Survival of SARS-CoV-2 and influenza virus on the human skin: Importance of hand hygiene in COVID-19

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Summary

The survival time of SARS-CoV-2 on the human skin was approximately 9 h, significantly longer than that of IAV (approximately 1.8 h). The longer survival of SARS-CoV-2 on the skin increases contact-transmission risk; however, hand hygiene can reduce this risk.

Abstract

Background: The stability of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) on human skin remains unknown, considering the hazards of viral exposure to humans. We generated a model that allows the safe reproduction of clinical studies on the application of pathogens to human skin and elucidated the stability of SARS-CoV-2 on the human skin.

Methods: We evaluated the stability of SARS-CoV-2 and influenza A virus (IAV), mixed with culture medium or upper respiratory mucus, on human skin surfaces and the dermal disinfection effectiveness of 80% (w/w) ethanol against SARS-CoV-2 and IAV.

Results: SARS-CoV-2 and IAV were inactivated more rapidly on skin surfaces than on other surfaces (stainless steel/glass/plastic); the survival time was significantly longer for SARS-CoV-2 than for IAV [9.04 h (95% confidence interval: 7.96–10.2 h) vs. 1.82 h (1.65–2.00 h)]. IAV on other surfaces was inactivated faster in mucus versus medium conditions, while SARS-CoV-2 showed similar stability in the mucus and medium; the survival time was significantly longer for SARS-CoV-2 than for IAV [11.09 h (10.22–12.00 h) vs. 1.69 h (1.57–1.81 h)]. Moreover, both SARS-CoV-2 and IAV in the mucus/medium on human skin were completely inactivated within 15 s by ethanol treatment.

Conclusions: The 9-h survival of SARS-CoV-2 on human skin may increase the risk of contact transmission in comparison with IAV, thus accelerating the pandemic. Proper hand hygiene is important to prevent the spread of SARS-CoV-2 infections.

Keywords

SARS-CoV-2; human skin; stability; influenza A virus; hand hygiene.

Introduction

Studies on the control of the novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have progressed rapidly worldwide. Numerous studies on the stability of SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) indicate that these coronaviruses have relatively higher stability compared to that of enveloped viruses, such as influenza A virus (IAV) [1-5]. Furthermore, the stability of SARS-CoV-2 on various surfaces was reported recently, providing essential information regarding the control of infection [6, 7]. Contact transmission through human skin is considered a significant risk factor in the spread of SARS-CoV-2 [8, 9]; hence, it is critical to have information about the stability (survival time) of SARS-CoV-2 on the human skin to develop approaches to prevent contact transmission. However, it is dangerous to apply highly pathogenic and infectious agents, including SARS-CoV-2, directly to human skin. Moreover, even though skin samples can be procured from surgical procedures, the amount of tissue obtained is small, which is a deterrent in the construction of models to conduct experiments with high reproducibility [10]. Owing to these limitations, the stability of highly pathogenic and infectious agents on the human skin remains unknown.

To overcome the abovementioned limitations, we generated a model to evaluate the stability of pathogens on human skin obtained from forensic autopsy specimens. Human skin (particularly the epidermis) is characterized by slower deterioration after death compared to other organs, and the collected skin can be used for grafting even 24 h after death [11-13]. Therefore, this evaluation model, using skin collected from autopsy specimens (approximately 1 day after death), could preserve skin functions and successfully model the *in vivo* conditions. Furthermore, at institutions performing forensic autopsy, fresh skin

samples collected up to 1 day after death can be stably supplied for research, thus, facilitating the generation of a high-quality and reproducible model [14].

In this study, we compared the stability of SARS-CoV-2 in the human skin, with that of IAV, which is a common virus transmitted through droplets and contact transmission worldwide. First, we confirmed whether the constructed model could replicate the *in vivo* skin conditions accurately. Thereafter, we evaluated the stability of SARS-CoV-2 and IAV in culture medium, on the surface of stainless steel, borosilicate glass, polystyrene, and human skin using this model. Moreover, the stability of SARS-CoV-2 and IAV, mixed with mucus from the upper respiratory tract, was evaluated because the viral particles of SARS-CoV-2 and IAV that adhere to objects and the skin are vehiculated by infectious body fluids (mucus). Finally, we evaluated the effectiveness of 80% (w/w) ethanol in the disinfection of human skin exposed to SARS-CoV-2 or IAV.

Methods

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% fetal bovine serum and standard antibiotics (penicillin/streptomycin). IAV (PR8; A/Puerto Rico/8/1934; H1N1) was cultured in MDCK cells and stored as a working stock at −80 °C. Virus titers were measured via focus forming assays in MDCK cells, as previously described, and expressed as focus forming units (FFU) [15, 16]. Specifically, at 12 h post-infection, cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.1% Triton X-100 for 30 min at room temperature. To detect influenza virus antigens, the cells were then stained with a rabbit polyclonal antibody which recognizes influenza virus NP and M1 proteins. Antibody binding

to viral proteins was detected with an Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Carlsbad, CA, USA) diluted 1:500 in PBS, and cells containing virus antigens were counted under a fluorescence microscope.

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% fetal bovine serum and G418 (Nacalai Tesque, Kyoto, Japan) [17, 18]. 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 titers of the virus were measured in terms of 50% tissue culture infectious dose ($TCID_{50}$) in VeroE6/TMPRSS2 cells. Specifically, 4 days after inoculation, the cytopathic effect in each well was scored under a microscope and the $TCID_{50}$ was calculated [3, 6, 17].

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 through a 20% (w/w) sucrose cushion in PBS through ultracentrifugation at 28,000 rpm for 2.5 h at 4 °C in a Beckman SW28 rotor [19].

Collection of sputum samples and preparation of mucus

Mucus samples (sputum samples ≥ 2 g) were obtained from three individuals diagnosed with an acute upper respiratory tract infection in 2019. Individuals < 20 years of age with chronic respiratory illness or those taking expectorants were excluded. The titration of viruses was performed on MDCK and VeroE6/TMPRSS2 cells to confirm that no active viruses (IAV or SARS-CoV-2) were present. Furthermore, to completely inactivate the infectious pathogens

derived from the individuals, the samples were irradiated with ultraviolet light (15 mJ/cm^2). Bubbles were eliminated from the irradiated samples through centrifugation at a low speed to avoid the destruction of the mucous structure [20, 21].

Collection of human skin from autopsy specimens and preparation of the virus stability evaluation model

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 4 × 8 cm^2 and used for subsequent evaluation [14].

Thereafter, we developed a model to accurately evaluate the stability of the viruses on human skin. This model was designed such that the skin sample did not deteriorate because of drying even after long-term incubation. Most subcutaneous adipose tissue was rapidly obliterated. The treated skin (mainly the epidermal and dermal layers) was rinsed with PBS and placed onto a culture insert with a membrane of 8.0 μm pore size (Corning, Corning, NY, USA). The culture inserts were placed into 6- or 12-well plates containing 1.0 mL of DMEM (Figure 1A).

Evaluation of viral stability on various surfaces

Viral survival was assessed on the surface of stainless steel, borosilicate glass, polystyrene, and the human skin model. IAV or SARS-CoV-2 were mixed with DMEM or mucus and applied in 5 μL aliquots to each surface (1.0×10^5 FFU or 1.0×10^5 $TCID_{50}$, respectively). Each sample was incubated in a controlled environment (25 °C and 45%–55% relative humidity) for 0–96 h. Thereafter, the residual viruses on the surface were recovered in 1 mL

of DMEM and titrated [3, 22-25]. Three independent experiments were performed for each measurement, and the results are expressed as the mean ± standard error of the mean.

To evaluate the reproducibility of the human skin model, the stability of IAV on the hands of six subjects, who provided informed consent, was analyzed. IAV was mixed with DMEM and applied in 5 µL aliquots to the skin of the subjects' hands (1.0×10^5 FFU). Each sample was incubated under a controlled room environment (25 °C and 45%–55% relative humidity) for 0–1.5 h. Thereafter, the residual viruses were recovered from the surface in 1 mL of DMEM and titrated [26].

Time measurements were started immediately after the virus aliquots were applied to the surfaces; zero-hour incubation indicates virus recovery immediately after applying the virus mixture to each surface.

Evaluation of the efficacy of an ethanol-based disinfectant against SARS-CoV-2 and IAV on human skin

SARS-CoV-2 or IAV were mixed with DMEM or mucus and applied in 5 µL aliquots to human skin (1.0×10^5 $TCID_{50}$ or 1.0×10^5 FFU, respectively). Skin samples were then incubated at 25 °C, under a relative humidity of 45%–55%, for 30 min. Thereafter, 95 µL of 80% (w/w) ethanol was applied to the skin and incubated for 15 s prior to neutralization by dilution with 900 µL of DMEM; the remaining viruses were then titrated [20]. Three independent experiments were performed for each measurement and the results are expressed as the mean ± standard error of the mean.

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 analyzed using the GraphPad Prism 7 software (GraphPad, Inc., La Jolla, CA, USA). The elapsed time was defined as the explanatory variable (X-axis), and the log virus titers of IAV or SARS-CoV-2 were defined as the explained variable (Y-axis). A least-squares linear regression analysis with a logarithmic link function was performed to generate regression curves for both viruses. The measurement limits of the titers of IAV and SARS-CoV-2 were 10^{1} FFU and $10^{0.5}$ $TCID_{50}$, respectively; therefore, the survival times of IAV and SARS-CoV-2 were defined as the X values when the Y values of the regression curves were 1.0 and 0.5, respectively. The half-life time of each log virus titer was determined from the slope of the respective regression line.

Results

To validate the model, first, we evaluated the stability of IAV on the skin using the newly generated skin model and compared it with that obtained in the context of live subjects' hand skin (Figure 1A). The 95% confidence interval of the viable virus titers on the model skin at each elapsed time (15, 30, 45, and 60 min) was within the 95% confidence interval of the viable virus titers on the subjects' skin [3.91 Log_{10}FFU (95% confidence interval: 3.68–4.06 Log_{10}FFU) *vs*. 3.93 Log_{10}FFU (3.06–4.20 Log_{10}FFU), 3.50 Log_{10}FFU (3.09–3.71 Log_{10}FFU) *vs*. 3.46 Log_{10}FFU (2.65–3.72 Log_{10}FFU), 3.06 Log_{10}FFU (2.83–3.21 Log_{10}FFU) *vs*. 3.01 Log_{10}FFU (2.23–3.27 Log_{10}FFU), and 1.87 Log_{10}FFU (1.68–2.00 Log_{10}FFU) *vs*. 1.93

Log_{10}FFU (1.08–2.20 Log_{10}FFU), respectively] (Figure 1B). These results indicate that the present evaluation model can replicate data obtained using the skin from live subjects' hands accurately.

Next, the stability of SARS-CoV-2 and IAV, mixed in DMEM, was evaluated on the surface of stainless steel, borosilicate glass, and polystyrene. The survival time of SARS-CoV-2 was approximately 8-fold the survival time of IAV on these surfaces; SARS-CoV-2 also displayed higher stability. However, SARS-CoV-2 and IAV were more rapidly inactivated on all human skin surfaces (HS1, HS2, HS3) than on stainless steel, borosilicate glass, and polystyrene surfaces; the survival time and half-life time of SARS-CoV-2 and IAV were significantly shorter on human skin than those on stainless steel, borosilicate glass, and polystyrene (Figure 2 and Table 1 and Supplementary Figure S1-3). Moreover, SARS-CoV-2 on all human skin surfaces (HS1, HS2, HS3) displayed higher stability than IAV, and the both the survival and half-life times of SARS-CoV-2 on the skin were significantly longer than those of IAV [9.04 h (95% confidence interval: 7.96–10.20 h) vs. 1.82 h (1.65–2.00 h) and 3.53 h (3.02–4.16 h) vs. 0.80 h (0.72–0.90 h), respectively] (Table 1 and Supplementary Figure S3).

Stability analysis of the viruses mixed with mucus from the upper respiratory tract revealed that on stainless steel, borosilicate glass, and polystyrene surfaces, IAV was more rapidly inactivated in mucus than in DMEM, whereas the stability of SARS-CoV-2 in mucus and DMEM was similar (Figure 2 and Supplementary Figure S1-2). Importantly, in the analysis of viruses mixed with mucus, the survival and half-life times of SARS-CoV-2 were also significantly longer than those of IAV on human skin [11.09 h (10.22–12.00 h) *vs.* 1.69 h (1.57–1.81 h) and 4.16 h (3.79–4.58 h) vs. 0.77 h (0.71–0.84 h), respectively] (Table 1 and Supplementary Figure S3).

Finally, SARS-CoV-2 both in the context of mucus and DMEM was completely inactivated on all human skin surfaces (HS1, HS2, HS3) within 15 s upon treatment with 80% (w/w) ethanol. Furthermore, IAV was completely inactivated under the same evaluation conditions (Figure 3).

Discussion

Since the collected skin can be used for skin grafting even 24 h after death [11-13], this evaluation model, using skin autopsy specimens collected approximately 1 day after death, has the potential to preserve skin function and to faithfully model *in vivo* conditions. Actually, the 95% confidence interval of the viable virus titers on the model skin at each elapsed time-point was within the corresponding 95% confidence interval of the viable virus titers in the context of live subjects' hand skin, indicating that the constructed evaluation model can accurately replicate the *in vivo* conditions. Moreover, IAV was inactivated on the skin in approximately 1 h, in concordance with previous reports [26, 27]. This model is, therefore, quite helpful for the evaluation of the stability of highly pathogenic and highly infectious agents, such as SARS-CoV-2, on human skin, as well as for the evaluation of their disinfection efficacy; we believe this new method has the potential to contribute greatly to the development of infection-control strategies in the future.

The survival time of SARS-CoV-2 was approximately 8-fold that of IAV on the surface of stainless steel, borosilicate glass, and polystyrene, displaying, consequently higher stability than IAV. These results are similar to those of previous studies [2, 3, 6]. However, the survival and half-life times of SARS-CoV-2 and IAV were significantly shorter on human skin than those on the other surfaces, indicating that human skin is less suitable for the survival of viruses. The survival time of SARS-CoV-2 on the skin was approximately 9 h and

was significantly longer than that of IAV (approximately 1.8 h). These results indicate that SARS-CoV-2 has a markedly higher stability on human skin than that of IAV.

IAV was inactivated more rapidly in mucus versus DMEM on the surface of stainless steel, borosilicate glass, and polystyrene; in fact, its survival time was drastically reduced. SARS-CoV-2 displayed the same level of stability in both mucus and DMEM, and the survival time and half-life were not significantly different. These results suggest that viral inactivators in the mucus from the upper respiratory tract are less effective against SARS-CoV-2 than they are against IAV.

Taken together, the determined long, 9-h survival time of SARS-CoV-2 on human skin may increase the risk of viral invasion in the body or its transmission from the skin to other surfaces, with a potential impact in the acceleration of the SARS-CoV-2 pandemic. However, SARS-CoV-2 in the mucus and DMEM was completely inactivated within 15 s of exposure to 80% (w/w) ethanol. Thus, appropriate hand hygiene using ethanol-based disinfectants leads to the quick viral inactivation and may reduce the high risk of contact-infections. On the contrary, since not only the virus stability but also the infectious dose and transmission route may greatly affect the risk of contact-transmission, future research needs to focus on factors other than virus stability.

In this study, the analysis was performed using a least-squares linear regression analysis with a logarithmic link function instead of the normal linear regression. There may be multiple phases in the context of loss of infectivity, with a recognized multifactorial character. The speed of infectivity loss may change over time due to many different factors; in fact, we speculate that this is the main reason because the regular linear regression did not fit well. We will focus on some of these potential factors, such as humidity and temperature, and carry out further research in the future.

There were three main limitations to this study. First, only one SARS-CoV-2 strain and only one influenza strain were used. Furthermore, the PR8 strain was used as the influenza strain instead of a relevant clinical isolate. It is necessary to further increase the number of virus strains and proceed with the research in the future, to understand if these results can be translated to human influenza and COVID-19. Second, only 3 skin samples from autopsy specimens and 3 mucus samples were used in this study. Since the number of samples is small, in future clinical studies, we plan to increase the number of samples and evaluate the clinical background of patients. Third, an evaluation model using human skin harvested in the context of a forensic autopsy was used for the evaluation of virus stability and disinfecting effectiveness. It is necessary to demonstrate in the future that the skin surface in this model is similar to that of live skin. However, the application of highly pathogenic and infectious agents to the skin of subjects is dangerous and not feasible clinically, and there is no evaluation system at this stage that is more accurate and reproducible than this model. Of note, the inactivation of IAV in the context of autopsy skin samples and of live skin was similar, supporting the validity of our model.

In conclusion, this study shows that SARS-CoV-2 may have a higher risk of contact transmission than IAV because the first is much more stable on human skin than the former. These findings support the hypothesis that proper hand hygiene is important for the prevention of the spread of SARS-CoV-2. Thus, this study may contribute to the development of better control strategies in the context of COVID-19 to prevent the occurrence of the second or third waves of this pandemic.

NOTES

Author contributions

Study concept and design: RH. Data acquisition: RH, HI, NW, TY, RB, TD. Data analysis and interpretation: RH, YN, YI and TN. Drafting of the manuscript: RH. Statistical analysis: RH. Secured funding: RH. Administrative/technical/material support: RH, and HI. Study supervision: RH and TN.

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Competing financial interests

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Figure legends

Figure 1. Outline of the pathogen stability evaluation model and its reproducibility. The pathogen stability evaluation model was constructed using human skin collected from forensic autopsy specimens (A). To evaluate the reproducibility of the model, influenza A virus (IAV) was applied to the six model skin samples and to the hand skin of six subjects (amount of virus: 1.0×10^5 FFU), and the titer of the remaining viruses on the skin was measured. The 95% confidence interval (red bar) of the viable virus titer on the model skin at each elapsed time was within the 95% confidence interval (blue bar) of the viable virus titer on the skin of live individuals (B).

Figure 2. (A–F) Fluctuations in the titer of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza A virus (IAV) surviving on the surface of stainless steel (A), borosilicate glass (B), polystyrene (C), and three skin samples [HS1 (D), HS2 (E), and HS3 (F)]. SARS-CoV-2/IAV was mixed with Dulbecco's modified Eagle's medium (DMEM) or mucus and applied in 5-μL aliquots to each surface (amount of virus: 1.0×10^5 FFU or 1.0×10^5 $TCID_{50}$, respectively). Each surface was incubated in a constant environment (temperature: 25 °C, humidity: 45–55%) for 0–120 h. The remaining viruses on the surface were then recovered in 1 ml of culture medium and titrated. For each measurement, three independent experiments were performed, and the results are expressed as the mean ± standard error of the mean. Bars referring to the data below the detection limit were omitted. See Supplementary Figure S1 and S2 for raw data.

Figure 3. Evaluation of the disinfection effectiveness of 80% (w/w) ethanol against SARS-CoV-2 (upper panel) and IAV (lower panel) on human skin. Thirty minutes after the mixture of the DMEM/mucus and SARS-CoV-2/IAV was applied to each skin surface (HS1/HS2/HS3), 80% ethanol was further applied to the skin surfaces for 15 s, followed by disinfectant inactivation via dilution with culture medium. The surviving viruses on the skin surfaces were then titrated. For comparison, the surviving viruses on the skin surfaces in the absence of ethanol were also titrated over time. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IAV, influenza A virus; DMEM, Dulbecco's modified Eagle's medium. For each measurement, three independent experiments were performed, and the results are expressed as mean ± standard error values.

Table 1. Survival time and half-life time of viruses on each surface.

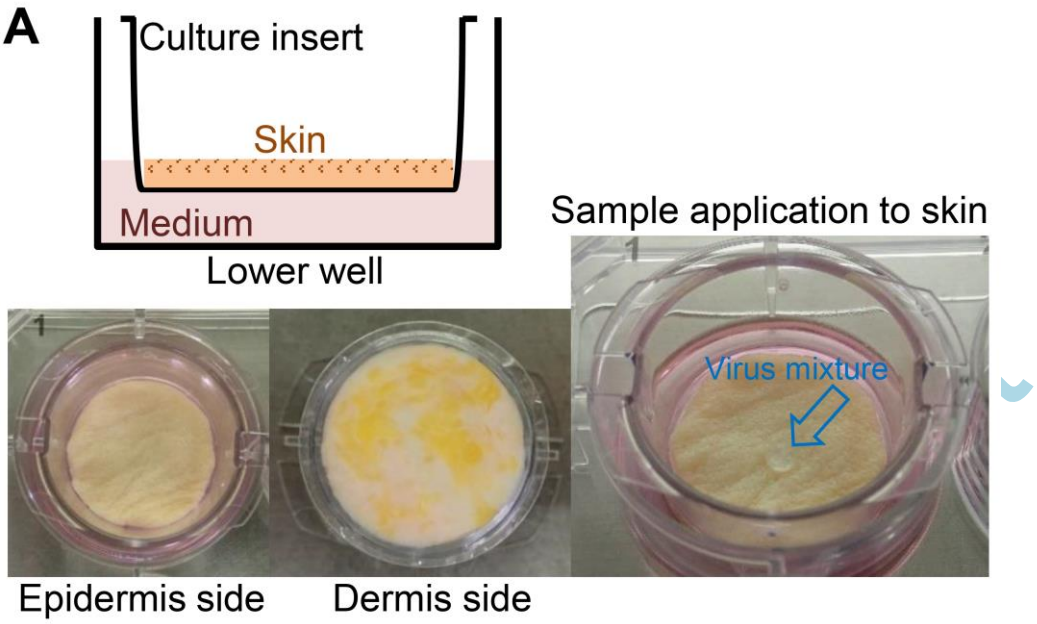
	Survival time[1], hour, median (95% CI)				Half-life time[2], hour, median (95% CI)			
	IAV (DMEM)	SARS-CoV-2 (DMEM)	IAV (Mucus)	SARS-CoV-2 (Mucus)	IAV (DMEM)	SARS-CoV-2 (DMEM)	IAV (Mucus)	SARS-CoV-2 (Mucus)
Stainless steel	11.56 (10.11-13.22)	84.29 (54.01-119.56)	1.73 (1.57-1.91)	64.51 (52.35-77.73)	6.78 (5.84-7.97)	32.62 (16.80-56.68)	0.86 (0.76-0.98)	25.53 (18.45-34.24)
Borosilicate glass	10.61 (9.18-12.27)	85.74 (56.27-119.80)	1.73 (1.58-1.88)	61.23 (49.03-74.44)	6.13 (5.22-7.29)	33.24 (17.59-56.49)	0.85 (0.76-0.96)	23.63 (17.16-31.86)
Polystyrene	6.07 (5.05-7.27)	58.07 (37.76-81.95)	1.96 (1.76-2.18)	35.92 (29.58-42.67)	3.04 (2.40-3.87)	22.58 (11.64-41.24)	0.91 (0.80-1.04)	13.17 (10.26-17.35)
Human skin (HS total)	1.82 (1.65-2.00)	9.04 (7.96-10.22)	1.69 (1.57-1.81)	11.09 (10.22-12.00)	0.80 (0.72-0.90)	3.53 (3.02-4.16)	0.77 (0.71-0.84)	4.16 (3.79-4.58)
Human skin (HS1)	1.81 (1.64-2.00)	10.93 (8.95-13.10)	1.66 (1.47-1.88)	12.24 (10.64-13.94)	0.82 (0.73-0.93)	4.13 (3.29-5.28)	0.77 (0.66-0.89)	4.47 (3.83-5.26)
Human skin (HS2)	1.79 (1.50-2.13)	9.45 (7.72-11.38)	1.71 (1.51-1.94)	12.2 (11.10-13.34)	0.78 (0.64-0.98)	3.75 (2.93-4.86)	0.78 (0.67-0.91)	4.51 (4.06-5.03)
Human skin (HS3)	1.86 (1.50-2.27)	6.14 (4.91-7.53)	1.69 (1.49-1.91)	8.13 (6.85-9.51)	0.79 (0.63-1.04)	2.36 (1.73-3.21)	0.77 (0.67-0.90)	3.13 (2.56-3.86)

The elapsed time was defined as an explanatory variable (X-axis), and the log virus titer of IAV or SARS-CoV-2 was defined as an explained variable (Y-axis). A linear regression analysis with logarithmic link function was performed for each virus to create a curve of regression (see also Supplementary Figure S3).

[1]The measurement limits of the titers of IAV and SARS-CoV-2 were 10^1 FFU and $10^{0.5}$ $TCID_{50}$, respectively; therefore, the survival times of IAV and SARS-CoV-2 were defined as the X values when the Y values of the regression curves were 1.0 and 0.5, respectively.

[2]The half-life time of each log virus titer was calculated from the slope of each regression line.

Figure 1



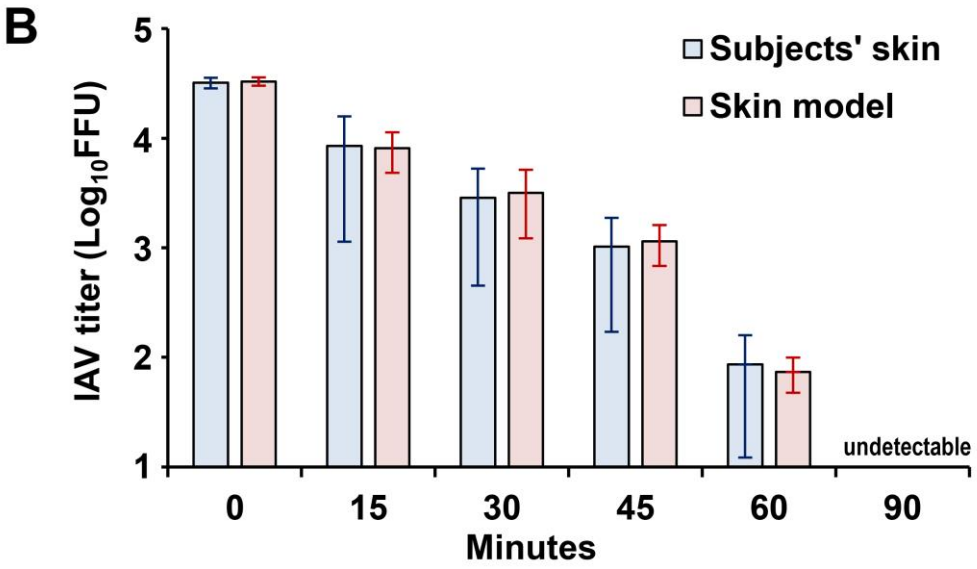


Figure 2

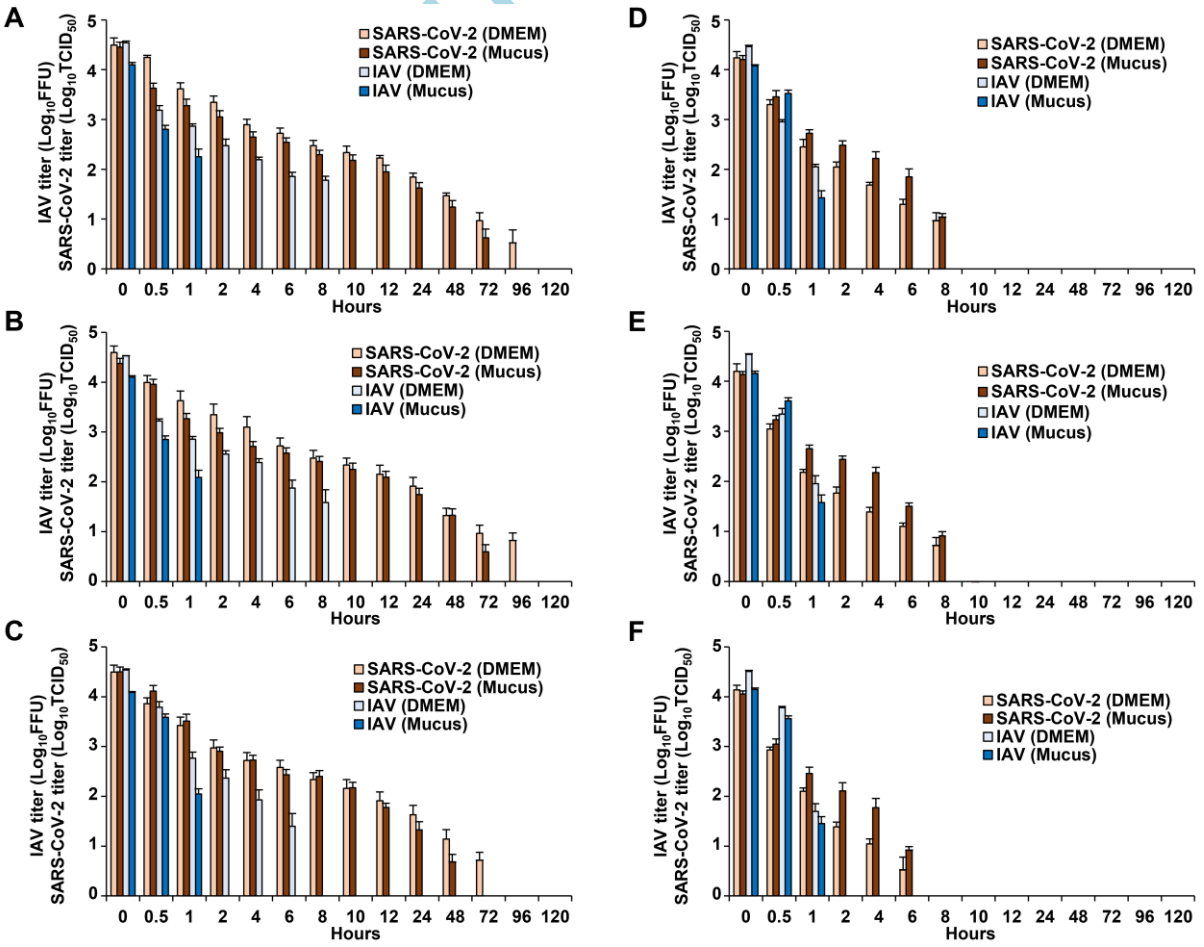


Figure 3