Development of a New Technique Using Glass Beads for Dry Dispersion of Airborne Fungal Spores

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To evaluate the removal of airborne microbes by air cleaners, a technique for generating airborne fungal spores in the dry state in a test chamber (dry dispersion) becomes necessary. The Society of Indoor Environment Japan (SIEJ) published SIEJ Standard Method No. 20110001 (SIEJ standard), in which an aerial ultrasonic oscillator was used as the device for dry dispersion. However, a more versatile apparatus is also necessary from a practical point of view. Therefore, we developed a new device using glass beads for the dispersion. Glass beads and a fungal sheet containing spores of Wallemia sebi were set in a midget impinger, which was connected to a compressor and a compact test chamber (1 m3). Air was blown into the impinger from the compressor. The spores on the fungal sheet were released by impingement of the glass beads when the beads were induced to float by the air blown into the impinger, and the spores were introduced to the chamber by the airflow. This newly developed technique can be used in a compact chamber system and could be applicable as an improved method for generating airborne fungal spores in the dry state in the SIEJ standard.

Key words : Airborne fungal spores / Dry dispersion / Glass beads / Impinger.

INTRODUCTION

Allergic diseases have long been an important national health issue. It has been reported that inhaling indoor allergens such as fungi or mites causes allergic rhinitis and asthma (Nambu et al., 2009; Salvaggio et al., 1981; Verhoeff et al., 1995). Thus, there is a consumer demand for equipment, such as air cleaners and vacuum cleaners, to effectively remove these indoor allergens.

The Society of Indoor Environment Japan (SIEJ) published a standard method to evaluate the removal of airborne fungi by air cleaners (SIEJ, 2012). In the SIEJ standard, a new technique for generating airborne fungi in the dry state was introduced, considering that fungal spores in the actual indoor space float in the dry state. Prior to the issuance of the SIEJ standard, test fungal spores were generally dispersed in a test chamber under the wet state. For example, spraying of a fungal spore suspension by a nebulizer has been historically used as a method for generating fungal spores (Lin and Li, 2003; Nakamura, 1987; Thomas et al., 2008). However, this wet state is different from the actual state of spores in the air. Thus, to reflect the actual conditions, a technique using both an aerial ultrasonic oscillator (referred to as the aerial ultrasonic technique) and fungal sheets containing ca. 107 spores of Wallemia sebi was introduced in the SIEJ standard.

However, the aerial ultrasonic technique is not easy to perform by non-skilled operators because the test operator must apply ultrasonic waves precisely at a
moist chamber. A saturated KNO₃ solution was used as a humidity control agent in the chamber. The cultured sheet contained 8.5 × 10⁶ CFU spores. To quantify the spores on the sheet, the sheet was washed in a stomacher and the spores in the wash solution were cultured for counting.

**Spore-dispersing device**

Fig. 1 shows the spore-dispersing device composed of a midget impinger containing glass beads and one fungal sheet. The fungal sheet was attached to the midget impinger using double-sided tape. Blowing of air into the impinger caused the glass beads to float. The spores were released from the sheet by the impingement of the floating beads.

![Fig. 1. Spore-dispersing device.](image)

The spore-dispersing device is composed of the midget impinger containing the glass beads and one fungal sheet. The fungal sheet was attached to the midget impinger using double-sided tape. Blowing of air into the impinger caused the glass beads to float. The spores were released from the sheet by the impingement of the floating beads.

In this study, we developed a new technique for the dry dispersion of spores with a simple device using glass beads. A device composed of a midget impinger containing glass beads and a fungal sheet of *Wallemia sebi* was prepared. Our developed technique is based on the idea that the shape of the impinger would be applicable for feeding the spores into a test chamber with the flow of air, although the apparatus (the impinger) has been until now primarily used for sampling aerosols in the air. Here, we report the appropriate conditions for the spore-dispersing device to introduce the fungal spores in the dry state in a compact test chamber (1 m³).

**MATERIALS AND METHODS**

**Test spores**

Spores were prepared according to the SIEJ Standard (SIEJ, 2012). A fungal sheet of *Wallemia sebi* J-155 strain (NITE BP-365) (Institute of Environmental Biology, Aikou-gun, Kanagawa) was cultured for 1 week at 25°C and 93.6% relative humidity (RH) in a moist chamber. A saturated KNO₃ solution was used as a humidity control agent in the chamber. The cultured sheet contained 8.5 × 10⁶ CFU spores. To quantify the spores on the sheet, the sheet was washed in a stomacher and the spores in the wash solution were cultured for counting.

**Spore-dispersing device**

Fig. 1 shows the spore-dispersing device composed of a midget impinger (diameter, 4 cm × height, 20 cm, based on JIS K 3800 : 2009) containing glass beads (10 g) and one fungal sheet (approximately 1.5 cm × 1.5 cm) after being cultured in a moisture chamber. The sheet was set with double-sided tape in the midget impinger at the central glass pipe, which had been closed. The bottle of the impinger was opened for blowing of the air. At the open site, a glass pipe to connect the tube from the air compressor and a glass filter to prevent the glass beads from falling were fused by heat. The glass beads and the midget impinger were subjected to dry heat sterilization for 30 min at 180°C prior to the experiments.

**Generating of spores in the test chamber**

Fig. 2 shows the arrangement of the test chamber and each device. Air from a compressor was fed into the midget impinger (spore-dispersing device) to disperse the spores in the test chamber. The airborne spores in the chamber were collected on the gelatin filter by actuating a suction pump.

![Fig. 2. Arrangement of the test chamber and each device.](image)

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In this study, we developed a new technique for the dry dispersion of spores with a simple device using glass beads. A device composed of a midget impinger containing glass beads and a fungal sheet of *Wallemia sebi* was prepared. Our developed technique is based on the idea that the shape of the impinger would be applicable for feeding the spores into a test chamber with the flow of air, although the apparatus (the impinger) has been until now primarily used for sampling aerosols in the air. Here, we report the appropriate conditions for the spore-dispersing device to introduce the fungal spores in the dry state in a compact test chamber (1 m³).
through the impinger was 5, 10, 15, or 20 L/min. The test was performed with each size of the glass beads (five types) and each airflow rate (four conditions), resulting in 20 trials. After the spores were dispersed, the compressor was stopped for 2 min. Subsequently, a suction pump was actuated to sample viable spores in the air. The airborne spores in the test chamber were collected on the gelatin filter.

A membrane filter (0.45 μm pore size) was connected to the test chamber for differential pressure control relative to the outside air. Two stirring fans were set at diagonally opposite corners inside the chamber. The particle counter and a fan filter unit were also set up in the chamber, and the tests were performed at 23—26°C and 50—60% RH.

**Particles in the test chamber**

To monitor the spreading of the spores in the test chamber, a light-scattering particle counter (MODEL 3886, Kanomax Japan, Osaka, Japan) was used. The particle counter received air at a flow rate of 2.83 L air/min and measured the number of particles with sizes of ≥0.3 μm, ≥0.5 μm, ≥1 μm, ≥3 μm, and ≥5 μm per 2.83 L of air, respectively. During all test periods, the particle counter monitored the concentration of particles in the test chamber. The number of particles in each size range was calculated by subtraction, i.e., 0.3—0.5 μm particles = ≥0.3 μm particles − ≥0.5 μm particles. Before the spores were dispersed, ≥1 μm particles were confirmed to have been removed from the test chamber by running the fan filter unit for approximately 10 min.

**Viable spores in the test chamber**

To determine the concentration of viable spores dispersed in the test chamber, a gelatin filter (12602-47-ALK, Sartorius, Gottingen, Germany) was used with a dedicated filter holder because this filter was reported to have high collection efficiency for microbial aerosols (Sudharsanam et al., 2012). The air suction rate of the suction pump connected to the filter holder for sampling of viable spores was 5 L/min for 4 min (20 L in total).

The gelatin filter on which the spores were collected was dissolved in 10 mL of saline supplemented with 0.05% Tween 80 (Polyoxyethylene (20) Sorbitan Monoooleate, Wako Pure Chemical, Osaka, Japan). This dissolved solution was diluted in ten-fold serial dilutions, and 0.1 mL of each serial dilution or undiluted solution was smeared onto each two plates of DG18 medium (Merck, Darmstadt, Germany). After cultivation for 7 days at 27°C, the fungal colonies on the plates were counted.

**RESULTS**

Table 1 shows the effects of changing the air flow rate and size of the glass beads. The glass beads were observed to be floating in the impinger under certain conditions.

In the case of glass beads with a diameter of 0.05 mm, floating was observed at an air flow rate of 5 L/min ("Floating" in the table). However, at air flow rates ≥ 10 L/min, the floating glass beads were observed to leak from the impinger into the test chamber ("Leaked" in the Table). For glass beads with a diameter of 0.1 mm, floating was seen at air flow rates of 5 L/min and 10 L/min, whereas the glass beads leaked into the chamber at air flow rates ≥ 15 L/min. In the case of glass beads with a diameter of 0.4 mm, floating was not observed ("Not floating" in the table) at an air flow rate of 5 L/min but floating was observed at air flow rates ≥10 L/min. For glass beads with a diameter of 0.8 mm, floating was also not seen at air flow rates ≤15 L/min, whereas floating occurred at 20 L/min. For glass beads with a diameter of 2 mm, floating beads were not

<table>
<thead>
<tr>
<th>Diameter of the glass beads (mm)</th>
<th>Rate of airflow into the spore-dispersing device (L/min)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>Floating**</td>
<td>Leaked***</td>
<td>Leaked</td>
<td>Leaked</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>Floating</td>
<td>Floating</td>
<td>Leaked</td>
<td>Leaked</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>Not floating**</td>
<td>Floating</td>
<td>Floating</td>
<td>Floating</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>Not floating</td>
<td>Not floating</td>
<td>Not floating</td>
<td>Floating</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Not floating</td>
<td>Not floating</td>
<td>Not floating</td>
<td>Not floating</td>
<td></td>
</tr>
</tbody>
</table>

*a* Floating: Glass beads were induced to float and impinge the fungal sheet by the blowing of air in the impinger. The floating of the glass beads could be confirmed by unaided visual inspection.  
*b* Not floating: Glass beads were not floating by the blowing of the air in the impinger.  
*c* Leaked: Glass beads leaked from the impinger into the test chamber.
of the fungal sheets were weakened similarly.

In the conditions when the glass beads were observed to float in the impinger, the concentration of 1–3 µm and 3–5 µm particles increased mainly in the test chamber (Fig. 4a). These data were obtained with 0.4 mm glass beads and an air flow rate of 10 L/min.

Table 2 shows the amount of viable spores (CFU/1,000 L of air) in the test chamber detected in each floating condition. The amount of the spores in these conditions reached 1.4–1.7 × 10^5 CFU/1,000 L of air in the chamber. The amount of spores under all conditions in Table 2 were higher than 0.6–3.0 × 10^4 CFU spores per 1,000 L of air in the SIEJ standard (Abe et al., 2007), i.e., it was sufficient to generate airborne fungal spores in the dry state in a test chamber. In contrast, the amount of viable spores was under the lower detection limit (<500 CFU/1,000L of air) in the conditions when the beads were not floating.

Fig. 3 shows a fungal sheet before and after impingement of glass beads, with a diameter of 0.4 mm at 10 L/min. The color on the surface of the fungal sheet of Wallemia sebi was weakened, which indicates that the spores were separated from the sheet. After impingement under other floating conditions, the colors of the fungal sheets were weakened similarly.

Therefore, we attempted to collect airborne fungal spores in the test chamber under the floating condition. Table 2 shows the amount of test spores detected (CFU/1,000L-air) in the test chamber detected in each floating condition. The amount of viable spores in these conditions reached 1.4–1.7 × 10^5 CFU/1,000 L of air in the chamber. The amount of viable spores in each floating condition in Table 2 was higher than 0.6–3.0 × 10^4 CFU spores per 1,000 L of air in the SIEJ standard (Abe et al., 2007), i.e., it was sufficient to generate airborne fungal spores in the dry state in a test chamber. In contrast, the amount of viable spores was under the lower detection limit (<500 CFU/1,000L of air) in the conditions when the beads were not floating.

![FIG. 3. Fungal sheet before and after impingement of glass beads. Before impingement, the fungal sheet contained spores of Wallemia sebi. After impingement with 0.4 mm glass beads at an air flow rate of 10 L/min, the spores were set free from the sheet.](image)

When glass beads were induced to float and to impinge the fungal sheet by the blowing of air into the impinger, the amount of test spores was measured by collecting the airborne fungal spores.

![FIG. 4a. Concentration of particles classified according to the particle size when the fungal sheet was impinged using the glass beads. Particles sized 1–3µm and 3–5µm were counted as major particles. These data were obtained with 0.4 mm glass beads and an air flow rate of 10 L/min.](image)

![FIG. 4b. Concentration of particles before the blowing of air classified according to the particle size. Particles sized 1–3µm, 3–5µm, and ≥5 µm were not counted in the test chamber before the blowing of air.](image)
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icles, the $0.5 - 1 \mu m$ particles and $\geq 5 \mu m$ particles were not highly associated with viable spores ($R^2 = 0.76$ for $0.3 - 0.5 \mu m$ particles, $R^2 = 0.77$ for $0.5 - 1 \mu m$, $R^2 = 0.65$ for $\geq 5 \mu m$ particles) (Fig. 5b).

**DISCUSSION**

Our results indicate that test spores were generated in the test chamber when floating of the glass beads was observed in the device (Fig. 1) and also that the most appropriate size of the glass beads for the dispersion was $0.4 \text{ mm}$, with an air flow rate of $10 - 20 \text{ L/min}$. In addition, spores in the test chamber were detectable in real-time using the particle counter. Therefore our developed technique using glass beads with an impinger was easy to operate and versatile. Moreover our glass bead technique has the advantage of a shortened preparation period for the fungal sheets because the drying step of $3 \text{ days}$ at $43 \% \text{ RH}$ specified in the SIEJ standard can be omitted.

It should be noted that the amount of viable spores in the chamber reached $1.4 - 1.7 \times 10^5 \text{ CFU/1,000 L of air}$ (1.4 $- 1.7 \times 10^5 \text{ CFU in one test chamber}$), regardless of the air volume that passed through the impinger during $2 \text{ min}$ of air flow with different airflow rates ($5, 10, 15, \text{ and } 20 \text{ L/min}$). Separation of spores from the fungal sheet by impingement with the floating glass beads in the impinger was thus completed in a short time. It could thus be considered that the airborne spores were generated rapidly and in a stable amount through monodisperse particles released by the spore-dispersing device.

We compared our glass bead technique to the aerial ultrasonic technique based on the SIEJ standard with regard to the amount of spores derived from the fungal sheet. In the SIEJ standard, $0.6 - 3.0 \times 10^2 \text{ CFU per 10 L of air}$ ($1.4 - 1.7 \times 10^5 \text{ CFU in one test chamber}$) that is, $0.6 - 3.0 \times 10^5 \text{ CFU}$ were generated from one fungal sheet by the aerial ultrasonic technique. Therefore, our technique using glass beads (generating $1.4 - 1.7 \times 10^5 \text{ CFU from one fungal sheet}$) provided a nearly identical amount of spores compared with the aerial ultrasonic technique. Additionally, it is possible to increase the amount of airborne spores in the chamber by increasing the number of spore-dispersing devices.

Moreover, we analyzed the size of the particles associated with the amount of viable test spores because the particles by the particle counter might have contained not only viable spores but also non-viable spores and debris from the spores. Fig. 5a and Fig. 5b show the correlation between the particle concentration and the amount of viable spores in the chamber (1,000L). These data were analyzed by simple linear regression. The $1 - 3 \mu m$ particles and the $3 - 5 \mu m$ particles were highly associated with viable spores ($R^2 = 0.90$ for $1 - 3 \mu m$ particles, $R^2 = 0.96$ for $3 - 5 \mu m$ particles) (Fig. 5a). In contrast, the $0.3 - 0.5 \mu m$ particles and $\geq 5 \mu m$ particles were not highly associated with viable spores ($R^2 = 0.76$ for $0.3 - 0.5 \mu m$ particles, $R^2 = 0.77$ for $0.5 - 1 \mu m$, $R^2 = 0.65$ for $\geq 5 \mu m$ particles) (Fig. 5b).
method, further investigation should be done to provide increased accuracy and reproducibility as well as statistical analysis when generating airborne fungal spores in the specified test chamber using our glass bead technique.

REFERENCES


