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RESEARCH

Microbial Contamination and Isolator Gloves: If It All Came Down to the Size of a Hole?

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ABSTRACT: Isolators play a critical role in protecting both the product and the environment, as well as the personnel involved in pharmaceutical manufacturing, analytical procedures, and sterility testing. Gloves attached to the windows and doors of the isolator are designed to facilitate intervention, testing, and safety. However, due to their inherent characteristics and vulnerability to puncture or loss of integrity, they are recognized as a significant potential source of contamination. In addition to the possible pathways of contamination transfer, the size of glove holes plays a critical role in determining the risk of contamination. In this study, chlorosulphonated polyethylene (CSM) gloves were exposed to an aerosol containing *Bacillus subtilis* or *Staphylococcus aureus*. This assessment aimed to ascertain the integrity of the gloves' seal. It was postulated that, below a certain aperture size, gloves used in isolator systems could establish an effective seal even if the external surface of the gloves exhibited modifications. Calibrated holes of different diameters (0.3, 0.5, 1, and 1.5 mm) were created using a femtosecond laser drilling technology. The holes were located on the tip of the middle finger. Based on the context of our study, passage of microorganisms through glove holes of a certain size does occur. Under the experimental conditions chosen, the cutoff for passage was determined to be a 0.5-mm hole, regardless of the microorganism evaluated. Although this study has some limitations, including the lack of a panel of microorganisms evaluated and the investigation of a single glove type called CSM, the high level of "worst case" challenge conditions provides compelling data to support our results. It would now be interesting to carry out studies at different production sites to assess their risk of contamination and relate this to their glove failure.

KEYWORDS: Gloves, Holes, Isolators, Microorganisms, Visual inspection, Cutoff.

Introduction

Barrier systems, such as isolators or restricted access barrier systems (RABS), play a fundamental role in protecting people and products in both pharmaceutical manufacturing and analytical and sterility testing (1). Gloves are a critical interface between the internal environment of the isolator and the operator. By providing such physical barriers, they are used to perform 'manual' inherent and/or corrective actions, creating the interactions within the Grade A environment, a zone for high-risk operations requiring the highest level of environmental control. Glove integrity is therefore essential to avoid any risk of contamination. Regular inspection of this integrity is required by many regulations. The US Food and Drug Administration (2), for example, stipulates that "a defective glove or sleeve represents a contamination pathway and a critical breach in the integrity of the isolator". In addition, employees must visually inspect gloves for damage before each use, and physical tests must be carried out regularly, including visual inspection. Similar requirements are set out in Annex 1 of the EU's Good Manufacturing Practice (GMP) (3).

Indeed, barrier technologies are widely recommended in this new version to avoid any direct interaction

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between operators—known to be the main source of potential contamination—and the critical steps in the sterile product manufacturing process. To this end, isolators require gloves to be inspected regularly according to their use, including a visual inspection each time they are used and after any handling that could affect the integrity of the system. In addition, isolation system integrity and tightness tests must be carried out at defined intervals. For RABS, gloves used in a Grade A area should be sterilized before use and sterilized or effectively biodecontaminated by a validated method before each manufacturing campaign. Gloves should be visually inspected at each use, and integrity tests should be performed at regular intervals.

One of the key challenges for pharmaceutical manufacturers is to define their own standard operating procedures (SOPs) for glove testing in line with these guidelines and to set up an appropriate process for their facilities based on a risk analysis. Microbiological surface testing of isolator gloves can be performed in real time during production, but the results are known only after production. Real-time decision-making must therefore be based on robust scientific data on critical glove attributes. To the best of our knowledge, scientific data on this subject is scarce. In fact, using the National Library of Medicine's PubMed site with the keyword "gloves, holes", only 83 publications have been published since 1960. By comparison, 120,232 articles have been published in the same period using the keyword "microbiota".

Previous studies on the risks of contamination transfer through holes in barrier isolator technology gloves have helped to understand the risks associated with realistic bioburden challenges of surface-to-surface contamination transfer and airborne contamination from handling contaminated test surfaces. The reference study is by Gessler et al. (1), which quantified the relevance of holes to the potential risk of contamination of work or product processed in isolators. At the same time, a comparison was made of different physical methods for testing glove integrity (flow test, pressure drop test, water breakthrough test, particle penetration test, diffusion test, and visual inspection). In this study, gloves made of chlorosulphonated polyethylene (CSM) were tested, with leaks prepared using a syringe needle of 0.4, 0.6, or 0.8 mm outer diameter, focusing on the microorganism *Brevundimona diminuta*. Although a high bioburden on the inside of defective gloves represents a contamination risk to the product as well as to the interior of the isolator (Grade A), the authors suggested that the bioburden found on the inside of gloves in practice is typically at low concentrations of < 20 CFU.cm^{-2}.

Some authors, such as Sandle (4) or Maier and Drinkwater (5), have shown that there is a momentary increase in positive pressure during glove entry. In fact, gloves can develop momentary pressure increases as the operator's hand passes through the wrist area and then enters the finger pockets (5). This momentary pressurization creates a jet stream of high velocity incoming air from the environment, potentially carrying both airborne particles and microbial contamination. Therefore, multiple pathways of contamination transfer play a critical role in determining the risk of contamination, suggesting that specific and accurate tests should be used to monitor the integrity of gloves during their use in critical applications such as pharmaceutical manufacturing to ensure that the environment remains sterile and free of contamination.

In a preliminary study initiated by the A3P Barrier Technology Interest Group (6), CSM gloves were exposed to an aerosol containing the microorganism *Geobacillus stearothermophilus* to test their seal. The authors hypothesized that isolator gloves could maintain a seal in the presence of a hole, provided it was below a certain size. *Geobacillus stearothermophilus*, a Gram-positive, spore-forming thermophilic bacterium, was chosen because it can grow and multiply at a selective temperature that is not conducive to the growth of any other organism, thus eliminating the possibility of false positives caused by contamination with other microorganisms (7). The results showed that below a hole size of approximately 0.6 mm, defective gloves had a low probability of presenting a high microbiological risk due to the passage of microorganisms from Grade C to Grade A. There is widespread expert agreement on the need for comprehensive glove integrity testing. While visual inspection remains a critical first step in glove integrity monitoring, it has limitations (8). The human eye can typically detect defects no smaller than 0.4–0.5 mm in diameter. In contrast, advanced glove integrity testing systems can detect holes as small as 0.1 mm, significantly improving detection capabilities.

Therefore, it seems important to complement our previous study by not only focusing on smaller glove holes (hardly detectable by operators), but also by increasing the size of glove holes to a range at which they could be detected by visual inspection, with the aim of this

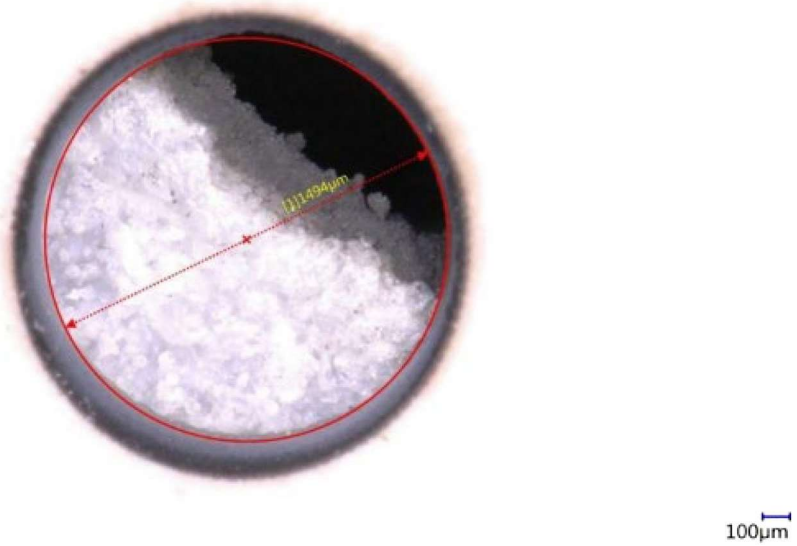

Figure 1

Keyence microscope image of a 1.5 mm hole (magnification x100).

investigation to be as close as possible to pharmaceutical production conditions, to assess the risk of microorganisms entering the inner surface of gloves. The selection of microorganisms should be representative of the production environment. Therefore, the aim of this study was to expose isolator gloves (CSM) to aerosol containing *Staphylococcus aureus* or *Bacillus subtilis* to test their impermeability. *Staphylococcus aureus* is representative of a well-known human-born type of Gram-positive cocci microorganism. The *Staphylococcus* genus can be a major cause of contamination of pharmaceutical products due to poor handling or aseptic processes and protocols. It can be transmitted in a variety of ways, including airborne droplets or aerosols and direct contact with contaminated objects (food, water, and so forth). *Bacillus subtilis* is a common source of environmental contamination in laboratories and is regularly found on environmental settle plates and in air monitoring. In addition, its ability to form highly resistant spores makes it ubiquitous and widely distributed in the environment (8).

Materials and Methods

Holes in Gloves

Eighty-two CSM gloves (size: 8; thickness: 0.6 mm) were tested. Four hole sizes were produced. The experiment was performed 10 times for a given hole size. Calibrated holes of different diameters (0.3, 0.5, 1, or 1.5 mm) were made using femtosecond laser drilling technology. The holes were positioned on the tip of the middle finger. Hole size measurements were verified after drilling using microscopic techniques (Zeiss Axio Scope Vario for 0.3 and 0.5 mm holes; Keyence VHX-5000 for 1 and 1.5 mm holes). Figure 1 shows a Keyence microscope image of a 1.5 mm hole (magnification x100). These holes, even if artificially created, represent a worst-case scenario. Typically, holes in gloves are more likely to be small cracks rather than perfectly round holes. Two gloves without holes were used as negative controls to ensure that the glove was watertight in the absence of a defect. One glove was tested for each strain.

Preparation of the Suspension of Staphylococcus aureus ATCC 6538 and Bacillus subtilis ATCC6633

A stock suspension of *Bacillus subtilis* spores at 10^9 CFU.mL^{-1} was diluted to 1/100th in physiological water (EPHY), which was used as the dilution medium; 0.1 mL of the suspension was added to 9.9 mL of EPHY to obtain a working suspension. Then 7 mL of this suspension was taken in a resealable tube and nebulized in one of the two isolator chambers (Figure 1), and the remaining 3 mL of suspension was kept for the counting steps, knowing that the final CFU count was 7×10^7. The mass median aerodynamic diameter (MMAD) indicated by the nebulizer used was of 4.7 µm, where MMAD means that 50% of the particles in the aerodynamic size distribution, based on mass, are above and below this diameter.

Staphylococcus aureus from a subculture was placed into a tube containing 9 mL of buffered peptone water

at a concentration of 10^7 CFU.mL^{-1}; 7 mL of this suspension was removed and used for nebulization in the isolator, while the remainder of the suspension was retained for counting.

Nebulization of the Suspension

The tests were carried out using "double isolators", which meet pharmaceutical industry qualification standards (Figure 2). The two chambers communicate via an airlock, with one of the two doors acting as a support for the glove being tested. The chamber on the right was used to nebulize the bacterial suspension, while the chamber on the left was used to sample any microorganisms that may have passed through. The glove to be tested was placed on the wrist ring of the left door of the transfer lock between the two isolators, with the glove hole facing the back wall. After predecontaminating the glove with isopropyl alcohol, the two isolators were decontaminated with vapor phase hydrogen peroxide (VPHP) for 20 min, maintaining a pressure of 20 Pa in each isolator and an air flow rate close to 50 m^3.h^{-1}. VPHP decontamination was then stopped, and a ventilation period was allowed to reduce the VPHP concentration to below 1 PPM. The nebulizer tank was filled with 7 mL of the *Staphylococcus aureus* ATCC 6538 or *Bacillus subtilis* ATCC 6633, and the nebulization was started in the right-hand side isolator. Once the nebulizer tank was empty, the pressure in the right chamber was increased to 500 Pa to mimic the overpressure induced by inserting the hand into the glove and to facilitate the passage of the bacterial cells through the glove hole.

During the nebulization, an Andersen cascade impactor (TCR TECORA) was connected to an air pump and placed under the glove being tested at the end of the middle finger in the left-hand chamber. Six Trypticase Soy agar (TSA) plates, corresponding to the six stages of the device, were placed in the impactor.

Microbiological Test

Swabs were taken around the hole on the index finger of the tested glove and spread on TSA. Air samples were taken using an Andersen cascade impactor on TSA plates. In the enclosure on the left, 9 surface samples were taken using contact agar plates and the applicator: 6 samples were taken from the floor and 3 samples were taken from the wall facing the end of the glove. After 72 h of incubation at 32.5°C ± 2°C, the presence or absence of colonies was evaluated.

Statistical Analysis

Data processing and statistical analysis were performed using SPSS software (version 29.0 [IBM]; SPSS Inc, Armonk, NY). Data are presented as mean ± standard error of the mean (SEM). Normality of data distribution was assessed using the Shapiro–Wilk test. The effect of glove hole size was analyzed by Kruskal–Wallis followed by U Mann–Whitney. The significance level was set at 5%.

Results

Data obtained for the microroganisms studied.

Staphylococcus aureus

Air Sampling: The results for the number of viable microorganisms such as *Staphylococcus aureus* likely to have been released into the air in relation to the size of hole on the glove are shown in Table I.

No statistical differences were found between the mean CFU obtained for a hole size of 1 and 1.5 mm. For a 0.5 mm hole, an average of 0.6 CFU (CFU/sample) was collected in the air. This value was significantly lower ($p < 0.01$) than that observed for a 1.5 or 1.0 mm hole.

This reduction was greatest for the 0.3 mm hole, as no colonies were collected in the air on the cascade impactor.

Surface Sampling: The mean number of *S. aureus* colonies collected by the contact plates on the surface (from the floor and wall facing the glove) after nebulization in the gloves ranged from 1.3 CFU/sample (SEM = 0.3) for the 1.5 mm glove hole and 0.4 CFU/sample (SEM = 0.1) for the 1 mm hole to < 0.1 for the 0.5 and 0.3 mm holes, with significant differences ($p < 0.05$) noted between the means found after nebulization.

There were no significant differences between the means found for the 0.3 and 0.5 mm holes.

***Staphylococcus aureus* Colonies Forming Unit Collected from the Glove Hole:** No statistical differences were observed between the mean CFU values obtained for a hole size of 1 and 1.5 mm (Table II). The same

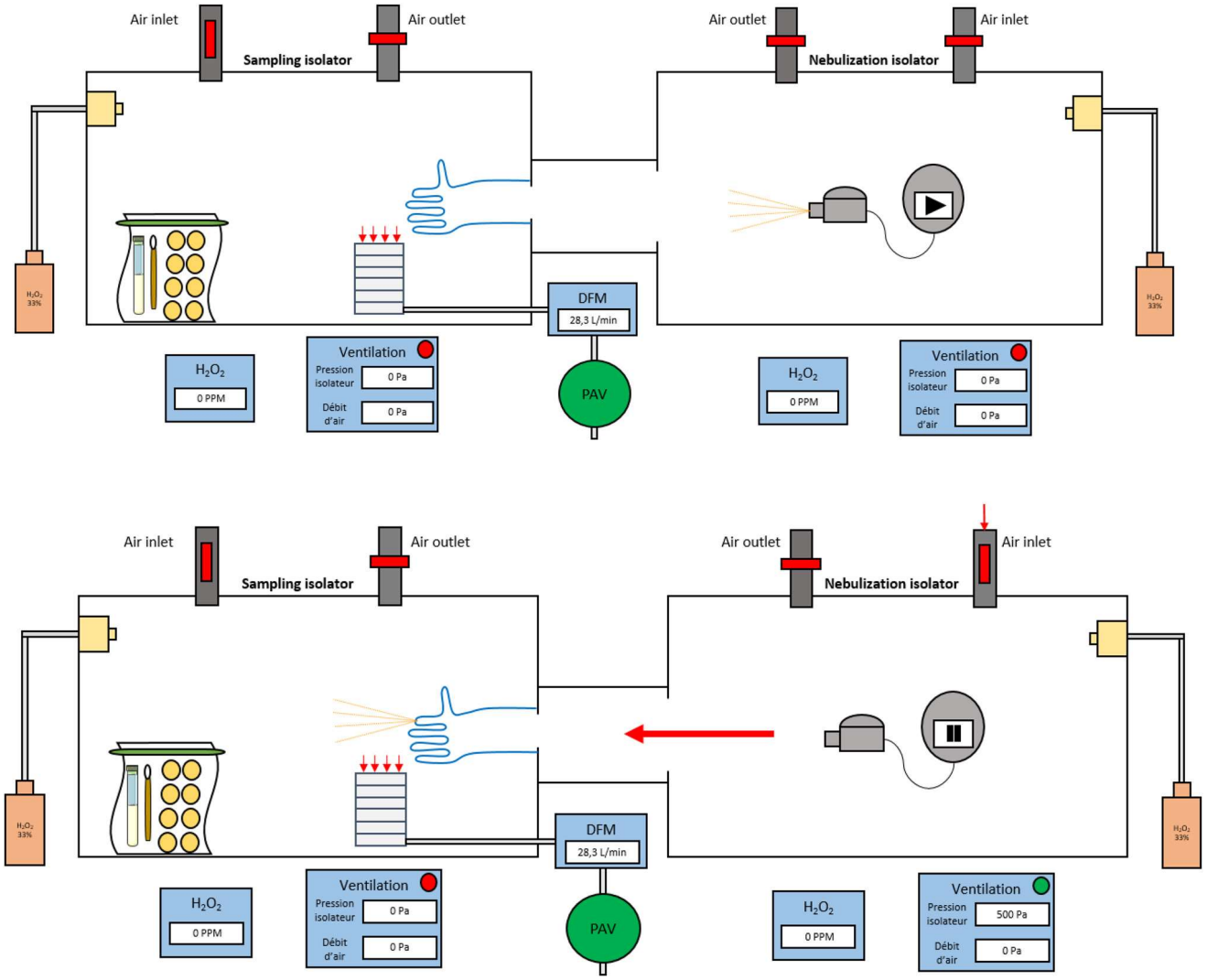

Figure 2

Nebulization of *Staphylococcus aureus* or *Bacillus subtilis* in the isolator.
Two isolators connected by a transfer lock and previously decontaminated with H_2O_2 were used. The glove to be tested was placed on the wrist ring on the left door of the transfer lock between the two isolators, with the glove hole facing the back wall. Nebulization of *Staphylococcus aureus* ATCC 6538 or *Bacillus subtilis* ATCC6633 was started in the right isolator. Once the nebulizer tank was empty, the pressure was increased to 500 Pa in the right chamber to mimic the overpressure induced by the introduction of the hand into the glove. Swabs were taken around the hole on the index finger of the tested glove and spread on trypticase soy agar (TSA). Surface sampling was also performed using Count Contact boxes on the walls of the left isolator.

observation was made between the values obtained for a hole size of 0.5 and 0.3 mm.

Bacillus subtilis

Air Sampling: Table III presents the mean (SEM) of *Bacillus subtilis* colonies collected in the air per assay after passing through the glove (CFU/sample).

The logarithmic reduction in the number of *B. subtilis* colonies observed forming a unit between 1.5 mm and 0.3 mm hole size tests was 2 log (Table III).

A significant difference ($p < 0.001$) was observed between the number of colonies forming a unit on air sampling after nebulization through a 0.5 mm hole compared to a 0.3 mm hole.

TABLE I
Mean (Standard Error of the Mean: SEM) of *Staphylococcus aureus* Colonies Collected in the Air per Assay after Passing Through the Glove (CFU/Sample)

Size of Hole (mm)	1.5	1.0	0.5	0.3
Staphylococcus aureus	33.0 (8.9)	34.4 (9.4)	0.6 (0.3) **	0 (0) $^{\$\$}$

** $p < 0.01$ versus hole size of 1 and 1.5 mm; $^{\$\$}$ $p < 0.01$ versus hole size of 1 and 1.5 mm.

Surface Sampling: The mean number of *B. subtilis* colonies collected by the contact plates on the surface (from the floor and wall facing the glove) after nebulization in the gloves ranged from 5.7 CFU/sample (SEM = 0.7) for the 1.5 mm glove hole and 1.4 CFU/sample (SEM = 0.5) for the 1 mm hole to 0.6 CFU/sample (SEM = 0.2) for the 0.5 and 0.1 mm holes (< 0.1), with significant differences ($p < 0.001$: 1 vs 1.5; 1.5 vs 0.3; 1.5 vs 0.5) noted between the means found after nebulization.

There were no significant differences between means found for 0.3 and 0.5 mm holes.

***Bacillus subtilis* Colonies Forming Unit Collected from the Glove Hole:** No significant differences were found between the mean CFU obtained for the 0.5 and 0.3 mm hole size (Table IV).

Discussion

The manufacture of sterile drugs and therapeutic products requires aseptic processing using technology such as isolators and RABS, which include gloves and sleeves, which are the weakest links in maintaining barrier integrity. In fact, the nature of gloves, due to their thickness and flexibility, creates a risk of tearing or piercing. However, there is still no consensus on the size of the hole that will cause a breach in the integrity of the isolator and allow microorganisms to pass through. This problem is not unique to the pharmaceutical industry. In fact, many studies have been carried out over the last 40 years in the food industry to determine the effect of holes on the microbial barrier properties of different packaging designs. These tests have been carried out using a wide range of protocols, and the results have been contradictory. Some laboratory tests have shown that submicron holes allow contamination, whereas studies of commercial packages with holes larger than 10 µm have been found to be free of microbial contamination (9, 10, 11). The aim of our study was to use bioburden conditions beyond industrial reality (worst case) to assess the transfer of microorganisms through hole sizes, some of which are visible to an operator. The aim was to provide arguments for manufacturers to develop their own SOPs and decisions based on quality risk management principles.

Based on the context of our study, the passage of the studied microorganisms does occur through CSM glove holes of a certain size, the cutoff of passage is determined to be 0.5 mm hole equivalent, regardless of the microorganism evaluated. Beyond this size, microbial contamination appears to increase. These results are in line with those obtained by Maier et al. (5). Indeed, the use of inoculated stainless-steel fingers (with a panel of isolates) and the assessment of the physical transfer of contamination to a contact plate (by touch) showed that if the hole was a capillary, that is the size of the hole was less than the thickness of the glove, this route did not represent a suitable pathway for the transfer of surface contamination (5). On the other hand, if the hole was the size of a pinhole, with a thickness equal to or greater than that of the glove, contamination transfer

TABLE II
Mean (Standard Error of the Mean: SEM) of Colonies Forming Unit of *Staphylococcus aureus* Collected on the Glove after Nebulization (CFU/Sample)

Size of Hole (mm)	1.5	1	0.5	0.3
Staphylococcus aureus	0.8 (0.24)	0.6 (0.3)	0.1 (0.1)*	0.1 (0.1)$^{\$}$

* $p < 0.05$ versus hole size of 1 and 1.5 mm; $^{\$}$ $p < 0.05$ versus hole size of 1 and 1.5 mm.

TABLE III
Mean (Standard Error of the Mean: SEM) of *Bacillus subtilis* Colonies Collected in the Air per Assay after Passing Through the Glove (CFU/Sample)

Size of Hole (mm)	1.5	1	0.5	0.3
Bacillus subtilis	174.0 (31.5)	73.9 (13.7)**	13.3 (3.8)	0.4 (0.3)$^{\$\$\$}$

** $p < 0.01$ versus hole size of 1.5 mm. $^{\$\$\$}$ $p < 0.001$ versus hole size from 1.5 to 0.5 mm.

levels were low. As CSM gloves have a thickness of 0.3 to 0.4 mm, the 0.5 mm limit we observed for a glove hole, beyond which the risk of contamination increases, is consistent with that of Maier et al. (5).

The recently published revision of EU Guidelines Annex 1 (3) specifies that glove testing must be carried out at defined intervals, at least at the beginning and end of each production batch. Visual inspection is limited in its ability to identify holes smaller than 0.8 mm. Therefore, a potential hole of 0.5 mm may not be detected, which could present a risk associated with the transfer of microorganisms from grade C to grade A, thereby affecting the environment, the process, and the product. The question is whether the justification could still support production continuity by assessing the impact on quality if a pinhole were to be detected in an isolator glove during production.

It's important to note that these results were obtained under worst-case conditions defined in the Materials and Methods section. Sterile pharmaceutical production areas comply with GMP guidelines for the manufacture of sterile products, which define the number of microorganisms that can be found in a volume of air of 1 m^3 or the number of CFU that can be found on a contact plate. (3). As grade C and D rooms commonly house isolators, they have a maximum of 100 and 200 CFU.m^{-3}, respectively. Furthermore, if we take the results given in Table III for the penetration of *Bacillus subtilis* through a 0.5 mm glove hole (13.3 CFU) from an environment containing 2×10^7 CFU.m^{-3}, we can estimate the expected penetration from a grade C environment (maximum 100 CFU.m^{-3}) to be close to 6.7×10^{-5} of *B. subtilis* for a 0.5 mm glove hole using these two formulae:

Penetration ratio = penetration/environmental concentration, that is in our case: $13.3/2 \times 10^7 = 6.65 \times 10^{-7}$

Expected penetration = Penetration ratio × grade C concentrations, that is, in our case: 6.7×10^{-5} CFU, which is practically negligible.

Due to the very low expected penetration value, it can be assumed that below a 0.5 mm glove hole, Grade A manufacturing environments are not compromised, which could potentially lead to sterile product contamination and a reduction in sterility assurance.

Conclusion

The results of the study highlighted the potential passage of microorganisms through holes in the fingers of CSM gloves, with a passage limit estimated to be around 0.5 mm. CSM gloves are typically used in isolators for filling lines where a high level of dexterity is not required and the thicker CSM gloves are used for added robustness in use. Thinner gloves (cuff change gloves) are used when manual manipulation and good dexterity are required, for example, ATMP processing, which is starting to be done in isolators after the revision of Annex 1, so this conclusion may not apply. Thus, although this study has limitations, such as the evaluation of only one glove type, the hole created in the finger rather than the shoulder, the limited

TABLE IV
Mean (Standard Error of the Mean: SEM) of Colonies Forming Unit of *Bacillus subtilis* Collected on the Glove after Nebulization (CFU/Sample)

Size of Hole (mm)	1.5	1	0.5	0.3
Bacillus subtilis	1.5 (0.5)	1.3 (0.3)	0.5 (0.3)$^{\$}$	0.3 (0.2)*

* $p < 0.05$ versus hole size of 1 and 1.5 mm; $^{\$}$ $p < 0.05$ versus hole size of 1 and 1.5 mm.

microbial panel, and the lack of measurements for other physical properties (e.g., tensile strength, elongation), the extensive investigation of worst-case challenge conditions provides compelling data to support our data.

Further studies need to be carried out under conditions more representative of actual production conditions to confirm these results. It would be interesting to carry out studies at different production sites to assess their risk of contamination and relate this to the defect in the gloves. This would allow us to confirm or refute our results.

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Conflict of Interest Declaration

The authors declare no competing interests.

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