Inactivation of Coxsackievirus B4, Feline Calicivirus and Herpes Simplex Virus Type 1: Unexpected Virucidal Effect of a Disinfectant on a Non-Enveloped Virus Applied onto a Surface

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Key Words

Stainless steel carrier · Feline calicivirus F9 · Quaternary ammonium · Disinfectant · Herpes simplex virus type 1 · Coxsackievirus B4

Abstract

Objective: To evaluate the effect of a disinfectant onto viruses in suspension on the one hand and applied onto a surface on the other. **Methods:** A system combining flocked swabs to recover viruses dried onto stainless steel carriers and gel filtration to eliminate cytotoxic products has been developed to study the virucidal effect of a quaternary ammonium-based disinfectant towards herpes simplex virus type 1 (HSV-1), coxsackievirus B4 (CVB4) and feline calicivirus F9 (FCV). The recovery of FCV has been estimated by RT real-time PCR. **Results:** HSV-1, CVB4 and FCV had a titer over 10^4 TCID₅₀ · ml⁻¹ after 2 h drying and were recovered from the carriers using flocked swabs. HSV-1 was inactivated in suspension and on stainless steel carriers by the disinfectant (a reduction factor of 4 and 2.83 log, respectively) whereas CVB4 was resistant. The reduction of infectious titer was moderate, 1.5 log in 30 min, when FCV was in suspension, whereas it was up to 4 log in 10 min when the virus was dried on a carrier. Dried FCV was efficiently recovered from carriers as demonstrated by RT real-time PCR. **Conclusion:** A non-enveloped virus, FCV, applied on a surface, but

not in suspension, was inactivated by a quaternary ammonium-based disinfectant. The resistance of viruses applied onto a surface to the effect of disinfectants should be investigated further.

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Introduction

Viruses can survive for extended periods on surfaces or medical devices, from a few hours to a few days for enveloped virus, like herpes virus type 1 to more than 60 days for non-enveloped viruses like rotavirus or poliovirus [1]. Besides the type of virus, humidity and temperature, the type of surface can play a role in the persistence of viruses. Furthermore, the influence of the inoculum size and biological components (feces) has been reported [1].

When no proper disinfection is done, it can lead to nosocomial spread of viral disease [1–3]. An effective disinfection of healthcare equipment and surfaces is a major step in preventing and controlling viral transmission [4]. In order to assess the virucidal activity of a product on viruses, two kinds of test can be done: suspension tests or surface tests [5–7]. Whether testing of disinfectants in suspension is appropriate when the intended use of the disinfectant will be to treat hard surfaces remains an open question [8]. The virucidal effect of products can be tested on viruses applied on a stainless steel surface [7, 9, 10]. Challenged viruses, in

these tests, must be resistant to drying, which is more likely with non-enveloped viruses than enveloped viruses [11].

In the present study we developed a system in order to study the virucidal effect of a commonly used disinfectant product towards viruses in suspension and dried on a stainless steel surface. In this system the resistance of non-enveloped viruses, feline calicivirus F9 (FCV) and coxsackievirus B4 (CVB4), and one enveloped virus, herpes simplex virus type 1 (HSV-1), was investigated. These viruses are relevant models to assess the virucidal effect of disinfectant products. They can be responsible for nosocomial infections [1, 12, 13] and viral preparations with infectious titers higher than 10^6 TCID₅₀ · ml⁻¹ are readily obtained in vitro. Moreover, enteroviruses like CVB4 and caliciviruses like FCV are known to be resistant to disinfectant products [14, 15].

Materials and Methods

Viruses and Cells

HSV-1 (ATCC VR-260) and FCV (ATCC VR-782) were obtained from the LGC Promochem (Molsheim, France). CVB4 E2 is a strain provided by Ji-Won Yoon, Julia McFarlane Diabetes Research Center (Calgary, Alta., Canada) [16]. The Hep-2 (ATCC CCL-23), Vero (ATCC CCL-81) and CRFK (ATCC CCL-94) cell lines were obtained from the European Collection of Cell Cultures (ECACC) (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France).

The viruses were propagated in a flask on the appropriated cell lines (HSV-1, CVB4 on Vero cells and FCV on CRFK cells) cultured in Eagle's essential medium (MEM; Invitrogen, France) supplemented with 2% fetal bovine serum (FBS), 1% non-essential amino acids and 1% L-glutamine at 37° in a 5% CO₂ atmosphere. When a cytopathic effect of at least 75% appeared, the cells were scratched and the cell suspension was centrifuged at 1,000 g for 10 min. The resulting supernatant was aliquoted and stored at -80° after one (for enveloped viruses) to three (for non-enveloped viruses) freezing and thawing cycles.

Disinfectant

The disinfectant (Surfa'Safe®) is an aqueous solution at pH 6 containing quaternary ammonium (didecylmethylammonium chloride 0.14%), biguanide (polyhexamethylene biguanide hydrochloride, 0.096%) and in addition a chelating agent (succinate), a foaming detergent (laurylamine), and a tenside agent (non-biocidal quaternary ammonium) (0.7% for the three compounds). The product was manufactured and provided by the Laboratoires Anios (Lille-Hellemmes, France). The disinfectant is a ready-to-use solution used for the disinfection of surfaces. The main components of this disinfectant, quaternary ammonium and biguanide, are supposed to be membrane-active products [17].

Inactivation of Virus in Suspension

To determine the residual infectious titer of viruses in virucidal suspension assay, the trimmed Spearman-Kärber method was used. The contact time was 0–30 min at room temperature (relative

humidity 30 ± 10%). For all these tests, 96-well plates were prepared 1 day before the titration. Briefly, four tests were performed: (1) The determination of the cytotoxicity of the product was done by testing various concentrations of product directly on an established cell monolayer. The cytotoxic effect was read after 5–7 days. (2) The elimination of the cytotoxicity of the product was obtained using a Sephacryl® column (S-400 High Resolution; GE Healthcare) in order to remove the cytotoxicity before testing it on an established cell monolayer. The cytotoxic effect was read after 5–7 days on the flow-through. Briefly, the fluids were distributed in six replicates in 96-well plates and serially diluted from 10⁻¹ to 10⁻⁸ in MEM (Invitrogen) supplemented with 2% FBS, 1% non-essential amino acids and 1% L-glutamine. The plates were examined under an inverted microscope to evaluate the extent of the cytotoxic effect in cell cultures. (3) The control of efficiency for suppression of disinfectant activity was done by filtrating the product through a Sephacryl column. The flow-through mixed with a virus preparation during half an hour before titration. (4) For testing the virucidal activity of the product, 1 part of virus supernatant was mixed with 9 parts of disinfectant or sterile water. The mix was then filtrated using a Sephacryl column and a titration was carried out on the flow-through. Briefly, the fluids were distributed in six replicates in 96-well plates and serially diluted from 10⁻¹ to 10⁻⁸ in MEM supplemented with 2% FBS, 1% non-essential amino acids and 1% L-glutamine, then the plates were incubated for 3 days (FCV), 5 days (CVB4 and HSV-1), in a 5% CO₂ atmosphere at 37°. Afterwards the plates were examined using an inverted microscope to evaluate the extent of the virus-induced cytopathic effect in the cell culture. Calculation of estimated virus concentration was carried out by the Spearman-Kärber method and expressed in log₁₀ TCID₅₀ · ml⁻¹ [18]. When appropriate, a reduction factor of infectious titer was calculated as the difference between the logarithmic virus titer in the absence and in the presence of disinfectant and expressed as log₁₀.

Inactivation of Virus on Carrier

Stainless steel discs (20 mm diameter), with grade 2B finish on both sides, were provided by Devos SA (Lille, France). Before use, the discs were sterilized with γ-rays. 50 µl of virus inocula (titer ≥ 10⁶ TCID₅₀ · ml⁻¹) were applied on the middle of each stainless steel disc and dried under the air flow of a class II biological safety cabinet at room temperature for 2 h (±15 min) until a dried aspect was observed. After drying, 100 µl of disinfectant (or water) were then put on the dried virus inocula and incubated for 0–60 min at room temperature. For testing the resistance of viruses to drying, control carriers were not recovered with disinfectant or water. After completion of contact time, the disc was scrubbed with a flocked swab (Copan). The swab was then centrifuged during 15 s in 1 ml of culture medium. The fluids were then harvested and passed on a Sephacryl column in order to remove the toxicity and the flow-through was titered and the results expressed as described above. Non-enveloped viruses were fully recovered after filtration in a Sephacryl column, whereas one log₁₀ reduction of infectious titer was observed as far as HSV-1 (data not shown).

Gel Filtration Column

Gel filtration was used in order to eliminate the cytotoxicity of fluids containing disinfectant. The Sephacryl slurry was washed three times in TE buffer (pH 7.6); each time a centrifugation of 1 min at 700 g was done. 10 ml of the washed Sephacryl slurry was then added to an empty Poly-Prep® chromatography column (Bio-

Rad) and centrifuged 1 min at 700 g to eliminate the washing buffer. Once the columns were prepared, 1 ml of the tested preparation was added to the Sephacryl column and centrifuged during 1 min at 700 g. The flow-through was then titrated as previously described.

RT Real-Time PCR

Viral RNA of FCV was extracted using a QIAmp Viral RNA Mini Kit® (Qiagen). Reverse transcription was performed using an AffinityScript cDNA Synthesis Kit® (Stratagene) using random hexamer. Primers (Eurogentec) for reverse transcription and amplification of FCV RNA were previously described [19]. For each reaction, 5 µl of cDNA was used in a real-time thermal cycler (MX3000P®; Stratagene) with 3 µl (7 pmol) forward primer (TAA TTC GGT GTT TGA TTT GGC CTG GGC T), 3 µl (7 pmol) reverse primer (CAT ATG CGG CTC TGA TGG CTT GAA ACT G) 12.5 µl SyberGreen Mix® (Stratagene) and water up to 25 µl. The PCR program had an initial denaturation step at 95° for 10 min followed by 40 cycles of denaturation for 30 s at 95°, 30 s of annealing at 42°, and 30 s of elongation at 72°. Immediately following PCR, a melting curve was performed by raising the temperature from 55 to 95°. Results were expressed as cycle threshold (C_t). The C_t value denotes how many PCR cycles are required for the sample fluorescence to reach the threshold level. A higher quantity of target molecule (retrotranscript RNA) gave a higher increase in fluorescence associated with a low C_t (inversely proportionate to RNA level). Thus in our system the amount of viral RNA was estimated through C_t values.

Results

Viruses in Suspension

The resistance of viruses in suspension towards the disinfectant has been investigated. To assess the efficiency of a virucidal product, a diminution of the virus titer of at least 4 log in suspension is needed [5]. The titers of the virus preparations were at least 10^6 TCID₅₀ · ml⁻¹ ($10^{7.17}$ TCID₅₀ · ml⁻¹, $10^{6.66}$ TCID₅₀ · ml⁻¹, and $10^{8.69}$ TCID₅₀ · ml⁻¹ for HSV-1, CVB4, and FCV, respectively). Clarified culture supernatant fluids from cells infected with non-enveloped viruses (FCV and CVB4) and enveloped viruses (HSV-1) were incubated in the presence of the disinfectant product. After 30 min a reduction of infectious titer lower than 1.5 log₁₀ was obtained in the case of FCV and CVB4. In contrast, a 4 log₁₀ reduction of infectious titer was observed in less than 1 min with HSV1 (fig. 1). As expected, the resistance of non-enveloped viruses in suspension was higher than the one of enveloped viruses when they were incubated with the disinfectant.

Viruses onto a Stainless Steel Surface

It was decided to apply viral preparations on stainless steel carriers and to recover the inoculum using flocked swabs. The yield of recovering virus has been assessed.

Fig. 1. Survival of viruses in suspension exposed to a disinfectant. Culture supernatant fluids containing FCV, CVB4 or HSV-1 were incubated in the absence or presence of a disinfectant for 0, 5, 15 or 30 min. The infectious titers of viral preparations were determined and the reduction factors were calculated as described in the Material and Methods section. The results are the mean ± SD of two independent experiments.

Table 1. Viral inocula recovered with flocked swabs

Virus	Initial inoculum	Recovered inoculum
HSV-1	7.33±0.50	7.17±0.33
FCV	9.00±0.50	8.69±0.50
CVB4	7.00±0.50	6.66±0.66

50 µl of clarified culture supernatant fluids from cells infected with FCV, CVB4 or HSV-1 applied onto stainless steel surfaces were immediately recovered with flocked swabs that were vortexed in 1 ml culture medium which was harvested for titration of infectious particles. The results are the mean ± SD of three independent experiments and are expressed as log₁₀ TCID₅₀ · ml⁻¹.

Clarified culture supernatant fluids from cells infected with FCV, CVB4 or HSV-1 applied onto stainless steel surfaces were immediately recovered with flocked swabs that were vortexed in 1 ml culture medium which was harvested for titration of infectious particles. The average amounts of infectious particles, in recovered and initial inocula, were very close: $10^{7.17}$ vs. $10^{7.33}$ TCID₅₀ · ml⁻¹ for HSV-1, $10^{8.69}$ vs. $10^{9.00}$ TCID₅₀ · ml⁻¹ for FCV, and $10^{6.66}$ vs. $10^{7.00}$ TCID₅₀ · ml⁻¹ for CVB4 (table 1). These results prompted us to use flocked swabs for recovering viruses in the rest of our studies.

Fig. 2. Viruses on a stainless surface. 50 μ l of culture supernatant fluid containing HSV-1, CVB4 or FCV were applied on a stainless carrier in duplicate. They were dried and thereafter recovered using wet flocked swabs at 0, 30, 60 or 120 min. The infectious titers of inocula and dried inocula were determined and expressed as $\log_{10}$ TCID₅₀ · ml⁻¹. The results are the mean $\pm$ SD of two independent experiments.

Clarified culture supernatant fluids from virus-infected cells were applied onto stainless steel carriers. Following drying, the carriers were scrubbed with flocked swabs humidified with culture medium and infectious titers of dried and fresh inocula were measured. A 1.86 $\log_{10}$ reduction of FCV infectious titer was observed ($10^{6.83}$ vs. $10^{8.69}$ TCID₅₀ · ml⁻¹) using swabs for recovering viruses. A more pronounced reduction of CVB4 and HSV-1 infectious titers was observed: 2.22 $\log_{10}$ ($10^{4.54}$ vs. $10^{6.66}$ TCID₅₀ · ml⁻¹) and 2.75 $\log_{10}$ ($10^{4.42}$ vs. $10^{7.17}$ TCID₅₀ · ml⁻¹), respectively. The infectious titers of dried inocula were roughly maintained up to 1 h after drying (fig. 2). The infectious titers were lower in the dried than in the fresh inocula, and the extent of the difference was higher in the case of HSV-1 (fig. 2).

Viruses onto Stainless Steel Surface Incubated with Disinfectant

Virus inocula (50 μ l) dried on a stainless steel surface were incubated with sterile water or ready-to-use disinfectant (100 μ l). After completion of the contact time (0–60 min) the surfaces were scrubbed with flocked swabs. The swabs were then vortexed in 1 ml culture medium in order to recover the viruses. Afterwards, the fluids were harvested.

The measurement of infectious viral titer in harvested fluid relies on its inoculation onto cell cultures. An approach based on gel filtration columns allowed the retention of small molecules (i.e. disinfectant) and displayed that the flow-through, at a 1:10 dilution, was no more cytotoxic. The successful removal of disinfectant, using gel filtration, enabled us to determine the residual infectious titer of virus previously applied on a stainless steel surface and treated with a virucidal product.

As shown in figure 3a, the infectious titer of a dried inoculum of HSV-1 was reduced up to 2.83 $\log_{10}$ in less than 15 s after addition of the disinfectant, whereas the reduction of infectious titer of a dried inoculum of CVB4 was only 1.00 $\log_{10}$ after incubation with disinfectant for 60 min. The infectious titer of a dried inoculum of FCV was dramatically reduced after incubation with disinfectant for 10 min (4 $\log_{10}$ reduction; fig. 3b). In contrast, the infectious titer of FCV maintained in suspension was not extensively reduced after incubation with disinfectant for 30 min (1.5 $\log_{10}$ reduction; fig. 1).

Subsequently, we determined whether drying impaired their resistance to the product. FCV inocula applied on carriers and dried were recovered using a wet swab then incubated with 200 μ l of disinfectant in a tube during 15 min, the resulting infectious titer was $10^{5.45}$ TCID₅₀ · ml⁻¹ (fig. 4). When dried inocula on carriers were either incubated with 100 μ l of sterile water or disinfectant, the infectious titers were $10^{6.37}$ and $10^{2.29}$ TCID₅₀ · ml⁻¹, respectively (i.e. around 4 $\log_{10}$ reduction) (fig. 4).

The 4 $\log_{10}$ reduction of infectious titer in recovered FCV inocula when disinfectant was added to spots of virus dried on stainless steel surface raised several issues. The efficiency of recovering viral particles in these conditions was questioned. Whether the disinfectant could prevent recovering viruses from the surface or could retain them in the swab was investigated. Therefore, on the one hand the infectious titer was determined and on the other, RNA was extracted to measure the amount of viral RNA by RT real-time PCR in order to estimate the level of viral particles. The amount of viral RNA in the recovered disinfectant-treated dried spots and in the recovered dried spots was closely similar as displayed by the pattern of C_t values obtained by RT real-time PCR (C_t = 16.3 vs. 18.1, respectively) whereas the infectious titer values were markedly different (4 $\log_{10}$ reduction) (fig. 5). These data brought evidence that viral particles dried on a stainless steel surface and treated or not with disinfectant were readily recovered and that the disinfectant reduced the survival of FCV dried on a stainless steel surface.

Fig. 3. Virucidal effect of a disinfectant onto viruses applied on stainless carriers. 50 μ l of culture supernatant fluids containing HSV-1, CVB4 (a) or FCV (b) were applied on stainless carriers in duplicate. They were dried and then covered with sterile water (100 μ l) or disinfectant (100 μ l) at room temperature for 0–60 min. Thereafter the carrier surface was scrubbed with a flocked swab and the infectious titer of recovered inoculum was determined and the reduction factor was calculated and expressed as $\log_{10}$ as described in the Material and Methods section. The results are the mean $\pm$ SD of two independent experiments.

Fig. 4. Virucidal effect of a disinfectant onto dried FCV. 50 μ l of culture supernatant fluid containing FCV was applied on stainless carriers in duplicate. Inocula were either dried and then immediately recovered by scrubbing the carrier surface with a wet flocked swab and incubated with 200 μ l of disinfectant during 15 min or dried and then covered with sterile water (100 μ l) or disinfectant (100 μ l) at room temperature for 15 min, and thereafter recovered using a flocked swab. The infectious titers of inocula and dried inocula were determined and expressed as $\log_{10}$ TCID₅₀ · ml⁻¹. The results are the mean $\pm$ SD of two independent experiments.

Fig. 5. Quantification of FCV RNA and level of infectious particles. 50 μ l of culture supernatant fluid containing FCV were applied on stainless carriers in duplicate. Inocula were dried and then covered with sterile water (100 μ l) or disinfectant (100 μ l) at room temperature for 15 min, and thereafter recovered using a flocked swab. The infectious titers of inocula were determined and expressed as $\log_{10}$ TCID₅₀ · ml⁻¹ (□). RNA was extracted from the harvested fluid and the level of viral RNA was measured by quantitative RT-PCR and expressed as C_t (■). The results are the mean $\pm$ SD of two independent experiments.

Discussion

An efficient surface disinfection is a key step in preventing the spreading of infectious agents [4]. In Europe, most disinfectants are tested in suspension, however developing tests to assess the efficiency of disinfectants in clearing infectious viruses from surfaces is crucial [8].

The present study is different in many respects from those of other investigators. There are several noteworthy

considerations for the system used in the present report. A critical issue was recovering viruses from the carrier. To this purpose, flocked swabs were successfully used in our experiments with viruses as they were previously used to recover infectious agents from various surfaces in clinical diagnosis and epidemiological studies [20–22]. Recovering a virus with flocked swabs, as described in the present study, opens up the possibility to test the virucidal effect of products on viruses applied on various surfaces what-

ever their size. One advantage of using flocked swabs to recover viruses is that they are vortexed in 1-cm diameter tubes with 1 ml culture medium, whereas carriers must be vortexed in 4-cm diameter flat-bottom containers with 1 ml culture medium, which, in other respects, produces a certain amount of foam. Another critical issue was the elimination of disinfectant before virus titration. Therefore, gel filtration columns were used to filter the disinfectant in experiments with viruses applied on a surface, as they can be used to eliminate disinfectant in virucidal assays towards viruses, as previously described [5, 23].

Our results show that when viruses are applied on a stainless steel surface they can be segregated into three categories as far as their resistance to drying and to the virucidal effect of a disinfectant. Enveloped and non-enveloped viruses can be incorporated in suspension and surface assays. In our experiments with HSV-1, the virucidal effect of disinfectant was displayed on this virus, on the one hand in suspension and on the other, dried on a carrier. Such a pattern of results has also been observed with CVB4, a non-enveloped virus which was as expected, in suspension and on a carrier, more resistant than HSV-1 to disinfectant. Moreover, an alternative pattern of resistance that was not reported previously, to the best of our knowledge, has been observed in our experiments with FCV. Indeed it appeared that FCV was more resistant to a disinfectant in suspension than on a stainless steel carrier, even though its infectivity was still maintained at a high level ($10^{6.5}$ TCID₅₀ · ml⁻¹) 1 h after drying.

In contrast to FCV, poliovirus, another non-enveloped virus, was shown to be able to resist the virucidal effect of a disinfectant (chlorine) when it was adsorbed to a porous surface whereas it was inactivated in 10 min by the same product when it was in suspension. However, it was not observed with other members of the *Enterovirus* genus. Indeed, coxsackievirus B5 and echovirus 6, in suspension and on a porous surface were resistant to disinfectant whereas in both conditions echovirus 25 was inactivated [24]. As a matter of fact, the type of surface might play a role in the resistance of viruses to a disinfectant, since dried poliovirus on a glass cup can be dramatically inactivated by chlorine in 1 min [25]. However, the virucidal effect of a disinfectant towards viruses applied on a surface is a complex issue insofar as it has been reported that FCV on non-porous surfaces can be inactivated or not by a disinfectant [10, 26].

Quaternary ammoniums are supposed to be membrane-active products, which can explain the effect towards HSV-1 and other enveloped viruses in our current studies and those of other groups, obtained with a quater-

nary ammonium-based disinfectant [27–29]. Non-enveloped viruses are known to be resistant to quaternary ammonium [17, 30], however a virucidal effect towards African horse sickness virus in suspension (member of the Reoviridae family) has been reported [28]. In our studies a limited or almost non-existent effect was observed in suspension on FCV and CVB4, respectively, whereas an important effect was obtained towards FCV dried on a stainless steel carrier, which is in agreement with a possible virucidal effect of quaternary ammonium towards FCV on a non-porous surface (Petri dish) that was already reported [10].

A biguanide is included in the disinfectant used in the current study, the virucidal effect of that chemical group against non-enveloped viruses is modest which by deduction argues against a role of this compound by itself in the inactivation of FCV applied on a surface [31]. One hypothesis is that the virucidal effect on FCV applied on a carrier, obtained in our studies, was due to the combination of quaternary ammonium in the presence of the other compounds encompassed within the product.

The discrepancy between the virucidal effect of a disinfectant on FCV in suspension and on a surface, as observed in the present study, is intriguing. The drying of particles on a carrier cannot account for the lower resistance of the virus applied on a stainless steel carrier, compared with virus in suspension, since drying did not impair its resistance to the disinfectant as demonstrated by measuring the viral titer and the amount of viral RNA in our experiments. It has been reported that the exposure to biguanides, through change of the virus surface hydrophobicity, can result in the formation of viral aggregates which hence can be involved in the resistance of viruses to disinfectants [31, 32]. Whether this effect of biguanides can play a role in the increased resistance of FCV particles to disinfectants in suspension in our experiments cannot be excluded. Further studies are needed to improve the understanding of the virucidal effect of a quaternary ammonium-based disinfectant towards FCV, particularly when the virus is applied on a surface.

In conclusion, it has been observed that HSV-1 appeared as a relevant candidate to test the virucidal activity of a disinfectant on surfaces. Whether other enveloped viruses are pertinent for this purpose remains to be investigated. FCV applied on a surface, but not in suspension, was inactivated by a disinfectant product whereas another non-enveloped virus, CVB4, was resistant. Further studies are needed to determine whether other non-enveloped viruses applied on a surface can be inactivated by this product. Together the results of this study suggest

that a system based on the combination of recovering virus using flocked swabs and disinfectant discarding through gel filtration columns has the potential of being used for evaluating the resistance of viruses applied onto a surface and for determining the effect of various disinfectants towards viruses in that condition. This system enabled us to display an unexpected virucidal effect of a disinfectant product.

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