INTRODUCTION

Various problems have emerged in the control of hospital-based viral infections, including nosocomial outbreaks of Ebola virus, Middle East Respiratory Syndrome Coronavirus, hepatitis B virus (HBV), and norovirus (Büchner et al., 2015; Hsieh, 2015; Kilmarx et al., 2014; Saegeman et al., 2015). Healthcare workers (HCWs) must wear appropriate personal protective clothing (PPC) when caring for infected patients in various situations. Therefore, it is important for HCWs to understand PPC performance including some evaluation methods for selection of appropriate PPC according to risk assessment.
Standard methods for evaluating PPC performance have been established by the International Organization for Standardization (ISO). ISO 16603 and ISO 16604 are standard test methods to evaluate the resistance of fabrics to synthetic blood and virus penetration with pressure considering direct contact transmission as the infection route (ISO 16603, 2004; ISO 16604, 2004). The penetration resistance of a fabric is determined based on leakage at different pressures increased step-wise. In Japan, similar methods have been published as the Japanese Industrial Standard (JIS) (JIS T 8060, 2007; JIS T 8061, 2010). The penetration resistance JIS test using synthetic blood with pressure (JIS T 8060, 2007), which is equivalent to ISO 16603, is commonly required for all types of PPC in JIS (JIS T 8122, 2015). PPCs made of various materials certified by JIS-recommended tests are used widely in hospitals and during emergencies (Shinohara and Shimasaki, 2012). HCWs or infection control teams usually select appropriate PPC on the basis of such information on protection performance.

However, penetration of liquid drops without pressure or splash through PPC is also a major route of blood or body fluid exposure. When HCWs care for patients, splash or liquid drops of patient’s blood or body fluids may come in contact with the HCWs’ PPC and can penetrate naturally into the fabric of PPC. Our previous paper has already reported that some protective clothing fabrics had different penetration resistance against splash of synthetic blood from that against synthetic blood with pressure by JIS T 8060 test (Shimasaki et al., 2017). Thus, further studies are needed to evaluate liquid-drops-route of transmission for barrier properties of PPC fabrics. As close studies, penetration resistance against bacterial liquid drops without pressure on hospital gowns or drapes has been studied previously (Blom et al., 2007; Lankester et al., 2002; Morimoto et al., 2005). However, no studies have examined the penetration resistance against viral liquid drops (VLDrop) without pressure, necessitating further studies because particles of virus are smaller and may penetrate more easily than that of bacteria.

In this study, we evaluated penetration resistance of PPC fabrics against VLDrop without pressure using phi-X174 phage, which is a virus used in ISO 16604 and JIS T 8061, to investigate the detailed penetration behaviors of VLDrop. After VLDrop was left on the fabric for a certain time, we performed culture assays to quantify the phage amounts leaking through the fabrics and migrating into the fabrics. Furthermore, we analyzed the physicochemical characteristics of PPC fabrics (thickness and contact angle) and the particle size distribution of phi-X174 in the test liquid to elucidate the factors affecting the penetration of VLDrop.

**Test fabrics**

Eight fabrics used in commercially available PPC and two fabrics (samples #1 and #6) as test controls were selected for this study (Figure 1). These samples (total ten fabrics) had been tested for penetration resistance to synthetic blood and grouped into specific classes in response to applied pressure according to ISO 16603 (ISO 16603, 2004), indicating that higher class fabrics withstand higher pressure, as follows: woven samples #1, #2, and #3 were class < 1, whereas #4 and #5 were class 1; nonwoven sample #7 was class < 1; #6, #8, and #9 were class 1; and sample #10 was class 3 (Shinohara and Shimasaki, 2012). In addition, multi-layer materials classified into class 6 such as laminated nonwoven or woven were not tested by a multifaceted leakage evaluation in this study because it has been confirmed that phage leakage of the class 6 samples was not detected at all under applied pressure in our previous paper (Shimasaki et al., 2016). Their surface images were observed using a stereoscopic microscope. Test samples were cut into squares measuring 3.5 x 3.5 cm² for test use.

**Phage preparation and assay of phage titer**

The phi-X174 phage (ATCC 13706-B1) and host *Escherichia coli* (ATCC 13706) were used as previously described (Shimasaki et al., 2016) with modifications. In brief, these two components were mixed and incubated in Nutrient Broth containing 0.5% NaCl and 2 mM CaCl₂ (NaCl-Ca-NB) at 35°C overnight. The lysate was filtered using a 0.22-µm membrane filter (MF). The filtrated suspension was diluted with NaCl-Ca-NB (1:10) to be used as challenge suspension (CS) containing about 5 x 10⁶ PFU/mL of phi-X174.

The phage titer was determined using soft-agar-overlaid plaque assays. The assay fluid was diluted 10-fold with NaCl-Ca-NB, and 0.2 mL of each serial dilution or 1 mL of undiluted assay fluid mixed with an equal volume of host *E. coli* were added to tubes containing NaCl-Ca-NB with 0.5% agar (10 volumes). After gentle mixing, the mixture was poured onto two plates of Nutrient Agar. The plaques on the plates were counted after cultivation for 18 h at 35°C.

**Test procedure**

Each fabric was tested independently five times at room temperature (RT) using CS maintained at RT. First, the test samples were placed on 6-cm petri dishes for tissue culture whose surface was physically subjected with hydrophilic treatment and has high wettability to VLDrop. Next, phi-X174 CS (100 µL) was dropped onto the samples. It seemed visually that there

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was no gap between the fabric sample and the surface of the petri dish owing to weight of phi-X174 CS drop. After 20 min, the VLDrop remaining on the sample was collected in a microtube using a micropipette, and the phage titer was assayed. The fabric sample was then transferred into a tube with 3 mL NaCl-Ca-NB and agitated by vortexing for 30 s to wash out the phage from the test fabric. The wash-out solution was filtered using a 0.22-µm MF to remove bacterial contamination derived from the fabric sample. The phage titer was then assayed as the amount of VLDrop that penetrated and migrated into the test fabric, which is described as migration amount. Finally, the dish was washed with 3 mL NaCl-Ca-NB by pipetting 10 times. The wash solution was also filtered with a 0.22-µm MF, and the phage titer was assayed as the amount of VLDrop that penetrated and leaked through the test fabric. The detection limit of the phage titer was less than 3 PFU per 3 mL of NaCl-Ca-NB. The contact time of VLDrop on the test samples was fixed at 20 min to match the maximum contact time for class 3 fabrics in ISO 16603 tests. The surface tension of NaCl-Ca-NB was 0.0502-0.0546 N/m at RT, similar to that of human blood (Centers for Disease Control and Prevention, 2018). In addition, it has been confirmed by preliminary experiments for sample #3 that VLDrop penetrated time-dependently into the test fabric since the average of leakage amounts (unit: log10 PFU/fabric) were examined twice to be < 0.48 at 10 s, < 0.48 at 5 min, and 3.0 ± 0.52 at 20 min. Furthermore, experimental conditions of this study are taken consideration that the blood from a Hemophilia patient during surgery penetrates into

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Five woven fabrics and five nonwoven fabrics were tested. The properties of the fabrics, including sample name, usage, fiber, type, treatment, and classification are shown. Classification was conducted according to resistance to penetration using synthetic blood based on ISO 16603.Thicknesses were measured, and the average (Ave.) and coefficient of variation (C.V.) were calculated. Surface images were observed. Scale bar: 5 mm.

| Construction | Woven | #1 | #2 | #3 | #4 | #5 |
|--------------|-------|----|----|----|----|----|
| Sample name  | Medical scrub | Surgical gown | Surgical gown | Surgical gown | Surgical gown |
| Usage        | Polyester 50% | Cotton 100% | Polyester 65% | Cotton 35% | Polyester 65% |
| Fiber        | Plain | Drill | Weather | Twill | Weather, High density |
| Type         | Untreated | Absorbance | Water repellent | Water repellent | Water Repellent |
| Treatment    | Class <1 (Leaked at 0 kPa) | Class <1 (Leaked at 0 kPa) | Class <1 (Leaked at 0 kPa) | Class 1 (No leaks at 0 kPa) | Class 1 (No leaks at 0 kPa) |
| Thickness    | Ave. (mm) | 0.292 | 0.468 | 0.314 | 0.421 | 0.221 |
|              | C.V.   | 1.1% | 1.8% | 1.5% | 1.0% | 0.4% |

![Surface image](image.png)

| Construction | Nonwoven | #6 | #7 | #8 | #9 | #10 |
|--------------|----------|----|----|----|----|-----|
| Sample name  | Drape | Isolation gown | Surgical gown | Surgical gown | Coverall |
| Usage        | Polynpropylene 100% | Polynpropylene 100% | PET45% Wood Pulp 55% | Polynpropylene 100% | Polynpropylene 100% |
| Fiber        | Spunbond/meltblown/s | Spunbond | Spunbond | Spunbond/meltblown/ | Flash spun |
| Type         | (SMS) | (SMS) | (SMS) | (SMS) | (SMS) |
| Treatment    | Untreated | Untreated | Water repellent | Untreated | Untreated |
| Classification of the penetration resistance to synthetic blood with pressure based on ISO 16603 | Class 1 (No leaks at 0 kPa) | Class <1 (Leaked at 0 kPa) | Class 1 (No leaks at 0 kPa) | Class 1 (No leaks at 0 kPa) | Class 3 (No leaks to 3.5 kPa) |
| Thickness    | Ave. (mm) | 0.420 | 0.200 | 0.284 | 0.314 | 0.158 |
|              | C.V.   | 8.1% | 17.0% | 5.4% | 6.3% | 15.4% |

![Surface image](image.png)
the fabric material of PPC without coagulation for 20 min (Nogami, 2014), as a risky case of hospital-based viral infections. It has been reported that not a few of Hemophilia patients treated with unheated blood products before 1985 were got iatrogenic HIV, HCV, and HBV infection (Nishida et al., 2007).

**Thickness of fabric samples**
The thicknesses of fabric samples shown in Figure 1 were determined as the total average of duplicate measuring the average of five values except maximum and minimum ones with a dial thickness gage SM-114 (TECLOCK Co., Nagano, Japan).

**Contact angle of the liquid on fabric samples**
The contact angles of the CS drops (approximately 4 µL) on the fabric sample were determined as the average by measuring more than three times with the sessile drop method using a contact angle meter CA-X (Kyowa Interface Science). Those of CS without phage (i.e., NaCl-Ca-NB only) were measured similarly. The contact angle was shown in Figure 2 (Sanuki, 1971).

**Transmission electron microscopy (TEM)**
As our previous paper described (Shimasaki et al., 2018), the phi-X174 of CS was negatively stained with 2% phosphotungstic acid. TEM images were obtained using a JEM-1400 transmission electron microscope (JEOL Ltd.).

**Dynamic light scattering (DSL)**
As our previous paper described (Shimasaki et al., 2018), the particle size distribution of phi-X174 in CS was measured at RT with a Model 802 DLS system using Omni Size 3.0 (Viscotek).

**Statistical analysis**
Statistical analysis was performed using GraphPad Prism version 6.0e (GraphPad Software, Inc.). The significance level was set at 0.05. The leakage and migration amounts of phage for the test fabrics were analyzed statistically using Dunnett’s multiple comparisons test. Correlations between the leakage amount and migration amount were analyzed using single regression analyses. Contact angles of CS with or without phage were analyzed by Student’s t-tests. Correlation analyses were performed for thickness and the cosine of the contact angle using Spearman’s rank method.

**RESULTS**

**Evaluation of phage leakage through the test fabrics**
For #1 and #2 fabrics, the VLDrop completely migrated into the fabric samples. For the other fabrics, the VLDrop remained on the samples, and the phage titers were 3.0-8.5 × 10^8 PFU/mL. The leakage amount was determined in five independent tests for each fabric (Figure 3). For woven materials, #1, #2, and #3 fabrics
exhibited average leakage amounts of over 2 log_{10} PFU/fabric, whereas those for #4 and #5 (class 1) were under 2 log_{10} PFU/fabric, respectively; thus, the fabrics of class 1 had higher penetration resistance than those belonging to class < 1. Notably, the leakage amount for #4 was not detected and was significantly lower than those for #1, a test control of woven. Incidentally, even though sample #2 had the highest leakage (higher than #1), liquid leakage through the sample was not observed visually on the dishes.

In contrast, for nonwoven samples, #6, #7, #8, and #9 (class < 1 or 1) showed leakage amounts ranging from 1 to 2 log_{10} PFU/fabric, similar to that of sample #10 which is a class 3 fabric. There was no significant difference among the samples, although the other samples had smaller leakage amounts than #6, a nonwoven test control fabric. Fabrics in lower classes based on ISO 16603 tests (with pressure) did not always have lower penetration resistance against VLDrop (without pressure).

Furthermore, we speculated that large variations in the leakage amount for nonwoven samples (#6, #7, #8, #9, and #10) were due to the large C.V. in thickness of these nonwoven samples (Figure 1). The leakage test in this study is considered to be sensitive to local differences of the sample because it does not evaluate the sample by a large area as ISO test. Especially sample #10 is the thinnest of all samples, however, has 15.4% of C.V. in thickness, and some unevenness in thickness of the sample could be observed visually. Moreover, we speculated that large variations in the leakage amount for #5 was due to the fiber structure of #5, whose fiber includes antistatic threads (Figure 1) that seems to pass through the both surface of the fabric partially in polyester. Such a fiber structure deferent from the other woven samples might cause variations in the amount of leak.

Correlation analysis of phage leakage and migration amounts

To investigate the factors associated with phage leakage, the migration amounts of the phage into the test fabrics were analyzed. The migration amounts for #3, #4, and #5 were significantly lower and those of #2 were significantly higher than those of #1, and there were significant differences among #6, #8, and #9 (Figure 4 (a)). These data suggested that differences among fabrics could be easily detected using the migration amounts. Furthermore, the leakage amount was significantly correlated with the migration amount by single regression analysis ($r = 0.73, p = 0.017$; Figure 4 (b)). These data revealed that the migration amount could be an important factor affecting the penetration resistance of fabrics against VLDrop without pressure. In addition, it has been confirmed in preliminary experiments that

**FIG. 4.** Correlation analysis of leakage amount and migration amount of phage. (a) Migration amount of phage absorbed into the test fabrics during the penetration test was also assayed by agitating the test fabric with NaCl-Ca-NB to wash out the phage from the test fabric. The bar graph represents the average ± standard deviation (n = 5) of the migration amount. Data were analyzed statistically by Dunnett’s multiple comparisons tests. **,** Significance probability $p < 0.01$, ****; $p < 0.0001$. (b) The correlation between the leakage amount and migration amount was analyzed by single regression analysis. r: Correlation coefficient, $p$: Significance probability.
the migration amount can be recovered approximately 100% from the material by our test procedure, even for the sample #2 with the highest migration amount of phage (data not shown).

Next, the thickness of the test fabrics (Figure 1) was analyzed. Thickness was not correlated with leakage amount (Spearman $r = 0.37$, $p = 0.30$; not significant).

**Physicochemical analysis of the test fabrics and the phi-X174 phage CS**

To identify textile factors associated with the migration of phage solution, we investigated the contact angle of the test liquid on each fabric (Figure 5(a)). For samples #1 and #2, which had the highest migration, the contact angles were zero. In contrast, the contact angles of the other samples were over 90°. Even nonwoven fabrics not treated with water-repellent (#7, #9, #10) had fluid-repellent effects. Moreover, correlation analysis showed that the cosine of the contact angle was significantly correlated with the migration amount (Spearman $r = 0.84$, $p = 0.0039$) as a previous study reported that the cosine of the contact angle was associated with wettability, inducing liquid penetration (Bico et al., 2002). Since the contact angle did not change significantly regardless of the presence or absence of the phage, these findings revealed that the contact angle mainly depended on the carrier (i.e., NaCl-Ca-NB) of CS.

In addition, to analyze how the phi-X174 phage exist in the carrier of CS, the dispersed state of the phage particles in the CS (e.g., any aggregation of the particles) was confirmed by DLS (Figure 5(b)). One major sharp peak (13.9 nm) and one negligible peak (60.9 nm) were observed in the hydrodynamic radius. Thus, the hydrodynamic diameter of the major peak particles was estimated to be 27.8 nm, which was similar to the particle size of the phi-X174 observed by TEM (25–30 nm; Figure 5(c)). The results showed that the phi-X174 particles were typically dispersed without aggregation in the carrier of CS.
DISCUSSION

In this study, we evaluated the protection performance of VLDrop on PPC fabrics under a different test condition from the conditions used by the ISO. Our results revealed that the penetration resistance on nonwoven fabrics without pressure was not always correlated with the ISO resistance class. Therefore, our results suggested that the penetration mechanism of VLDrop without pressure might be different from that with pressure, as is evaluated in ISO tests.

Our findings showed that the migration amount affected the leakage amount without pressure and was correlated with the cosine of the contact angle of the phage CS on the fabrics. It indicated that penetration resistance of the fabric against VLDrop without pressure was indirectly affected by fluid repellence, as was highlighted as an important factor in a previous review (Mitchell et al., 2015). Since there was no significant difference in the contact angle in the presence and absence of the phage, these findings suggested that the interaction between the fabrics and the carrier itself (NaCl-Ca-NB) could contribute to the penetration of VLDrop into the fabric. Furthermore, the carrier was thought to physicochemical mimic human body fluids for evaluation of PPC performance because it had a surface tension similar to that of human blood (Centers for Disease Control and Prevention, 2018). The DLS result (Figure 5(b)) and the surface images of test fabrics (Figure 1) speculated that the penetration phenomenon of the phage might be driven by the movement of the carrier of CS containing the monodispersed phage particles into gaps of the fibers, which may be larger than the phi-X174 particles. Bico described that penetration movement of liquid on a textured surface was induced by a force associated with the cosine of the contact angle (Bico et al., 2002).

Our findings can be useful for PPC manufacturer because improvement of penetration resistance against VLDrop was due to the effects of low migration of human body fluids and fluid-repellency on PPC fabric. Moreover, our evaluation method is simple and applicable to actual performance checks by HCWs. In fact, HCWs cannot use ISO methods to confirm the performance of PPC by referring to catalogue information because ISO methods require dedicated test devices for applying pressure and lack versatility. Our test method of VLDrop penetration resistance can be used by HCWs to evaluate the reduction of barrier properties of the fabrics during re-use in their hospitals, because Schwartz reported that the barrier properties of the textiles were reduced (Schwartz and Saunders, 1980).

Additionally, this evaluation by HCWs of PPC protective performance could provide HCWs with insights into the selection of appropriate PPC to mitigate the risk of infection. For example, the phage inoculum amount on the fabrics in this study ($3.0-8.5 \times 10^7$ PFU/100 µL) is similar to HBV concentrations ($7.5 \times 10^5 - 4.3 \times 10^6$ copies/mL) in whole blood of infected patients (Cao et al., 2011), considering 1 PFU of phage has one copy. If some PPCs are exposed to infected blood of patients with HBV infection, HCWs can predict a risk that contaminated blood may leak through PPC made of fabric #10 even if it was classified to ISO class 3.

This study has some limitations. The phi-X174 phage used in our evaluation is a surrogate virus, on the assumption that phi-X174 phage particles would penetrate into the fabric similarly to real pathogens in human body fluids including blood. However, pathogens may not always show similar penetration behavior to the phage in CS in biological sight. Because human body fluids have various protein components and cells, the real pathogen with the various viral surface proteins that are directed to cellular receptors may interact cells in human body fluids and may show the different dispersed state in human body fluids. Thus, these properties of the pathogens may also affect the penetration behaviors of pathogens in the fabrics, mentioning possibility of the need to validate our findings using human body fluids and real pathogens with similar particle size to the phage, such as Hepatitis A virus (Volkin et al., 1997; Shimasaki et al., 2009).

In conclusion, our multifaceted evaluation may help to improve our understanding of the characteristics of PPC fabrics, thereby enabling HCWs to select the appropriate PPC according to the specific hazardous situation and infection risk.

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