit (goodness-of-fit of simple linear regression; $R^2 = 0.9942$). The detection limit for this ELISA using rM1 was calculated to be 2.5 ng/mL on the basis of the cut-off value.

Linearity and repeatability of the assay for inactPR8

To investigate the linearity and repeatability of the ELISA, the dose of inactPR8 was varied (total protein content: 7.5, 15, 30, 60, and 100 ng/mL) and was assayed using this ELISA in 6 independent experiments (Fig. 4-4). The M1 protein concentration determined by the assay was proportional to the dose of inactPR8 (goodness-of-fit of simple linear regression; $R^2 = 0.9981$). The repeatability of the assay ranged between 6.1% and 11.5% relative standard deviation (RSD), and the mean of RSD was 9.7%.

Accuracy of the assay for inactPR8 in the presence of environmental microbes

When an aerosol performance test is carried out, environmental bacteria and viruses might be present as contaminants on the object being assayed. Therefore, I investigated whether environmental microbes influence the accuracy of the ELISA. As models of environmental microbes, I used *B. subtilis* (2.3×10^2 CFU/mL), *S. epidermidis* (1.5×10^2 CFU/mL), and *human adenovirus 3* (10^6 $TCID_{50}$/mL). Nonspecific reactions toward these microbes were not observed in the ELISA. Then, the test solution of each microbe and

inactPR8 test suspension (final total protein content: approximately 60 ng/mL) were mixed in equal volumes, and the mixed solutions diluted with 9 volumes of gelaDPBS-TX were assayed 6 times by the ELISA (Table 4-2). The assay could detect exactly the M1 protein concentration of the inactPR8 virus regardless of the presence of these microbes, exhibiting no significant differences in the detected values (T-test: $P > 0.2$). This result indicates that the ELISA retains its accuracy for PR8 even in the presence of environmental microbes.

Correlation between the amount of infectious particles and the M1 protein concentration

The M1 protein concentration detected in the ELISA should be validated before it may be regarded an accurate indicator of the amount of infectious influenza virus. Therefore, I investigated the correlation between the virus titer of live PR8 virus by a plaque assay, which estimates the amount of infectious viral particles, and the M1 protein concentration determined in the ELISA (Fig. 4-5). I used live crude PR8 virus propagated in egg allantoic fluid and live purified PR8 virus with 2-fold serial dilution. These data were analyzed by simple linear regression through zero. The results revealed that the detected M1 protein concentration of PR8 was highly associated with the virus titer; the coefficient of determination (R^2 value) was 0.96449, suggesting that the M1 protein concentration determined in the ELISA may be considered as a good indicator of the amount of infectious intact particles of the influenza virus.

Furthermore, the M1 protein amount of the PR8 virus containing 1 PFU was estimated to be 0.2 pg using the equation for linear approximation (y =

0.0002x), as the M1 protein concentration is proportional to the virus titer.

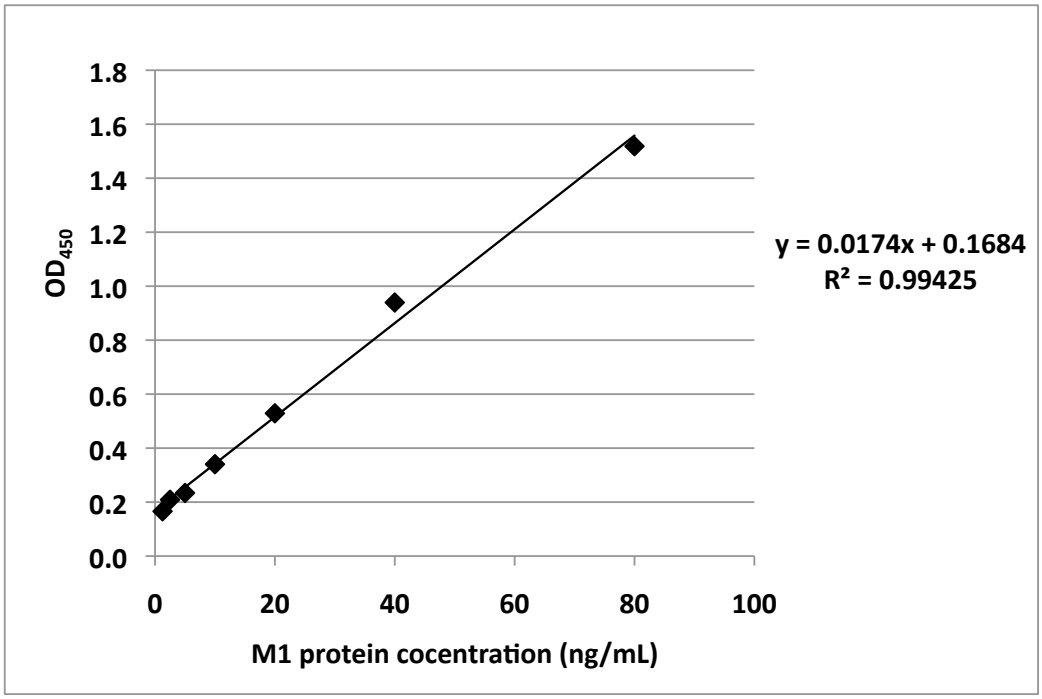

FIG. 4-3. Standard curve of the M1 protein in antigen-capture enzyme-linked immunosorbent assay (ELISA).

The figure shows a representative standard curve for the M1 protein using recombinant H1N1 (A/Puerto Rico/8/34/Mount Sinai) M1 protein (rM1) in the antigen-capture ELISA. The standard curve between OD_{450} and the M1 protein concentration was approximated by a linear function.

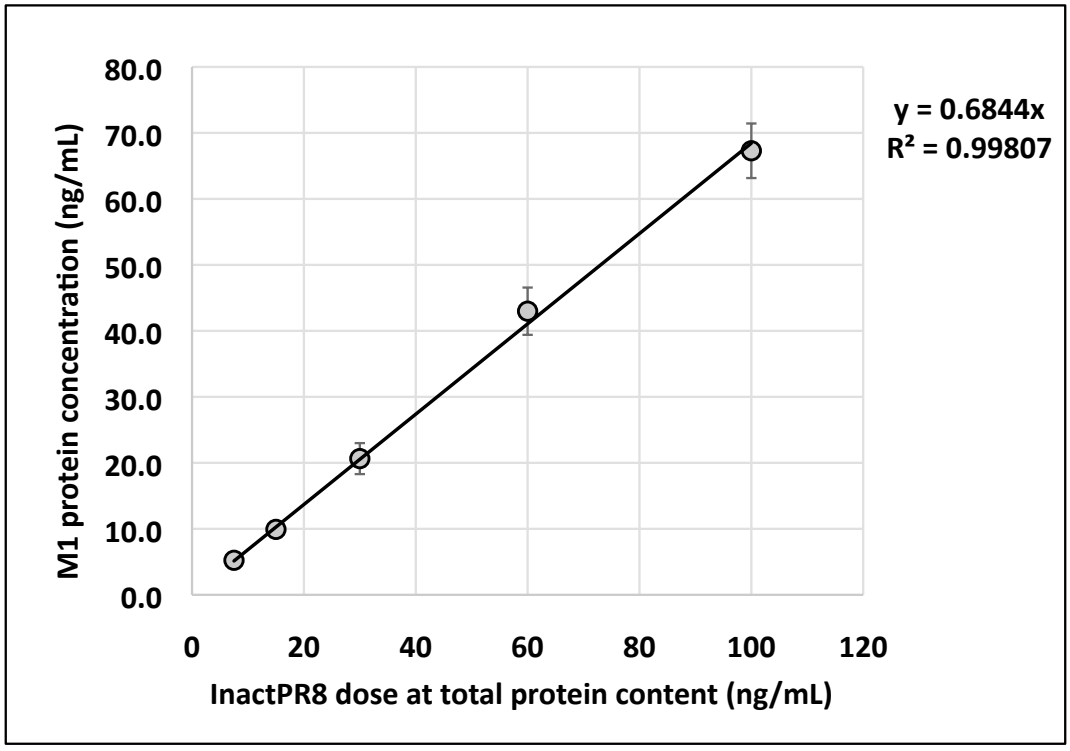

FIG. 4-4. Linearity and repeatability of the antigen-capture ELISA for inactPR8.

The dose of inactPR8 was varied (total protein content: 7.5, 15, 30, 60, and 100 ng/mL) and was assayed by ELISA in 6 independent experiments. The average for each dose is shown. Error bars denote standard deviation. These data were analyzed by simple linear regression through zero.

Table 4-2. Accuracy of the antigen-capture ELISA for inactPR8 in the presence of environmental microbes

	M1 protein concentration (ng/mL) in the ELISA			
	inactPR8	inactPR8 + *B. subtilis*	inactPR8 + *S. epidermidis*	inactPR8 + *Human Adenovirus 3*
Ave.(n = 6)	38.1	37.8	37.9	36.4
S.D.	2.6	1.5	1.7	2.4
RSD	6.9%	4.0%	4.5%	6.7%
T-test	–	$P > 0.2$	$P > 0.2$	$P > 0.2$

As models of environmental microbes, we used *Bacillus subtilis* (2.3×10^2 CFU/ml), *Staphylococcus epidermidis* (1.5×10^2 CFU/ml), and *human adenovirus 3* (10^6 $TCID_{50}$/ml). In the presence of these microbes, the M1 protein concentration of the inactPR8 virus (final total protein content, approximately 60 ng/mL) was assayed by the ELISA. The values in the table are the average (Ave.), the standard deviation (S.D.) and relative standard deviation (RSD) of six measurements. The detected values were analyzed by the T-test between inactPR8 alone and inactPR8 together with each microbe. In the presence of these microbes, there were no significant differences in the M1 protein concentration of the inactPR8 virus (T-test: $P > 0.2$).

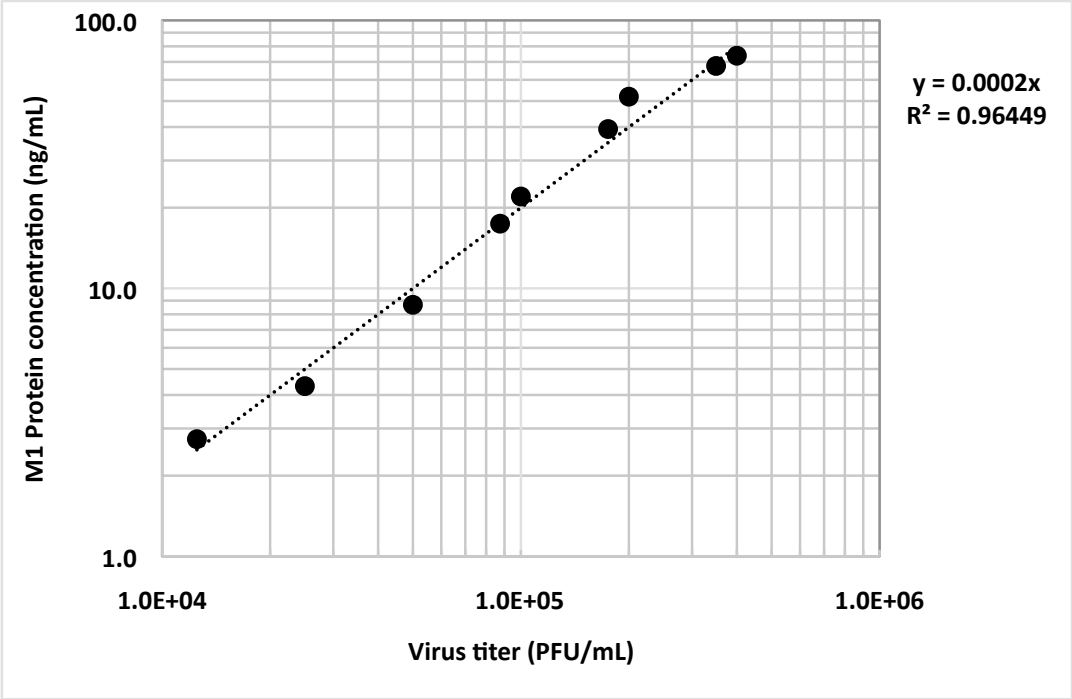

FIG. 4-5. Correlation between the virus titer (intact virus particle amount) and the M1 protein concentration, as estimated by the antigen-capture ELISA.

The virus titer and the M1 protein concentration were assayed using the live crude PR8 virus propagated in egg allantoic fluid and live purified PR8 virus. The detected M1 protein concentration of PR8 was highly associated with the virus titer forming plaques, as evidenced by simple linear regression analysis through zero ($R^2 = 0.96449$).

4.5 Filter performance test against the Influenza virus aerosol using the ELISA

Finally, to confirm the applicability of this new detection method, I carried out a performance evaluation of a filter material against inactivated influenza virus aerosol (Fig. 4-1). I tested a medical nonwoven fabric currently used in hospital which was certified the performance of the barrier against bacterial aerosol, made of polypropylene 100% and a three-layer structure; spunbond-meltblown-spunbond (Shinohara et al., 2012). I tested each sample 3 times (the blank experiment [no filter] was repeated 4 times). I assayed the M1 protein amount of the virus aerosol penetrating through the test materials by multiplying the dilution volume with the M1 protein concentration determined in the ELISA. The penetration ratio was calculated using the following equation: penetration ratio (%) = (the M1 protein amount in the case of each sample)/(the M1 protein amount in case of the blank experiment [no filter]).

Fig. 4-6 shows the M1 protein amount of the inactPR8 aerosol penetrating through the test filter. The penetration ratio of the nonwoven fabric filter was 15.0%, significantly different from that obtained in the control experiment (T-test: $P < 0.001$).

The test virus suspension was sprayed at a volume of 0.24 ± 0.02 mL per test. The M1 protein concentration of the test virus suspension was 109.5 μg/mL, as determined by ELISA. The average M1 protein amount was 3072 ng in the control experiment. The collection ratio of virus aerosol in the control experiment was calculated to be 12% (collection ratio = 3072/[109.5 × 0.24 × 1000]), which is comparable to that reported

previously (Nishimura, 2011). For reference, the virus titer of the inactPR8 test suspension (M1 protein concentration of 109.5 μg/mL) was estimated to be 5×10^8 PFU/mL by the plaque assay before inactivation.

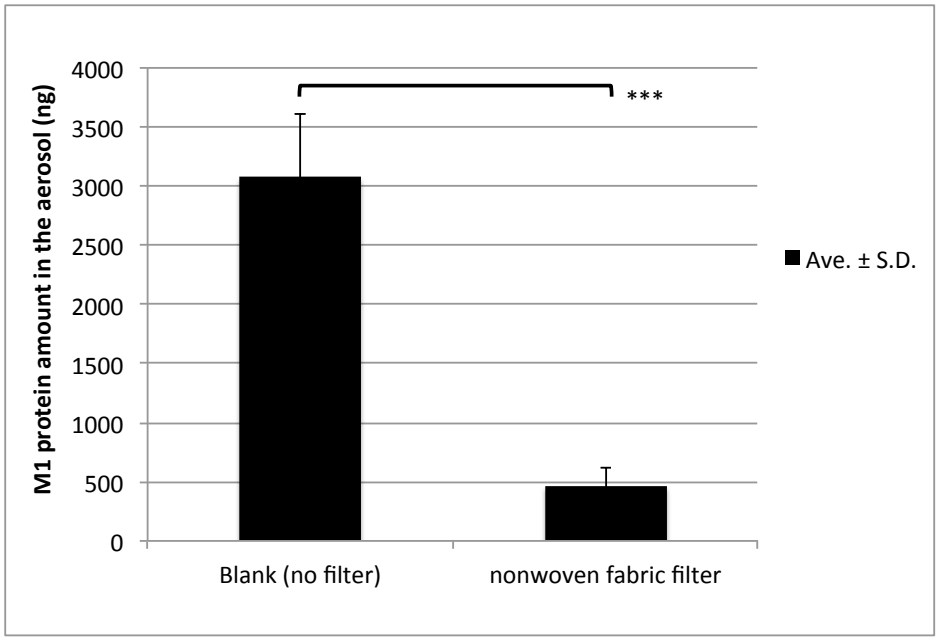

FIG. 4-6 The M1 protein amount of inactPR8 aerosol penetrating through the test materials, as detected by antigen-capture ELISA.

The performance test of a nonwoven filter sample against the inactPR8 virus aerosol was performed. The test sample was tested three times; blank was tested 4 times. The amount of virus aerosol penetrating through the test materials was assayed using our ELISA-based method. The average (Ave.) for each dose is shown. Error bars denote standard deviation (S.D.). ***$P < 0.001$ by T-test.

4.6 Discission and Conclusion

In this chapter, I developed a new method for safely measuring influenza virus particles from an aerosol using antigen-capture ELISA for the performance evaluation of filter materials. Because the test virus is inactivated, the aerosol test can be carried out without risk of infection at any normal laboratory. Moreover, it can be easily performed by operators who do not have specialized knowledge of virology. This method may be employed to safely measure the amount of virus aerosol particles that penetrate filter materials.

The results in this chapter revealed that the amount of virus aerosol was removed by physical decrease; the filter efficiency was calculated to be 85.0 % (filter efficiency = 100% − penetration ratio).

Moreover, the PR8 strain used here was not filamentous but spherical. Previous reports showed that influenza viruses isolated from patients frequently form filaments that can be many microns long (Chu et al., 1949; Elleman et al., 2004). Because the particles of PR8 may be smaller than that of clinical isolates, PR8 can be considered to be a proper strain for use in the evaluation of aerosol penetration in terms of particle-size. Additionally, the PR8 virus can be purchased from ATCC and is easily cultured. InactPR8 particles could be prepared for aerosol tests by inactivating the PR8 virus.

Compared to the method of culturing live influenza virus, the detection limit of the ELISA was 2.5 ng/mL and that of the plaque assay was 0.2 pg/mL (Fig. 4-5). Thus, methods that involve cultivation of live viruses may be more sensitive than the ELISA-based method. However, our method has

the following advantages: test operators can safely measure the protection performance against the influenza virus aerosol, and the virus aerosol amount can be estimated in a short time (within one day).

In the method described here, the influenza virus was used merely as a model particle for viral aerosol penetration of a filter. The viral aerosol of non-enveloped viruses in the picornaviridae family or phi-X174 phage, whose particle size are smaller than that of the influenza virus, needs to be investigated in future studies. Previous studies have suggested that nanoparticles (not viral particles) whose size is approximately 0.1 μm can easily penetrate surgical masks (Huang et al., 1998; Lee et al., 2008). Therefore, it is possible that influenza virus particles (size, approximately 0.1 μm) can also easily penetrate nonwoven fabric filter. In next chapter, I aim to compare the filter efficiency of nonwoven fabrics against phi-X174 phage with Influenza virus particles developed in this chapter.

In conclusion, this chapter could provide the developed method may be safely used to filter materials at any normal laboratory, thereby enabling manufacturers of air-cleaning product to select the appropriate filter materials for the product, depending on the hazardous situation and infection risk.

Chapter 5

Comparison of the Filter Efficiency against Three Different Microbe Aerosols

The manuscript related to chapter 5 was submitted to a journal "Biocontrol Science", and will be expected to publish within two years. Until the paper is published, web-based publication of this chapter is impossible. Therefore, chapter 5 is abridged as follows;
Using the ELISA developed in chapter 4, I tested the collection efficiencies for aerosols of three types of spherical microbes (phi-X174 phages, an inactivated influenza virus, and *Staphylococcus aureus*), characterized by DLS. The experimental penetration ratio for each microbe was compared with theoretical calculation. These results suggests that the standard test for evaluating the reduction of airborne viruses using phi-X174 phages may overestimate the performance of filters compared to the penetration ratio for real pathogens such as the influenza virus.

Incidentally, chapter 5 in the full version of my doctorate thesis consists of as follows;

5.1 Purpose of this chapter

5.2 Materials and Methods

5.3 Characterization of three different microbe suspension using DLS

5.4 Comparison of the filter efficiency of nonwoven materials against three different microbe aerosol

5.5 Theoretical calculation of the filter efficiency of the nonwoven material against the three-microbe aerosol

5.6 Discussion and Conclusion

Chapter 6

Comparison of the Inactivation Resistance of Four Microbes to Chemical or Physical Treatments to Evaluate Phages as Virus Surrogates

The paper related to chapter 6 (Shimasaki et al., Vox Sang. 2009 Jan;96(1):14-9. doi: 10.1111/j.1423-0410.2008.01113.x.) is not able to be agreed with web-based publication of doctorate thesis by all co-authors because some co-authors passed away. Therefore, chapter 6 is abridged as follows;

In order to verify whether the phage is a good surrogate virus when inactivating airborne viruses captured with a filter, three pathogens (influenza virus, hepatitis A virus, and *Staphylococcus aureus*) were compared with the phage by chemical or physical inactivation treatments. As a result, the phage was in some cases more resistant to inactivation by heat treatment, UV irradiation, or ethanol treatment than the real pathogen. It was also found that the inactivation efficiencies of each method varied with different pathogen strains. This result suggests that it is important to carefully evaluate the inactivation efficiency of each method, so as to select an appropriate virus strain as the inactivated pathogen for the standard inactivation efficiency test. It needs to be sufficiently resistant to the inactivation treatment.

Incidentally, chapter 6 in the full version of my doctorate thesis consists of as follows;

6.1 Purpose of this chapter

6.2 Materials and Methods

6.3 Inactivation by heat treatment

6.4 Inactivation by UV irradiation

6.5 Inactivation by ethanol

6.6 Discussion and Conclusion

Chapter 7

Development of a new technique using glass beads for dry dispersion of airborne fungal spores

The paper related to chapter 7 (Shimasaki et al., Biocontrol Sci. 2015;20(1):53-8. doi: 10.4265/bio.20.53.) is not able to be agreed with web-based publication of doctorate thesis by all co-authors because a co-author passed away. Therefore, chapter 7 is abridged as follows;

A new device was developed in order to improve the dry dispersion technique of airborne fungal spores in the current standard test, SIEJ Standard Method No. 20110001. The device was composed of a midget impinger containing glass beads and a fungal sheet of *Wallemia sebi*. I investigated the appropriate conditions, the size of glass beads and the rate of airflow into the impinge, for the spore-dispersing device to introduce the fungal spores in the dry state in a compact test chamber. The new device using glass beads for dry dispersion was successfully trialed in this study as a more versatile apparatus for use with the compact test chamber. Therefore, this newly developed technique may present an improved method for generating dry airborne fungal spores to use in the current standard performance test for air-cleaning products.

Incidentally, chapter 7 in the full version of my doctorate thesis consists of as follows;

7.1 Background

7.2 Purpose of this chapter

7.3 Materials and Methods

7.4 Result

7.5 Discussion and Conclusion

Chapter 8

Summary

The purpose of this study is to improve current standardized methods and to improve our understanding of the reduction mechanisms of airborne microbes, especially viruses. An evaluation method is proposed that correlates the performance of a safe bacteriophage against real pathogens.

In chapter 2 and 3, I could clarify the characterization of the aerosol test system using airborne *S. aureus* and airborne phi-X174 phage in a small chamber ($1m^3$). A polymerase chain reaction (PCR) was used to quantify the number of phi-X174-specific particles in the aerosol. In the small chamber system, a behavior analysis using this quantitative PCR method and a culture assay identified a humidity range within which viable airborne phages were inactivated. This observation may help to identify mechanisms that differentiate between the physical decrease and inactivation of viable airborne phages.

In chapter 4 and 5, to compare the collection efficiency of the filter for the phage used in the current standardized method with that of influenza virus, a safe method was developed. It involved quantitating an inactivated influenza virus aerosol with an antigen-capture enzyme-linked immunosorbent assay (ELISA). Using this ELISA, the collection efficiencies for aerosols of three types of spherical microbes (phi-X174 phages, an inactivated influenza virus, and *Staphylococcus aureus*) were tested. These results suggests that the standard test for evaluating the reduction of

airborne viruses using phi-X174 phages may overestimate the performance of filters compared to the penetration ratio for real pathogens such as the influenza virus.

In chapter 6, to verify whether the phage is a good surrogate virus when inactivating airborne viruses captured with a filter, some pathogens were compared with the phage by heat treatment, UV irradiation, or ethanol treatment. It was found that the phage was more resistant to inactivation than the real pathogen. The inactivation efficiencies of each method were also found to vary with different pathogen strains. This suggests that it is important to carefully evaluate the inactivation efficiency of each method, so as to select an appropriate virus strain as the inactivated pathogen for the standard inactivation efficiency test. It needs to be sufficiently resistant to the inactivation treatment.

In chapter 7, to improve the dry dispersion technique of airborne fungal spores in the current standard test, a new device was developed. A new device using glass beads for dry dispersion was successfully trialed in this study as a more versatile apparatus for use with the compact test chamber. Therefore, this newly developed technique may present an improved method for generating dry airborne fungal spores to use in the current standard performance test for air-cleaning products.

I believe that this study will help to accurately evaluate the performance of air-cleaning products against microbial aerosols and will contribute to a cleaner and safer indoor environment.

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