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Determination of anti-herpes simplex virus type 1 activity of the air filter coated with silver ions using a modified method of ISO 21702:2019

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ABSTRACT

Background: Since severe acute respiratory syndrome coronavirus 2 causing coronavirus disease 2019 pandemic has emerged, various air membrane filters with antimicrobial agents have been explored and investigated for inactivating virus particles. **Objectives:** The purpose of this study was to determine an antiviral activity of the air filter coated with silver ions using the method of ISO 21702:2019. **Materials and Methods:** Herpes simplex virus type 1 (HSV-1), KOS strain, an enveloped virus causing skin, mucous membrane, and respiratory tract infection (RTI) were tested with the non-porous surface coated with or without silver ions (control filter) for 0, 24 h at 25°C. The viral infectivity titers were determined on Vero cells by a plaque assay. After 24 h, the silver filter showed high anti-viral effect with logarithmic reduction >3.91 or 99.92% reduction compared to that of the control filter. Validation of the method demonstrated that the filters were nontoxic, the cell was sensitive to the virus and the neutralizer could inactivate the antiviral activity. **Conclusion:** The air filter coated with silver ions possessed anti-HSV-1activity. Further study with other viruses causing RTI would be helpful for the prevention of viral spread.

Keywords: Air filter, antiviral activity, herpes simplex virus type 1, ISO 21702:2019, silver ion-coating

INTRODUCTION

Since severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped RNA virus, causing coronavirus disease 2019 pandemic, has emerged, various air membrane filters coated with antimicrobial agents have been explored and investigated for inactivating viral particles under airflow conditions that can be used in an indoor environment.[1-7] Jung *et al*.[1] prepared silver/carbon nanotubes hybrid nanoparticles using an aerosol process to use for antimicrobial air filtration against *Staphylococcus epidermidis* and *Escherichia coli* bioaerosols. Joe *et al*.[6,7] fabricated an anti-viral filter using an aerosol coating of silica hybrid particles decorated with silver nanoparticles and performed an anti-viral ability test against the aerosolized bacteriophage MS2 virus. They also evaluated the anti-viral efficiency of a silver nanoparticle coated air filter against the bacteriophage MS2 virus through the process of dust loading. Silver ions are very reactive and are popular used for coating on air filters because their strong biocidal effects.[1]

We are interested in investigating an antiviral activity of the air filter coated with silver ions against human herpes simplex virus type 1 (HSV-1) because it is an enveloped virus causing skin, mucous membrane, and respiratory tract (RT) infections, it is suitable for our biosafety level (BSL) II laboratory and little is known about anti-HSV effect of silver ion-coating air filter. Hu *et al*. demonstrated that the mixture of silver-nanoparticle suspension and HSV-2 prior to infecting cells could significantly inhibit the production of progeny viruses.[8] A silver nanoparticles-coated polyurethane condom developed by Mohammed Fayaz *et al*. showed anti-HSV-1 and anti-HSV-2 activities by preventing the virus-induced cytopathic effects (CPE) in Vero-E6 cells.[9]

HSV is an enveloped spherical medium-size (155–240 nm) DNA virus and a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Simplexvirus*. This genus is consist of HSV type 1 (HSV-1) and HSV serotype 2 (HSV-2).[10] Human HSV was first recalled as a pulmonary pathogen.[11] HSV-1 usually infects upper RT including gingivostomatitis and pharyngitis. However, lower RT infections, especially pneumonia caused by this virus, have been reported since 1949.[12] HSV-2 usually infects genital tract including genital herpes).[10]

In this study, we determined the antiviral activity of the air filter using a modified method of ISO 21702:2019.[13] ISO 21702:2019 is a proper method for measuring antiviral activity on plastics and other non-porous surfaces of antiviral-treated products against specified viruses. We demonstrated the antiviral efficiency of the air filter against an airborne infectious virus, HSV-1.

MATERIALS AND METHODS

Preparation of Test Air Filters

Air filters in our study were non-porous surface made from microglass fiber with or without silver ion-coating and were manufactured by M-Plus filtration Company Limited. M-ION® was the silver-coating tested filter and the one without silver was the control filter. The color of the control filter was cream with 0.25 mm-thickness and of the tested filter was white with 0.3 mm-thickness. According to the method of ISO 21702:2019,[13] the filter was cut into a square of (50 ± 2) mm × (50 ± 2) mm, 12 specimens of the control filter (untreated test specimens) and nine specimens of M-ION® (treated test specimens). Each piece of specimens was put on a sterile Petri dish [Figure 1a]. In the test procedure, film (Parafilm M®, Bemis USA) was also cut into a square of (40 ± 2) mm × (40 ± 2) mm (three untreated test specimens after incubation time and three treated test specimens after incubation time) for covering the test inoculum on the air filters [Figure 1b]. All of the prepared air filters and films were separately sterilized by UV radiation for 15 second/side before the testing.

Preparation of Test Virus and Host Cell

HSV-1, KOS strain obtained from the National Institute of Health of Thailand, was used as a test virus and suitable for our BSL II laboratory. Vero cell (kidney cell of an African green monkey) is a tumor cell line and susceptible host cell for the test virus. The host cells were propagated confluently in a growth medium (GM) (minimum essential medium [MEM, Gibco®] supplemented with 10% heated fetal bovine serum, Gibco®) on the surface of a 75 cm^2-tissue culture flask in an incubator with 37°C and 5% CO_2 for 24 h. One milliliter (mL) of the virus suspension was inoculated on Vero cells with a multiplicity of infection ≤0.01. After viral adsorption for 1 h, 20 mL of MEM was added and the cells were reincubated for 2–3 days or until 90% of CPE was observed under an inverted microscope. The virus was then harvested and collected as the stock virus and kept at −20°C until used. Quantification of the stock virus was checked by a plaque assay.

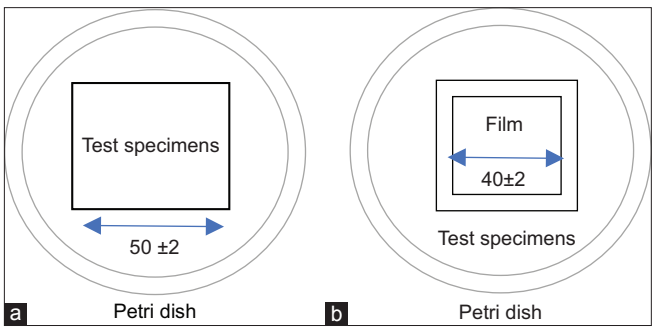

Figure 1: (a) Preparation of test specimen and (b) placement of cover film after virus inoculation

Plaque Assay

A fresh confluence cell monolayer of Vero cells prepared on each well of a 6-well tissue culture plate. HSV-1 stock solution was 5-fold diluted with MEM and then 0.1 mL of each dilution was added on Vero cells in each well (2 wells/dilution) after removing the GM. The plates were put in the incubator for 1 h, in the meantime, they were tilted every 15 min. Three ml of the overlayer medium (MEM containing 0.8% methyl cellulose) were added on the monolayer in each well and the plates were then reincubated for 3 days. After the incubation, 1 mL of fixing solution (38% formalin: Normal saline, 1:2) was added to and mixed with the overlayer medium in each of the wells. The plates were kept at room temperature for more than 1 h to fix the cells. The mixture solution was removed and the cells were stained with 1% of methylene blue with 10% of methanol solution at room temperature for more than 1 h. The extra methylene blue solution was washed with tapped water and plaques were counted in each well under an inverted microscope.[14] The viral stock concentration was calculated in plaque forming unit (PFU) numbers/ml by selecting the wells containing 6–60 PFU.[13]

Verification of the Test

Cytotoxicity of the test air filter

Soybean casein digest broth supplemented with 0.1% lecithin and 0.05% polysorbate 20 (SCDLP20) was prepared as a neutralizer and used to perform this test. Ten milliliters of SCDLP20 were added to either the untreated test specimen (U) or the treated test specimen (T). Then, the specimen was washed at least 4 times by pipetting the broth onto the filter. Three U and three T plates were used for this test. The recovered broth was collected from the specimen into a new sterile tube (6 tubes, 3 U, and 3 T). A fresh confluence cell monolayer of Vero cells prepared on each well of a 6-well tissue culture plate. At least three plates were needed (12 wells for the filters, two wells for the neutralizer control (neutralizer, no filter washing) and two wells for the cell control (no neutralizer, no filter washing). The GM was removed from each well and the monolayer on the well was washed once with 3 mL of MEM. After removing the extra MEM, 0.1 mL of the broth in the tube was added immediately in the well (2 wells/tube). The plates were put in the incubator for 1 h, in the meantime, they were tilted every 15 min. Three milliliters of the overlayer medium were added on the monolayer in each well and the plates were then reincubated for 3 days. After the incubation, the cells were fixed and stained as the same conditions used in the plaque assay. The CPE in each well was observed. If any CPE (cytotoxicity) was observed in the neutralizer control wells, the neutralizer formulation would be modified. If no CPE was observed in the tested filter wells, the next step would be proceeded.[13]

The inactivation of antiviral activity

SCDLP20 was prepared as a neutralizer used to inactivate antiviral activity. Ten milliliters of SCDLP20 were added to each specimen (3U, three treated test specimen [T]). Then, the specimen was washed at least 4 times by pipetting the broth

onto the filter. Five milliliters of the recovered broth were collected from the specimen into a new sterile tube (six tubes, 3 U and 3 T) and 5 ml of the new SCDLP20 into three new sterile test tubes as negative controls (N). Then, 50 µL of a virus suspension prepared to be a concentration of 5×10^4 PFU/ml were added into each of the nine tubes (3U, 3T, and 3N). A fresh confluence cell monolayer of Vero cells prepared on each well of a 6-well plate. At least three plates (18 wells) were needed (2 wells/tube). The rest of the procedure was performed as described in the plaque assay. The viral concentration in each tube was calculated in PFU/mL.[13]

Test Procedure

Inoculation of test specimens

Surface of each filter specimen prepared in the sterile Petri dish (6 U, 3T) was inoculated with 0.4 mL of the stock virus suspension. The test inoculum was then covered with the prepared film in 2.1 and the film was gently pressed down so that the inoculum spreaded to the edges and did not leak beyond the edges of the film. After the film was applied, the lid of the Petri dish was closed. The dishes were kept at (25 ± 1)°C and a relatively humidity of not <90% for 24 h.[13]

Recovery of virus from test specimen immediately after inoculation

Immediately after inoculation, the three untreated test specimens were processed by adding 10 mL of SCDLP20 broth to each Petri dish. The virus was completely recovered from the specimens by pipetting the broth at least 4 times. The number of the infectivity titer of the virus was determined by the plaque assay. The infectivity titer of virus recovered was in accordance with formula (1).[13]

$$N = (10 \times C X D X V)/A \quad (1)$$

N was the infectivity titer of virus recovered per cm^2 of the test specimens.

C was the average number of plaque counted for the duplicate wells.

D was the dilution factor for the wells counted.

V was the volume of the SCDLP20 added to the specimen (mL).

A was the surface area of the cover film (cm^2).

Recovery of Virus from Test Specimens Immediately after Contact

After the contact time (24 h), the three untreated test specimens and three treated test specimens were processed with the same procedure described before. The viral concentration in each specimens was calculated in PFU/ml by selecting the wells containing 6–60 PFU. If the plaque numbers in the wells containing the 0.1 mL aliquots of the test suspension were <6, they were counted and recorded for the calculation. If there was no plaque recovered in any of the wells in the dilution series, the number of plaque would be <1. The antiviral activity was calculated using formula (2).[13]

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \quad (2)$$

R was the antiviral activity.

U_0 was the common logarithmic (log) of the number of plaques recovered from the three untreated test specimens immediately after inoculation (PFU/cm^2).

U_t was the common log of the number of plaques recovered from the three treated test specimens immediately after 24 h (PFU/cm^2).

A_t was the common log of the number of plaques recovered from the three treated test specimens immediately after 24 h (PFU/cm^2).

Validation Tests

The test procedure was deemed validly when the three conditions given below were satisfied. If any condition was not met, the test was considered as invalid and the specimens should be retested.[13]

The logarithmic (log) value of the number of plaques recovered immediately after inoculation from the untreated specimens should satisfy the requirement of this formula.

$$(L_{max} - L_{min})/(L_{mean}) \leq 0.2$$

L_{max} was the common log of the maximum number of plaques recovered from a specimen.

L_{min} was the common log of the minimum number of plaques recovered from a specimen.

L_{mean} was the common log of the mean number of plaques recovered from the three specimens.

The average number of plaques recovered immediately after inoculation from the untreated specimens should be within the range of 2.5×10^5–1.2×10^6 PFU/cm^2.

The number of plaques recovered from each untreated test specimen after contacting for 24 h should not be $<6.2 \times 10^2$ PFU/cm^2.

Statistical Analysis

Data were analyzed using SPSS 28 (Wilconxon signed-rank test and *t*-test) and are reported as means ± SD of three independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Cytotoxicity Effect

CPE was not shown in all wells of the cells contacting with washing broth from the air filters indicating that the air filters coated with or without silver ions were safe to Vero cells and could be tested in the next experiments. According to the method of ISO 21702:2019,[13] soybean casein digest broth with 0.1% lecithin and 0.7% polysorbate (SCDLP) broth was suggested as a neutralizer. We found that 0.1–0.7% polysorbate 20 in SCDLP formulations destroyed Vero cells and HSV-1. HSV-1 is an enveloped virus which is generally sensitive to a surfactant with high concentration. The highest concentration of polysorbate 20 non-toxic to the tested cells and virus was 0.05%. Consequently, modified SCDLP20 was used as the neutralizer throughout our experiments.

Effects of the Inactivation of Antiviral Activity

The washing neutralizer broth could inactivate anti-HSV-1 activity of the silver-coated filter as shown in the results in Table 1. The log viral titers recovered from M-ION filter (S_t) and control filter (S_u) were determined by plaque assay. In addition, the neutralizer was nontoxic to Vero cells as demonstrated by the infection capability of the virus recovered from the neutralizer (S_n). Difference of log viral titer between of neutralizer broth and of M-ION filter (S_n-S_t) was 0.05. Difference of log viral titer between of neutralizer broth and of control filter (S_n-S_u) was 0.02. Based on ISO 21702:2019 requirement, if both of the log differences were less than 0.5, the test procedure conditions including the neutralizer, host cell and virus were verified and suitable. Low concentration of non-ionic surfactant such as 0.05% polysorbate 20 and 0.1% lecithin could inactivate anti-viral effect of silver ion-coating on the air filter as shown in the result in Table 1, the viral titers on the treated specimen were not different significantly (S_n–S_t <0.5)[13](Wilconxon signed-rank test, $P > 0.05$) from that on the negative control. In addition, the control filter had no effect on the viral titer (S_n–S_u <0.5).[13] Lecithin as a biological lipid and was used to enhance the diffusion of silver ions. Silver ions can be absorbed by lecithin molecules because of electrostatic interaction of positive charge of silver ions and negative charge of phosphate group.[15] Polysorbate 20 is a non-ionic surfactant containing ether-ester linkages which can inactivate a virus by solvating the viral envelope and disrupting the nucleocapsid.[16] In addition, polysorbate can be used as a neutralizer when combined with lecithin.[13] However, 0.05% polysorbate 20 could inactivate anti-viral effect of silver ion-coating without destroying the virus. The inactivation of the antiviral activity by the neutralizer demonstrated that we measured the actual viral concentration at the collected time.

Anti-viral Effect of Non-porous Surface Air Filters

Immediately after inoculation on the three untreated test specimens (control filter), the virus was completely recovered from the specimen and was quantified by the plaque assay. The initial viral titer was 1.4×10^7 PFU/mL. The log average viral titer of the three untreated test specimens (U_0) was 5.75 PFU/cm^2 [Table 2]. After 24 h, the log average viral titer of them (U_t) was a little lower (5.10 PFU/cm^2) according to natural survival. Interestingly, no plaque observed in any of the wells in the dilutions of the three treated test specimens. Consequently, the log mean viral titer recovered from M-ION filter after 24 h would be less than 1.19 PFU/cm^2 (A_t). M-ION filter showed high anti-viral effect with log reduction (R) more than 3.91 or more than 99.92% reduction compared to that of the control filter at the same time. A_t was less than U_t significantly ($P < 0.05$, *t*-test).

Possible mechanisms of antimicrobial activities of silver or silver ions had been proposed. Sondi and Salopek-Sondi investigated that *E. coli* cells treated with silver nanoparticles were destroyed. Scanning and transmission electron microscopy demonstrated the formation of "pits" in the bacterial cell wall and the accumulation of silver nanoparticles in the bacterial membrane resulting in increasing the membrane permeability.[17] Silver ions bind to tissue proteins, inducing structural changes in the bacterial cell wall and nuclear membrane that lead to cell distortion and death.[1] Baram-Pinto *et al*. designed silver nanoparticles that were capped with mercaptoethane sulfonate. These capped nanoparticles were predicted to target HSV-1 and to compete for its binding to cellular heparan sulfate, leading to the blockage of viral entry into the cell and to the prevention of subsequent infection. Inhibition of HSV-1 infection in cell culture by the capped silver nanoparticles was observed. Their results suggested that capped silver nanoparticles might serve as useful topical agents for the prevention of HSV infections.[18] HSV-1 KOS strain was originally isolated from a cold sore and has been passaged 12 times in tissue culture. KOS is frequently used to investigate HSV-1 gene function and pathogenesis.[19] Lara *et al*. suggested that silver nanoparticles exerted an anti-HIV activity at an early stage of viral replication by binding to gp120 in a manner that prevented CD4-dependent virion binding. Silver nanoparticles were acting as an effective virucidal agent against cell-free and cell-associated HIV.[20] Speshock *et al*. proved that pre-treatment of the cells with silver nanoparticles had no effect on Tacaribe virus replication; therefore, they concluded that the silver nanoparticles could be inactivating the virus before entry into the cell.[21] Silver nanoparticles were presumed to inhibit extracellular SARS-CoV-2. Luciferase-based pseudovirus entry assay revealed that silver nanoparticles potently inhibited viral entry step through disrupting viral integrity.[22] Most of the published literatures described the antiviral activities of silver nanoparticles or ions against enveloped viruses, with both a DNA or an RNA genome. In our research, we did not study the mechanism of antiviral action of the silver ions coated on the filter. We did observe that the viral solution on the silver-coated filter was disappeared after 24 h incubation whereas the viral solution on the control filter was still available. Since the antiviral effect of silver ions/nanoparticles was broad-spectrum on enveloped viruses, the mechanism of them might be the same manner in the preventing the viral entry through destroying the viral

Table 1: Result of the inactivation of antiviral activity

Test sample	Common log viral titer* (PFU/ml)(S)				Difference of log value
	Sample 1	Sample 2	Sample 3	Mean±SE	
Neutralizer broth** (negative control, *n*)	2.23	2.45	2.40	2.36±1.02 (S_n)	-
Control filter (untreated test specimen, u)	2.20	2.43	2.39	2.34±1.02 (S_u)	S_n–S_u=0.02***
M-ION filter (treated test specimen, t)	2.23	2.35	2.35	2.31±0.87 (S_t)	S_n–S_t=0.05***

*Herpes simplex virus Type 1 (KOS strain), initial titer=2.5×10^3 PFU, **Soybean casein digest broth with 1% lecithin and 0.05% polysorbate 20, ***Based on ISO 21702:2019 method, if the log differences<0.5, the neutralizer was suitable, Based on Wilconxon signed-rank test, they were not different significantly ($P > 0.05$)

Table 2: Anti-viral effect of non-porous surface with (M-ION filter) or without silver ion-coating (control filter)

Test sample	Common log viral titer (PFU/cm²)*				Antiviral activity (R)
	Sample 1	Sample 2	Sample 3	Mean±SE	
Control filter at 0 h	5.77	5.74	5.74	5.75±2.51 (U_0)	-
Control filter at 24 h	5.20	5.16	4.85	5.10±2.70 (U_t)	-
M-ION filter at 24 h	<1.19	<1.19	<1.19	<1.19±0 (A_t)	>3.91 (Ut–At)**

*Calculated from the formula in 2.5.2, the filter area exposed to virus was 16 (4 × 4) cm² and tested virus was herpes simplex virus type 1 (KOS strain), initial titer=1.4 × 10^7 PFU/mL, **Reduction>99.92% and they were different significantly ($P < 0.05$, *t*-test)

Table 3: Results of validation tests (based on ISO 21702:2019 method)

Conditions	Test results	Validation tests
$(L_{max}–L_{min})/(L_{mean})$* ≤0.2	(6.97–6.94)/6.95=0.004	Passed
U_0** range 2.5×10^5–1.2×10^6 PFU/cm²	U_0 = 5.60 × 10^5 PFU/cm²	Passed
3. U_t*** ≥6.2×10^2 PFU/cm²	Ut = 1.25 × 10^5 PFU/cm²	Passed

*L_{max} was the common log of the maximum number of plaques recovered from a specimen, L_{min} was the common log of the minimum number of plaques recovered from a specimen, L_{mean} was the common log of the mean number of plaques recovered from the 3 specimens, **U_0 was the average number of plaques recovered immediately after inoculation from the untreated specimens, ***U_t was the number of plaques recovered from each untreated test specimen after contacting for 24 h

envelopes. Further study about time killing, the mechanism of action and the lifespan of this coated filter should be undertaken.

Results of Validation Tests

The test procedure was deemed validly because the three conditions given in the validation tests were satisfied as shown in Table 3. In condition 1, our result was <0.2, 50 times indicating that the maximum number and the minimum number of plaques recovered from specimens were very close. U_0 was 5.60 × 10^5 PFU/cm² and was in the range (2.5 × 10^5–1.2 × 10^6 PFU/cm²). U_t was naturally lower (1.25 × 10^5 PFU/cm²) than U_0 after 24 h but was much higher than the lowest requirement (6.2 × 10^2 PFU/cm²) indicating that the control filter had no antiviral effect and the virus could survive in an environment for 24 h and suitable for the test.[13]

CONCLUSION

The results in this study indicated that the air filter coated with silver ions, M-ION filter possessed, anti-HSV-1activity. Further study with other viruses causing RT infections would be helpful for prevention of viral spread.

DECLARATION OF COMPETING INTEREST

The authors declare that we have no financial interests or personal relationships that could be influent the results in this study.

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