Supplementary Material

Disinfectant effectiveness against SARS-CoV-2 and influenza virus present on the human skin

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This PDF file includes:

Supplementary Materials

Viruses and cells.

Madin-Darby canine kidney (MDCK) cells were purchased from the RIKEN BioResource Center Cell Bank (Ibaragi, Japan) and were cultured in minimal essential medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% foetal bovine serum and standard antibiotics (penicillin/streptomycin). IAV (clinical strains H3N2) was isolated from MDCK cells inoculated with sputum from influenza-infected patients in 2012 and stored as a working stock at −80 °C. Virus titres were measured via focus forming assays in MDCK cells and expressed as focus forming units (FFU) [1, 2].

VeroE6/TMPRSS2 cells, expressing the transmembrane serine protease, TMPRSS2, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 5% foetal bovine serum and G418 (Nacalai Tesque, Kyoto, Japan) [3, 4]. SARS-CoV-2 (JPN/TY/WK-521) was generously provided by the National Institute of Infectious Diseases (Tokyo, Japan). The virus was cultured in VeroE6/TMPRSS2 cells and stored as a working stock at −80 °C. The virus titres were measured in terms of 50% tissue culture infectious dose ($TCID_{50}$) in VeroE6/TMPRSS2 cells. Specifically, 4 days after inoculation, each well's cytopathic effect was scored under a microscope, and the $TCID_{50}$ was calculated [3, 5, 6].

Both viruses were concentrated and purified as follows: 96 h post-infection, the culture medium was harvested and centrifuged for 10 min at 2,500 g at 4 °C to eliminate the cellular debris. Virions in the supernatant were sedimented with a 20% (w/w) sucrose cushion in

phosphate-buffered saline (PBS; Nacalai Tesque) through ultracentrifugation at 28,000 rpm for 2.5 h at 4 °C in a Beckman SW28 rotor [7].

Collection of human skin from autopsy specimens and preparation of the evaluation model for disinfectant effectiveness

Human skin was sampled from forensic autopsy specimens obtained from the Department of Forensic Medicine, Kyoto Prefectural University of Medicine. Abdominal skin autopsy samples from subjects aged 20–70 years, with a post-mortem duration of approximately 1 day, were excised into rectangles with dimensions greater than 5×10 cm^2 and used for subsequent evaluation [8]. Thereafter, we developed a model to evaluate disinfectant's effectiveness against the viruses on human skin. Most subcutaneous adipose tissue was rapidly obliterated. The treated skin (mainly the epidermal and dermal layers) was rinsed with PBS and placed into a culture insert with a membrane of 8.0-μm pore size (Corning, Corning, NY, USA). The culture inserts were placed into 12-well plates containing 1.0 mL of DMEM in each well [9].

***In vitro* evaluation of disinfectant effectiveness against SARS-CoV-2 and IAV**

As evaluation target disinfectants, we used 80%, 60%, 40%, and 20% (w/w) ethanol (Nacalai Tesque), and 70% (w/w) isopropanol (Nacalai Tesque), and 0.2% and 1.0% (w/v) chlorhexidine gluconate (Saraya, Osaka, Japan), and 0.05% and 0.2% (w/v) benzalkonium chloride (Yakuhan Pharmaceutical, Hokkaido, Japan). The evaluation protocol is as follows [10].

IAV or SARS-CoV-2 was mixed with DMEM before each disinfectant exposure (viral titre: 1.0×10^8 FFU/mL or 1.0×10^8 TCID 50/mL, respectively). Next, 95 μL of each disinfectant was added to 5 μL of the virus mixture in 24-well plates and incubated at 25 °C for 5, 15, or 60 s before neutralization by dilution with 900 μL of Soybean-Casein Digest Broth with Lecithin &

Polysorbate 80 (SCDLP; Nihon Pharmaceutical, Tokyo, Japan) and 3000 µL of DMEM. Finally, the titration of IAV/SARS-CoV-2 was then performed. A nonpolar polystyrene adsorbent (Bio-Beads SM-2 Resin; Bio-Rad, Hercules, CA, USA) was used in combination as needed to neutralize these disinfectants.

The measurement limits of the titres of IAV and SARS-CoV-2 were 10^1 FFU/mL and $10^{0.5}$ $TCID_{50}$/mL, respectively. The log reduction value was calculated using the virus titres with PBS addition instead of disinfectant addition as a control to evaluate the disinfectant effectiveness under each condition. Three independent experiments were performed for each measurement, and the results were expressed as the mean ± standard deviation.

Evaluation of disinfectant effectiveness against SARS-CoV-2 and IAV on human skin

To evaluate target disinfectants, we used 80%, 60%, 40%, and 20% (w/w) ethanol, and 70% (w/w) isopropanol; 0.2% and 1.0% (w/v) chlorhexidine gluconate; 0.05% and 0.2% (w/v) benzalkonium chloride. The evaluation protocol is as follows (Supplementary Figure S1) [9]. IAV or SARS-CoV-2 were mixed with DMEM and applied in 5 µL aliquots to human skin (viral load: 2.0×10^5 FFU or 2.0×10^5 $TCID_{50}$, respectively). Skin samples were then incubated at 25 °C, under a relative humidity of 45%–55%, for 5 min to dry the viral mixture on the skin completely. After that, 95 µL of each disinfectant was applied to the skin samples, incubated for 5 s, 15 s, or 60 s, and then air-dried for 5 min. After drying, the remaining viruses on the skin were recovered with 250 µL of SCDLP and 750 µL of DMEM and then titrated.

The measurement limits of the titres of IAV and SARS-CoV-2 were 10^1 FFU/mL and $10^{0.5}$ $TCID_{50}$/mL, respectively. The log reduction value was calculated using the virus titres on the skin with PBS application instead of disinfectant application as a control to evaluate the

disinfectant effectiveness under each condition. Three independent experiments were performed for each measurement, and the results were expressed as the mean ± standard deviation.

Ethical considerations

The study protocol, including the sample collection procedures, was reviewed and approved by the Institutional Review Board of the Kyoto Prefectural University of Medicine (ERB-C-1593).

Statistical analysis

Data were analysed using the GraphPad Prism 7 software (GraphPad, Inc., La Jolla, CA, USA). The virus titre values were all logarithm-transformed; the log reduction value under each condition was calculated.

Limitations

There were two limitations to this study. First, it is difficult to evaluate the mechanical effect with this human skin model; therefore, further improvement of this model is needed. Second, the disinfection effectiveness of BAC and CHG on SARS-CoV-2 might be overestimated because neutralization of BAC and CHG is more difficult than that of EA and IPA.

Supplementary References

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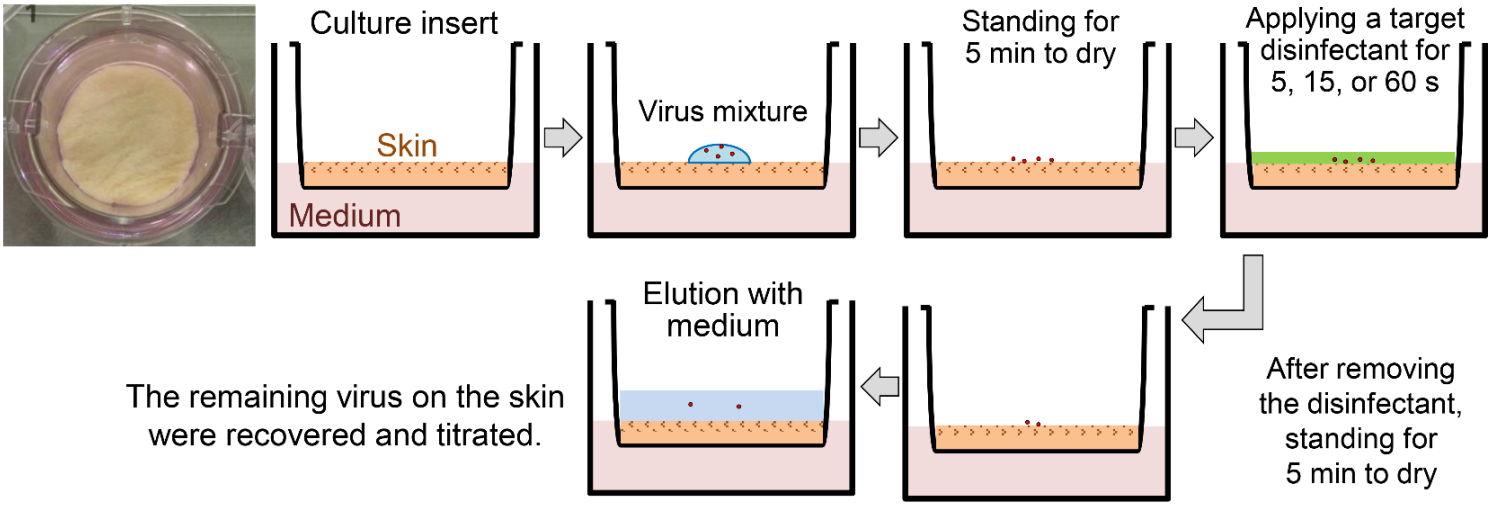

Supplementary Figure S1. The protocol for evaluation of disinfectant effectiveness against SARS-CoV-2 and IAV on human skin. SARS-CoV-2 and IAV were mixed with Dulbecco's modified Eagle's medium (DMEM) and applied in 5 µL aliquots to human skin (viral load: 2.0×10^5 $TCID_{50}$). Skin samples were then incubated at 25 °C, under a relative humidity of 45–55%, for 5 min, to dry the viral mixture on the skin completely. Then, 95 µL of each disinfectant was applied to the skin samples, incubated for 5 s, 15 s, or 60 s, and air-dried for 5 min. After drying, the remaining viruses on the skin were recovered with 250 µL of SCDLP and 750 µL of DMEM and then titrated.