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Original Article

Cleaning methods for biosafety cabinet to eliminate residual mycoplasmas, viruses, and endotoxins after changeover

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ABSTRACT

Introduction: Cell-processing operations can potentially contaminate biosafety cabinets, which should be maintained sterile. However, unintended contamination can occur owing to the presence of viruses, mycoplasmas, and bacteria in the raw materials. Moreover, although several methods for expunging these contaminants have been proposed, an optimal method has not yet been determined. Additionally, the effectiveness of conventional methods for eliminating these contaminants remains unclear owing to their unique characteristics and potential resistances to cleaning. Therefore, this paper proposes a risk-based approach to identify appropriate cleaning methods and reduce the likelihood of cross-contamination in biosafety cabinets by these contaminants.

Methods: Various cleaning methods for eliminating mycoplasmas, viruses, and endotoxins from biosafety cabinets were evaluated, including ultraviolet (UV) irradiation at 200 mJ/cm^2 for 20 min and wiping with disinfectants such as distilled water, benzalkonium chloride (BKC), and 70 % ethanol (ETH). The effectiveness of each method was evaluated by applying the contaminants on stainless steel plates and cleaning them using each method. *Mycoplasma orale* was cultured for 2 weeks in a liquid medium after cleaning. *Feline calicivirus* (FCV) was used for evaluating the virus-cleaning effectiveness and its presence was tested using the TCID50 test, whereas endotoxins obtained from the dried extract of *Escherichia coli* were measured via endotoxin testing.

Results: UV irradiation and wiping with BKC inhibited the growth of mycoplasma and significant decreased their presence compared with the other cleaning methods. Notably, mycoplasma were detected after wiping all SUS304 plates with ETH, which is a widely used cleaning method. Additionally, the cleaning efficacy for virus showed that the $TCID_{50}$ of the wet group was 132,000 $TCID_{50}$/plate, whereas those after UV irradiation or cleaning with BKC or DW were below the detection limit. Finally, UV irradiation did not significantly reduce the endotoxin production compared with that in the dry group. Additionally, wiping with ETH did not significantly reduce endotoxins compared with the dry group and their residues were higher than those detected after wiping with BKC or DW.

Conclusions: The changeover protocols currently employed in most cell-processing facilities may be ineffective as pathogenic or nonpathogenic materials may remain even after ETH wiping, leading to unintended cross-contamination. To the best of our knowledge, this is the first study to provide reference data of different cleaning methods for mycoplasmas, viruses, and endotoxins in cell-product

Abbreviations: BKC, Benzalkonium chloride; CFU, Colony formation unit; CPEs, Cytopathic effects; CRFK, Crandell Rees feline kidney; CV, Coefficient of variation; DW, Distilled water; EMEM, Eagle's minimum essential medium; ETH, Ethanol; FBS, Fetal bovine serum; FCV, Feline calicivirus; IQR, Interquartile range; LOD, Limit of detection; NC, Negative control; PCR, Polymerase chain reaction; UV, Ultraviolet.

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manufacturing facilities, and can potentially support the development of evidence-based management strategies for ensuring safe cell-product processing.

1. Introduction

Cell-processing operations involve various risks that can potentially contaminate biosafety cabinets, which should be maintained sterile [1]. Particularly, for cell products whose raw materials cannot be sterilized, residues of manufacturing-related substances can lead to various issues. As cell products involve manual processing [2], contaminants can inadvertently persist in the manufacturing area. For example, the presence of many viruses in raw materials [3] and the introduction of mycoplasmas and bacteria from operators or the environment can lead to cross-contamination [4]. Even after being destroyed, Gram-negative bacteria leave behind thermogenic substances such as endotoxins, in biosafety cabinets; therefore, reliable methods for removing these contaminants are required. However, an optimal method for removing these residual pathogens and substances has not yet been developed.

Ultraviolet (UV) irradiation has reported to be extremely effective for destroying microorganisms such as *Bacillus subtilis*, an endospore-forming bacterium, and *Aspergillus brasiliensis*, a spore-forming fungus that typically remain in biosafety cabinets [5]. However, it cannot eradicate non-living organisms, such as proteins and DNA, derived from culture media and cells [6]. Wiping with distilled water (DW) or surfactants has been reported to be effective for destroying both living and non-living organisms [6,7]. However, its effectiveness against mycoplasmas, viruses, and endotoxins, whose thresholds are defined in the cell-product shipping protocols of many countries [8–13], is unclear. Owing to the unique characteristics of mycoplasmas, including their propensity to coexist with cells, their mobility and adhesiveness have been extensively investigated [14]. Additionally, their adhesion to and persistence on biosafety-cabinet work surfaces, as well as their resistance to cleaning, may differ from those of bacteria. Moreover, the characteristics of viruses and endotoxins differ and they may exhibit unique and unexpected cleaning resistances owing to synergistic interactions with proteins specifically found in cell-product manufacturing areas.

In this study, the effects of various cleaning methods for biosafety cabinets, which serve as direct cell-manufacturing areas within aseptic processing environments, were analyzed by assuming that mycoplasma, viruses, and endotoxins remain within them. Specifically, the results of UV irradiation and wiping with DW, ethanol (ETH), and benzalkonium chloride (BKC) were compared and verified. This study focuses on “changeover” cleaning, defined as the removal of contaminants to the extent necessary for subsequent processing or intended use (ISO 11139:2018). This ensures that the residues from preceding operations do not compromise subsequent ones. It is important to distinguish this from “line clearance,” which entails establishing a sterile environment through initialization procedures. Based on our findings, we propose a risk- and evidence-based cleaning approach for biosafety cabinets to mitigate the risk of cross-contamination. Specifically, we focused on the elimination of mycoplasmas, viruses, and endotoxins, which are crucial contaminants that have not yet been comprehensively analyzed owing to the complexity of the analytical methods. However, appropriate contamination-control strategies are imperative to enhance the safety and quality of cell products that cannot be sterilized.

2. Materials and methods

2.1. Cleaning procedures

First, we employed the typical cleaning procedures for mycoplasmas, viruses, and endotoxins, as shown in Fig. 1. UV irradiation was conducted in a biosafety cabinet equipped with a 15 W UV-C germicidal lamp (Sankyo Denki Co., Kanagawa, Japan). The irradiation dose was measured using a UV-intensity meter (UVC-254SD; SATOTECH, Kanagawa, Japan) at 254 nm (range, 220–280 nm). The equipment was irradiated with a dose of 200 mJ/cm^2 for 20 min. Disinfectant treatments included wiping with DW (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), BKC with a corrosion inhibitor (Zalkonin N solution; 0.1 % w/v BKC containing 0.5 % w/v dicyclohexylamine nitrite as an anticorrosive and 8 % w/v ethanol; Kenei Pharmaceutical Co., Ltd., Osaka, Japan), and 70 % disinfectant ETH (76.9–81.4 % w/v ethanol and 3.7 % w/v isopropyl alcohol; Yamazen Pharm Co., Osaka, Japan). Two milliliters each of DW, BKC, and ETH were used simultaneously while wiping using a 7 × 7 cm piece of BEMCOT wipe (Asahi Kasei Co., Tokyo, Japan) with the relevant solution immediately after applying 500 g of one-way force at 50 mm/s, similar to previous studies [6,7].

2.2. Preparation for mycoplasma experiments

Mycoplasma orale (NBRC 14477), which was used as the target microorganism, was frozen with a colony formation unit (CFU) value adjusted to 1 × 10^9 using Hayflick Agar (Merck KGaA, Darmstadt, Germany). Subsequently, it was thawed and diluted to 1 × 10^6 CFU/200 μL with saline (Otsuka Pharmaceutical) and dispensed onto a SUS304 stainless steel plate (5 × 5 cm; AS ONE Co. Osaka, Japan) using a micropipette and air-dried.

2.3. Cleaning effectiveness for residual mycoplasma

To analyze whether mycoplasma remained after cleaning, its presence was determined using a liquid culture medium with the following composition, which was prepared according to a previous report [15]: 21 g of Difco PPLO broth without crystal violet (Becton, Dickinson and Company; BD Co., NJ, USA), 2 g of L-arginine (Fujifilm Wako, Osaka, Japan), 5 mL of 0.4 % phenol red (Fujifilm Wako), 700 mL of DW mixed and sterilized in an autoclave. After cooling, 200 mL horse serum (Sigma-Aldrich, Merck), 100 mL of 25 % fresh yeast extract, 10 mL of 2.5 % thallium acetate solution (Fujifilm Wako), 1 M units of potassium penicillin G (Sigma-Aldrich, Merck) were added. The 25 % fresh yeast extract prepared by dissolving 500 g of Nitten Dry East (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan) in 1500 mL of DW and boiled for 20 min. After cooling, the mixture was centrifuged at 7000 rpm for 20 min, the supernatant was collected, and its pH was adjusted to 7.6 using a 4 % sodium hydroxide solution (Fujifilm Wako). Thereafter, the supernatant was again immersed in a boiling water bath for 10 min, cooled, and centrifuged at 7000 rpm for 20 min. This supernatant stored at −80 °C and sterilized through filtration (0.2-μm filter) before being used as 25 % fresh yeast extract.

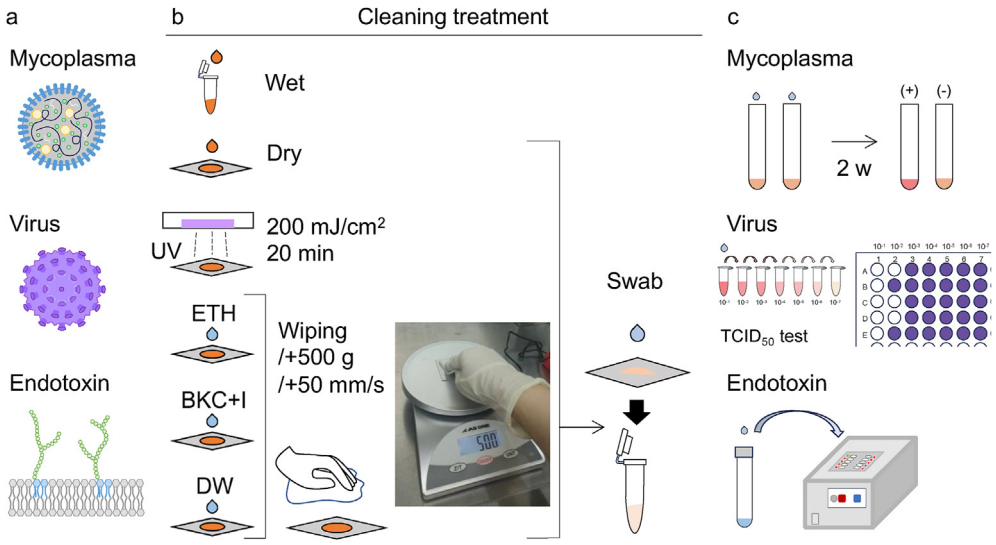

Fig. 1. Illustrations of the experimental conditions. (a) Contaminants tested. (b) Cleaning treatments employed. Wet groups were used as positive controls in mycoplasma and virus experiments. (c) Evaluation methods for cleaning effectiveness. For mycoplasma, color change after 2 weeks of incubation was observed. For viruses, $TCID_{50}$ test using CRFK cells was employed. For endotoxins, the gelatinization method with changes in light transmission at 430 nm was used.

The swab method was used to collect 200 μL of saline from each cleaned SUS304 plate, seeded into a liquid medium for mycoplasma, and incubated at 32.5 °C. Each cleaning method was implemented on four SUS304 plates and repeated thrice (N = 12). Some supernatant was collected and centrifuged after one and two weeks of incubation. The presence of mycoplasma was determined using an absorbance spectrophotometer (Infinite M200; Tecan, Männedorf, Switzerland) based on the changes in the color tone of the medium as mycoplasma grew. Preliminary validation confirmed that this culture method was suitable for detecting the presence of a single-digit CFU after two weeks of incubation (Fig. S1). The boundary line between positive and negative detection was defined as the midpoint between the positive and negative values in the preliminary validation (Fig. S1).

2.4. Preparation for virus experiments

Feline calicivirus (FCV) F4 strain, a non-enveloped virus that is highly resistant to disinfection and used as an alternative to norovirus, was used to determine the cleaning effect for viruses [16–19]. FCV cultures and viral infection titers were determined from stock cells derived from feline kidneys (Crandell Rees feline kidney cells; CRFK). FCV and CRFK cells were kindly provided from the Laboratory of Veterinary Microbiology, Nihon University College of Bioresource Sciences, Kanagawa, Japan. CRFK cells were grown at 37 °C under a 5 % CO_2 atmosphere in Eagle's minimum essential medium (EMEM; Fujifilm Wako) supplemented with heat-inactivated 10 % fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA), 1 % antibiotic and antimycotic (Thermo), 1 % l-glutamine (Fujifilm Wako), and 100 × non-essential amino acids (Fujifilm Wako). To infect and replicate the virus, FCV was absorbed into 80 % confluent CRFK cells for 1 h with several tilts, and incubated at 37 °C in a 5 % CO_2 atmosphere. After infection, EMEM with 1 % FBS was added to a flask and cells were incubated at 37 °C in a 5 % CO_2 atmosphere. After observing the cytopathic effects (CPEs), the supernatant was collected and cell debris were removed via centrifugation at 2000*g* at 4 °C for 5 min. Finally, the supernatant was stored at −80 °C and used as the stock virus solution.

2.5. Determination of cleaning effectiveness for residual virus

FCV stock solution was diluted and titrated using the 50 % tissue culture infectious dose ($TCID_{50}$) test using CRFK. A working virus solution diluted with 200 μL of EMEM with 1 % FBS was placed on SUS304 plates and allowed to dry before being subjected to cleaning treatments. Subsequently, 200 μL of saline solution was collected from each treated SUS304 plate using the swab method and stored at −80 °C until titration. Each cleaning method was implemented on four SUS304 plates and repeated twice (N = 8).

The stock virus solution and viruses were titrated after cleaning by calculating the $TCID_{50}$ after infecting CRFK cells grown in 96-well plates (BD Co.) [20]. The specific procedure was as follows: Cultured CRFK cells were seeded at a density of 6000 cells/well in seven rows × five wells of the 96-well plate. Virus recovery solution of 200 μL of EMEM with 1 % FBS was stepwise diluted 10-fold to 10^{-7} and added to the 96-well plate with the seeded and incubated cells. After 2–5 days, the CPEs were observed in each well through a microscope and recorded. Based on the determined CPEs, the viral infection titer was calculated using the Reed–Muench method as follows:

$$\log \mathrm{TCID50} = \log(\text{dilution with } >50\% \text{ positive}) + \mathrm{PD} \times (-\log(\text{dilution factor})).$$

where PD = (percent positive above 50%−50%)/(percent positive above 50%−percent positive below 50%)

To ensure the accuracy of the CPEs determined via microscopy, the medium containing the cells was collected from the wells both with and without CPEs, and RNA was extracted, reverse transcribed, and confirmed through polymerase chain reaction (PCR). The RNA was extracted using the QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and reverse transcription was performed using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). PCR was conducted using TaKaRa Ex Taq (TAKARA BiO Inc., Shiga, Japan) with forward primer 5′-AGCTGCGTTCAAGGGTGTTA-3′ and reverse primer 5′-TCCAACGGGACTTGTCAACCC-3'. All procedures were performed according to the manufacturers' instructions. After PCR, electrophoresis was performed to detect the presence of bands.

To visualize the $TCID_{50}$ test, both cells that were denatured by the virus and those that were not were stained with crystal violet. Specifically, they were fixed with formalin (Fujifilm Wako) for 5 min, stained with a crystal-violet solution (Fujifilm Wako) for 10 min, rinsed, and photographed using a camera.

2.6. Preparation for endotoxin experiments

The endotoxin used in the experiment was a dried extract of *Escherichia coli* (Fujifilm Wako). It was dissolved in DW to 1000 EU/mL and diluted with EMEM containing 1 % FBS to 5 EU/mL. The solution was seeded on SUS304 plates at 1 EU/200 μL, air-dried, and subjected to each cleaning treatment. From each cleaned SUS304 plate, dripped 200 μL of DW was collected using the swab method and stored at −30 °C until the endotoxin concentration was determined. Each cleaning method was implemented on four SUS304 plates and repeated twice (N = 8).

2.7. Determination of cleaning effectiveness for residual endotoxin

Endotoxin levels in the solutions were determined using the Limulus ES-II single test (Fujifilm Wako), according to the manufacturer's instructions, under a toxinometer (ET-6000; Fujifilm Wako).

2.8. Statistical analyses

Statistical analyses were conducted using Prism version 9 (GraphPad Inc., La Jolla, CA, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). All data are presented as medians and interquartile ranges (IQRs). Each statistical test is described in the corresponding figure legend. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Cleaning efficacy for mycoplasma

The cleaning efficacy for mycoplasma was evaluated using *Mycoplasma orale*. Two weeks after cleaning and seeding the liquid medium with mycoplasmas, a color change was observed (Fig. 2(a)). The media in which the mycoplasmas were observed tended to be acidic. These results indicated that the UV- and BKC-treated specimens exhibited no mycoplasma growth and a significant reduction in their content compared with the other cleaning groups. Similar to the samples from the wet group, which were used as the positive control, the samples from the dry group, which were air-dried on an SUS304 plate, also showed mycoplasma growth. Surprisingly, mycoplasma was detected in all SUS304 plates cleaned with ETH. Additionally, 66.7 % of the plates from the DW group contained mycoplasma, showing no significant differences from the other groups (Fig. 2(b)).

3.2. Cleaning efficacy for viruses

The cleaning efficacy for viruses was evaluated using FCV. In samples where FCV remained after cleaning, CPEs such as cell spheroidization and loss of adhesiveness were observed in CRFK cells (Fig. 3(a), left), whereas no CPEs were observed in those from which FCV was successfully eliminated (Fig. 3(a), right). Additionally, PCR analysis confirmed that these CPEs were not caused by the disinfectants but by the virus (Fig. 3(b)). $TCID_{50}$ visualization via crystal-violet staining showed that dead cells were exfoliated and unstained, whereas those that did not induce CPEs were stained purple (Fig. 3(c)). The $TCID_{50}$ of the wet group, which was used as the positive control, was 132,000 $TCID_{50}$/plate (200 μL; Median, IQR = 65,000–273,000) before seeding onto the SUS304 plate. The $TCID_{50}$ of UV irradiated as well as BKC and DW cleaned samples were below the limit of detection (LOD) of 58.7 $TCID_{50}$/plate (200 μL). Compared with the wet group, viral titers were significantly lower in the cleaned plates, and no significant differences were observed between the results of dry samples and those cleaned with ETH (Fig. 3(d)). The $TCID_{50}$ test may exhibit high variability, as indicated by the coefficient of variation (CV). Even under wet conditions, the CV was 1.17, with values of 0.99 and 0.80 observed for dry and ETH conditions, respectively (Fig. S2).

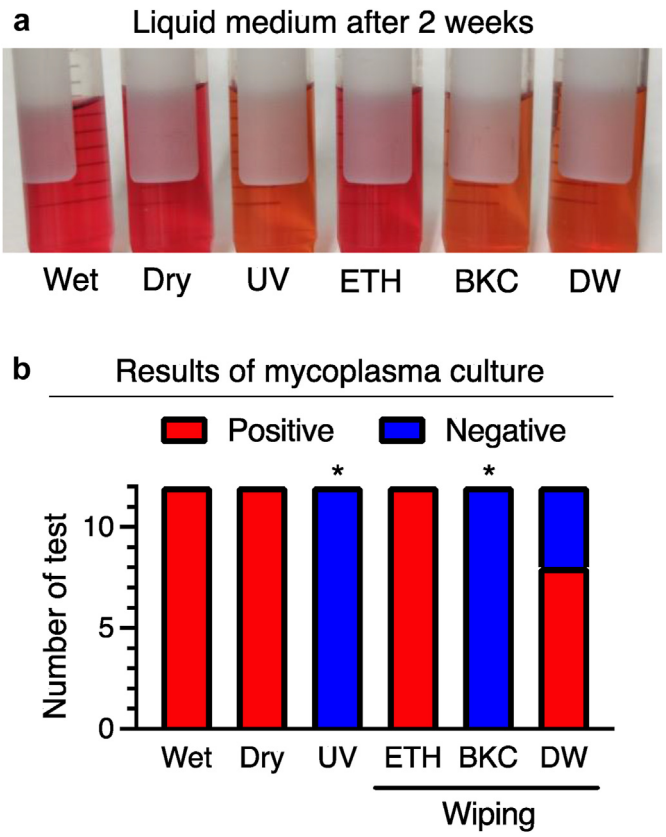

Fig. 2. Cleaning efficacy for mycoplasma. (a) Two weeks after seeding, the solutions were recovered into a liquid medium after implementing cleaning treatments for mycoplasmas seeded on SUS304 plates. (b) Positive and negative results for each test (N = 12). *P < 0.05. P-values were calculated using Pearson's chi-squared test with pairwise comparison using the Bonferroni correction multiple comparison test.

3.3. Cleaning efficacy for endotoxins

The cleaning efficacy for endotoxins was evaluated using a dried extract of *Escherichia coli* (Fig. 4(a)). UV irradiation did not significantly reduce the endotoxin levels compared with those in the dry group, whereas ETH cleaning led to a substantial reduction in endotoxin levels; however, they were significantly higher than those in the plates wiped with BKC and DW. Additionally, although DW was more effective, it exhibited a high CV of 1.02, whereas BKC achieved a stable cleaning effect, with a CV of 0.17 (Fig. 4(b)).

4. Discussion

In this study, we analyzed the effects of various cleaning treatments on the residual presence of mycoplasmas, viruses, and endotoxins in biosafety cabinets. UV irradiation was significantly

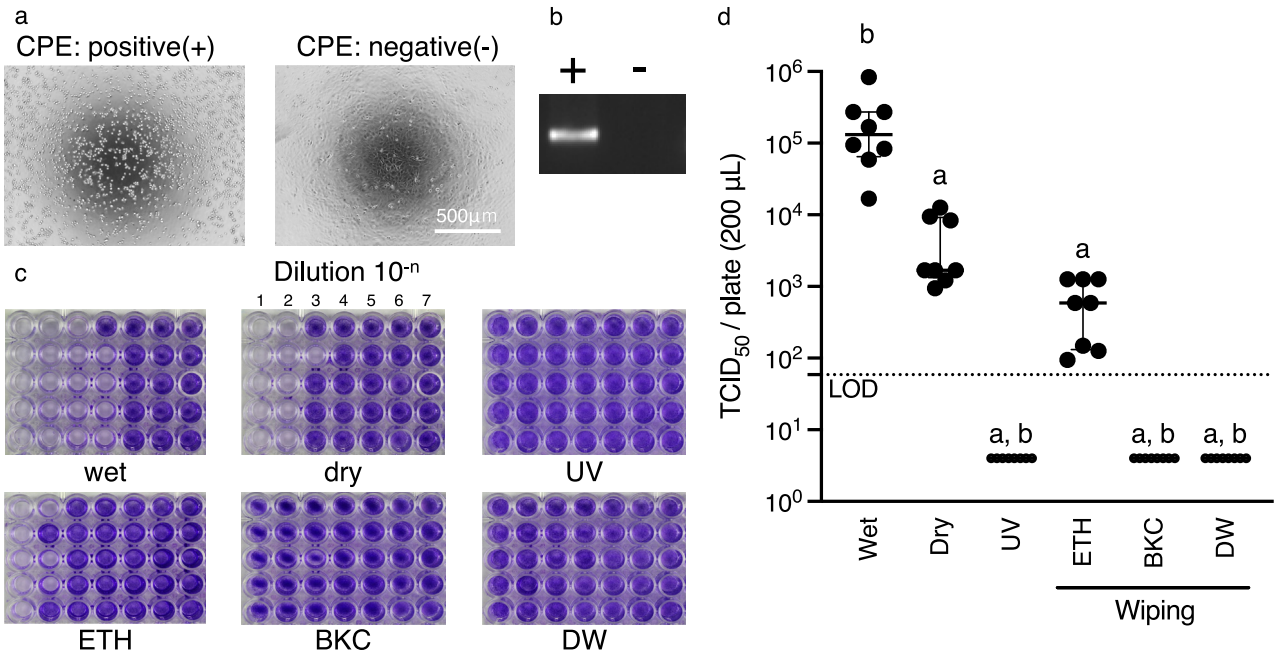

Fig. 3. Cleaning efficacy for virus. (a) CPEs of CRFK cells infected with FCV were observed. (b) Virus was detected via PCR in wells with CPEs, whereas it was not detected in those without CPEs. (c) Crystal violet staining of 96-well plates after $TCID_{50}$ test. (d) Quantitative results of $TCID_{50}$ test (N = 8). Data are presented as medians with IQRs. a: p-valuse <0.05 compared to the Wet group and b: p-values <0.05 compared to the Dry group. P-values were determined using the Kruskal–Wallis test with Steel–Dwass's multiple comparison test. LOD: limit of detection.

effective against organisms, mycoplasmas, and viruses; however, it was ineffective against non-living organisms, i.e., endotoxins. ETH wiping, which is the most commonly used method for cleaning biosafety cabinets, reduced the mycoplasma content but did not completely remove those cultured in liquid medium for 2 weeks. Additionally, it did not significantly reduce the virus or endotoxin content. Notably, wiping with DW was remarkably effective for viruses and endotoxins; however, it exhibited unstable results for endotoxins and some scattered tests showed that it had no effect on mycoplasmas. Wiping with BKC was substantially effective for both living organisms, i.e., viruses and mycoplasmas, and non-living organisms, i.e., endotoxins. These results suggest that the current changeover protocols employed in cell-manufacturing facilities may not be appropriate. Thus, various pathogenic or nonpathogenic materials may be introduced from nonsterile cell-product raw materials and remain in the production areas, leading to unintended cross-contamination.

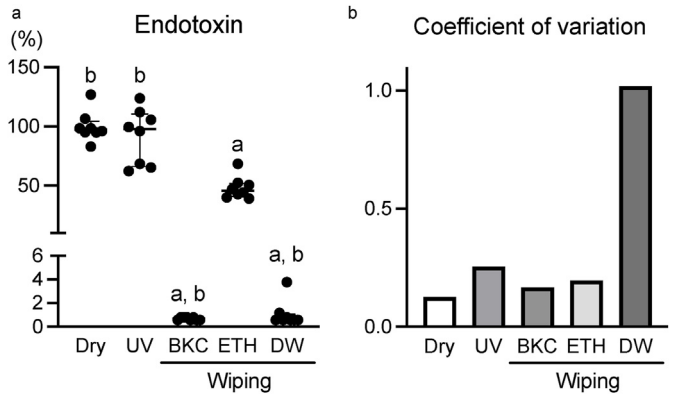

Fig. 4. Cleaning efficacy for endotoxins. (a) Quantitative results of endotoxin test (N = 8). Data are presented as medians with IQRs. a: p-values <0.05 compared to the Dry group and b: p-values <0.05 compared to the ETH group. P-values were determined using the Kruskal–Wallis test with Steel–Dwass's multiple comparison test. (b) Coefficient of validation for endotoxin values after cleaning.

Biosafety cabinets and production areas used for cell-processing operations that employ nonsterile raw materials may be at high a risk of contamination. Additionally, the cleaning effects for bacteria, fungi, and patient-derived contaminants, such as proteins and DNA, vary based on the method employed. For example, UV irradiation is effective against disinfection-resistant *Bacillus subtilis*, which forms endospores [5]; however, it is not effective against non-living organisms such as proteins and DNA [6]. Nevertheless, wiping with DW or surfactants is effective for both living organisms and non-living organisms [6,7]. Thus, the cleaning effects differ depending on the method employed and the target organism. Mycoplasma, viruses, and endotoxins, which are defined in the shipping control protocols for cell products, may also remain in biosafety cabinets; however, the effectiveness of the various cleaning methods on these contaminants is unclear owing to their unique properties and potential resistance to cleaning. Furthermore, these contaminants can act synergistically with contamination in protein-rich cell-product manufacturing areas. Additionally, the methods for evaluating the cleaning effectiveness of approaches are complicated and require comprehensive knowledge. For example, mycoplasma cultivation requires considerable amounts of various nutrients as they can exist in symbiosis with cells. Therefore, a mycoplasma-culture technique is necessary to determine whether they have been eliminated. However, the preparation of these culture media is expensive. Even if a custom culture technique can be developed, substantial effort and time are required to obtain the relevant information and establish a suitable method. Additionally, PCR entails high costs and preliminary validations are labor-intensive; therefore, it is difficult to conduct multiple experiments. Additionally, the pathogenicity of viruses poses significant risks to operators and present various technical obstacles; therefore, selecting an appropriate detection method is crucial. Similarly, determining and employing an appropriate detection method for endotoxins may

entail substantial costs. Therefore, we carefully selected the most cost-effective method that can be validated across numerous cell-processing facilities, thereby facilitating its verification at the facilities itself. Thus, the comprehensive elucidation of the cleaning methods provided in this paper is expected to assist stakeholders in selecting and validating the appropriate cleaning methods for their facilities.

The *Mycoplasma orale* employed in this study is isolated from the oropharynx of humans and is generally not pathogenic. Moreover, it is one of the most common species detected in cell lines [21]. Mycoplasma contamination is presumed to be caused by humans, and mycoplasmas dispersed from humans are believe spread owing to their strong tendency to persist under dry conditions [21]. Additionally, the effect of desiccation in this study was minor, which differs from the characteristics of desiccation-sensitive bacteria [7]. Furthermore, the lack of a cell walls limits the effectiveness of antibiotics and the conditions are such that penicillin, which is commonly used in cell cultures, is ineffective and the bacteria tend to persist [22]. However, under appropriate cleanroom operating conditions, residues are unlikely to remain in biosafety cabinets used for cell-product manufacturing. However, it is necessary to predetermine how changes, such as in the unlikely event of a positive result in shipping tests, will be implemented. This study suggests that caution should be exercised when wiping with ETH under certain conditions. The results of our analysis indicated that no positive media were observed after one week when ETH was used for wiping; however, positive media were found after two weeks. This was considered a reduction effect because the color change was comparable to the contamination of 10-digit CFUs in the preliminary validation. Thus, the results suggest that contamination in small amounts is not an issue. However, large amounts of residues may be problematic.

The FCV used in this study is pathogenic to cats but not humans, which makes it easy to handle in laboratories. Furthermore, it is often used as an alternative to norovirus because of its high resistance to disinfection as it lacks a viral envelope [16–19]. Although it is unlikely to contaminate the production of human-cell products, it can approximately reflect the disinfection-resistance of parvovirus B19, which is particularly common in raw materials [3]. For example, the ethanol sensitivity of FCV is lower than those of hepatitis A virus, human rotavirus, and adenovirus, and similar to that of porcine parvovirus, which is also a non-enveloped virus [23–26]. Furthermore, as parvovirus B19 is extremely difficult to culture, FCV is a useful substitute for evaluating cleaning methods for parvovirus B19. Viruses mixed with raw materials may remain in biosafety cabinets when liquid droplets are formed during cell-product processing [5,27]. When using bone marrow, which is frequently contaminated with parvovirus B19, as a raw material, it is necessary to consider the presence of invisible droplets and design changes in advance based on the assumption that it will cause contamination. This study suggests that ETH, which is commonly used for wiping, reacts with 1 % FBS that is included as a loading substance and adheres to the working surface of biosafety cabinets. Nevertheless, the $TCID_{50}$ was reduced by two orders of magnitude by drying, and a single digit reduction was observed after wiping the dried group with ETH. These results indicate that small amounts of residual virus are not problematic. However, wiping with ETH may not be optimal for eliminating this virus. Therefore, the risks posed by raw materials and residue amounts must be properly assessed in advance and an optimal cleaning method must be formulated.

This study used a dried extract of *Escherichia coli* as the positive target in the endotoxin test. Endotoxins are lipopolysaccharides that constitute the cell walls of gram-negative rods and released as thermogenic substances when bacteria are killed or mechanically destroyed. Shipping protocols specify a standard threshold of endotoxins for cell products, as well as pharmaceuticals and medical devices [28,29]. Gram-negative bacteria are less frequently detected in cleanroom environments than gram-positive bacteria, whose detection frequency may reach 97.8 % [30]; however, these gram-positive bacteria is possible to unintentionally brought into the biosafety cabinet in adhering with raw materials. For example, raw materials for cell products, such as the epidermis and intestinal epithelium, are introduced into Grade A environments in a live state; therefore, gram-positive bacteria within them can potentially contaminate the environment. Autoclaving at 121 °C for 20 min is insufficient for removing these endotoxins, and dry heat treatment at 250 °C for 30 min is required [31,32]. However, these treatments cannot be applied in biosafety cabinets. Therefore, it is necessary to develop and validate an endotoxin removal method that can be applied in cell-product manufacturing environments. This study suggests that, similar to mycoplasmas and viruses, cleaning with ETH is inadequate for endotoxins. Moreover, DW, which is highly effective against other contaminants, also exhibits unstable effects probably because DW is less slippery than BKC, which showed stable cleaning effects [7]. The residual amount of 1 EU/plate verified in this study is unlikely to occur in the real world as it is substantial when converted to the number of bacteria. However, similar to other contaminants, it is necessary to design a change-over method assess its effectiveness in advance if residues are expected.

Our findings suggest that an optimal cleaning strategy involves a combination of methods. This is because UV effectively eliminates living organisms but not non-living organic matter such as endotoxins. Furthermore, although benzalkonium chloride is highly effective, it is non-volatile and may persist within the biosafety cabinet. Although water residues are generally benign, wiping with water alone yields inconsistent results against mycoplasma. Additionally, the efficacy of ethanol was diminished in the presence of protein components such as serum, likely owing to fixation. Hence, it is necessary to understand the strengths and weaknesses of each cleaning method to employ a combination that leverages the strengths of each method. We believe that a reliable cleaning will involve thorough decontamination with benzalkonium chloride, removal of residual benzalkonium chloride with water, volatilization with ethanol, and UV irradiation of unwiped areas. However, this approach relies heavily on the efficacy of manual wiping, which can vary considerably based on the operator skill. Therefore, future studies should focus on establishing an objective evaluation method for wiping effectiveness and developing technologies for automated wiping or cleaning.

Furthermore, the evaluation method used in this study has some limitations. Specifically, the $TCID_{50}$ test exhibited substantial variability in virus-cleaning evaluations, potentially because this was an exploratory study and multiple cleaning scenarios were employed. Although the $TCID_{50}$ test is inherently unstable, and researchers have attempted to improve its stability by combining it with other tests [33], the stability observed in this study may be slightly lower compared to those in previous reports. Nevertheless, because the median was calculated over multiple tests, we believe that the test results themselves are reliable. Similarly, some variation was observed in the results of using UV for endotoxins; however, it was unclear whether this was due to the testing method or a factor that enhanced the UV effect. Therefore, researchers or stakeholders replicating or adopting the proposed methods should consider appropriate strategies to minimize variability. Additionally, future studies should focus on refining the propose experimental design based on the issues identified this research and conducting multi-site verification studies to obtain more stable results.

5. Conclusion

This study targeted contaminants such as mycoplasma, viruses, and endotoxins, whose thresholds have been specified in shipping protocols but their decontamination methods have not been comprehensively examined. The results suggested that the combination of wiping with various disinfectants and UV irradiation can reliably remove these contaminants. Nevertheless, the characteristics and environments of cell-manufacturing facilities differ, and appropriate risk assessments must be conducted individually. Therefore, the quantitative data provided in this study should not be interpreted as definitive and applicable to all facilities. However, it is noteworthy that this is the first study to provide reference data for mycoplasmas, viruses, and endotoxins to establish operational policies for cell-product manufacturing facilities. Thus, it offers fundamental information to support the development of evidence-based management strategies for safe cell-product manufacturing. However, continuous scientific evaluations are crucial to ensure safe cell-product manufacturing. Such studies are expected to facilitate the establishment of appropriate risk-based operational policies to mitigate contamination in cell-production facilities, which is a major concern for stakeholders and workers.

Author contributions

MM, SK, HI, NI, YN: Data acquisition, Data analysis, Interpretation; **MM:** Writing- Original draft; **MM, MN, IS:** Writing- Revisions for important intellectual content. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.11.020.

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