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Silver Ion (Ag^+) Formulations with Virucidal Efficacy against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

Yutaka Nishihara, Hideo Eguchi and Sifang Steve Zhou

Abstract

This chapter focuses on viral efficacy evaluations of silver ion (Ag^+) formulations against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus associated with the COVID-19 pandemic and feline calicivirus (FCV), a surrogate for human norovirus. The chapter discusses the proposed mechanism of inactivation, with reference to some previously published articles. In addition, it discusses the background/current trend/future view of Ag^+ products that have been used widely as surface/environment disinfectants in daily life all over the world. In efficacy studies performed by using the standardized ASTM E1052 methodology, it was found that Ag^+ formulated with a low concentration (26% w/w) of ethanol displayed virucidal activity against SARS-CoV-2 and FeCV. These formulations might be useful for preventing the transmission of such viruses and limiting the outbreaks of emerging infectious diseases caused by coronaviruses and caliciviruses. To our knowledge, this is the first report describing the virucidal efficacy of an Ag^+ formulation, evaluated by using the standardized ASTM E1052 methodology, for inactivating SARS-CoV-2. Some characteristics of Ag^+ -based virucides are discussed in this research report/minireview.

Keywords: COVID-19, feline calicivirus, liquid inactivation, SARS-CoV-2, silver ion (Ag^+), virucidal efficacy evaluation

1. Introduction

For over 6000 years and prior to the introduction of penicillin in the early 1940s, silver has been the main antimicrobial used by mankind [1]. Few people today are aware that, by 1940 prior to the introduction of penicillin, in the USA alone more than 50 silver-based antimicrobial products had been marketed in different formulations (solutions, ointments, colloids, or foils) for topical, oral, and intramuscular injections [2]. In brief, between 1900 and 1940, tens of thousands of patients were treated with colloidal silver, with several million doses of silver administered intravenously [1]. Since the early 2000s, antibiotic resistance of microbes has been of increasing concern. For both antiviral and antimicrobial

applications, silver ion (Ag^+)- and silver nanoparticle (AgNP)-based formulations have displayed an advantage in this respect, attacking bacteria and viruses in multiple ways and thereby limiting the chances of both viruses and bacteria to develop resistance to such formulations [2, 3].

This chapter describes the virucidal efficacy of a silver ion formulation, evaluated by using the standardized ASTM International (ASTM) E1052 methodology [4], for inactivating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and feline calicivirus (FeCV). The chapter also includes a discussion of the proposed mechanism of inactivation, with reference to some previously published articles. Some characteristics of Ag^+ - and AgNP-based virucides also are discussed in this research report/minireview.

2. Rediscovery of the antimicrobial potential of silver ion (Ag^+) and AgNP

The discovery that the antibacterial activity of AgNP is chiefly due to Ag^+ nonetheless led Xiu et al. [5] to recommend the use of AgNP in antimicrobial formulations because AgNP are less prone than Ag^+ to binding and sequestering by naturally occurring ligands. For this reason, it was thought that AgNP might better deliver Ag^+ to the bacterial cytoplasm via the acidic cell membrane.

The rediscovery of silver as a powerful and broad-spectrum antimicrobial since the early 2000s has several lessons to teach us. The demonstration of the efficacy of silver, this time in the form of AgNP, against drug-resistant bacteria such as *Pseudomonas aeruginosa*, ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Streptococcus pyogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA) is encouraging, in view of the continuous increase in multidrug-resistant human pathogenic microbes [6]. Research advances suggesting new medical uses of silver, including nanocrystalline silver, have been rapid and numerous products have been marketed, especially for healing wounds. The rediscovery of the medical uses of silver provides a noticeable example of the interface of chemistry and medicine in enhancing the real (and nonlinear) progress of scientific research.

The use of AgNP for various biological and biomedical applications, such as antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, and anti-angiogenic has now been described [7]. Under these circumstances, there have not been many efficacy studies for Ag^+ compared to AgNP. In our development work, we have conducted a series of virucidal tests of Ag^+ formulations against SARS-CoV-2 and FeCV. These are enveloped and non-enveloped viruses, respectively, which are human pathogens or surrogate viruses for human pathogens that continue to impact health. Additional virucidal agents with broad-spectrum efficacy might be useful for infection prevention and control (IPAC) during the present or future viral epidemics/pandemics.

3. Virucidal efficacy evaluation of Ag^+ formulations against SARS-CoV-2 and FeCV

3.1 Materials and methods

1. Challenge viruses

- Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): Isolate USA-WA1/2020, BEI Resources, NR-1586.

- Feline calicivirus (FeCV): ATCC VR-782 (used as a surrogate for human norovirus).

2. Host (detector) cells

- For SARS-CoV-2: Vero E6 cells (African green monkey kidney); ATCC CRL-1586, grown in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS).
- For FeCV: CrFK cells (Crandell-Rees Feline Kidney); ATCC CCL-94, grown in RPMI 1640 medium containing 5% FBS.

3. Test formulations

- 5 ppm Ag⁺ solution in water; pH 6.1.
- 5 ppm Ag⁺ solution in water containing 26% w/w ethanol, pH 4.2.

4. Synopsis of the ASTM E1052 testing methodology (Suspension Time-Kill Test for Virus) [4]

- Each stock challenge virus had a titer of $\geq 6 \log_{10}$ infectious units/mL, in a culture medium containing 5% fetal bovine serum as the organic soil challenge.
- The test product was prepared for the use-dilution and an equal volume of the dilution medium (minimum essential medium containing 2% newborn calf serum) was prepared to serve a virus control.
- The prepared viral inoculum was added to the test formulation and the virus recovery control at a ratio of virus (one part) + test formulation or dilution medium (nine parts).
- Upon completion of the contact time, the test and recovery suspensions were neutralized by dilution into a chemical neutralizer (minimum essential medium +10% newborn calf serum +0.5% lecithin +1 mM EDTA).
- For the cytotoxicity control, an aliquot of the use-dilution of the test formulation was mixed with the dilution medium (in lieu of the virus) and then neutralized in an identical manner to the test suspension.
- For the neutralization control, an aliquot of the use-dilution of the test formulation was mixed with the dilution medium, neutralized, and then spiked with a low level of virus.
- The neutralized test suspension, virus recovery control, cytotoxicity control, and neutralization control suspensions were serially diluted in the dilution medium. Each diluted solution was plated in quadruplicate to host cell monolayers in a 24-well plate. Maintenance medium was then added to each well, and the host cells with the inoculated virus were allowed to incubate for 4–9 days at 37°C with 5% CO₂.

- Infectivity assay: The residual infectious virus in both test and control conditions was determined by viral-induced cytopathic effect (CPE) that was observed by light microscopy. Cytotoxicity control wells were examined for cytotoxicity to host cells caused by the test formulation. The resulting virus-specific CPE and test formulation-specific cytotoxic effects were scored by examining both test and control cultures.
- Determination of efficacy (calculation): The virus titers in 50% tissue culture infectious doses per mL (TCID₅₀/mL) were determined by using the method of Spearman-Kärber [8] and the amounts of infectious virus present prior to and after treatment were quantified as shown below.

The virus load was calculated according to Eq. (1):

$$\text{Virus Load (Log}_{10} \text{ TCID}_{50}) = \text{Virus Titer (Log}_{10} \text{ TCID}_{50}/\text{mL}) + \text{Log}_{10} [\text{Volume (mL)} \times \text{Volume correction (e.g., neutralization)}] \tag{1}$$

The Log₁₀ Inactivation was calculated according to Eq. (2):

$$\text{Log}_{10} \text{ Inactivation} = \text{Virus Recovery Control (Log}_{10} \text{ TCID}_{50}) - \text{Test (Log}_{10} \text{ TCID}_{50}) \tag{2}$$

5. Virucidal test acceptance criteria [4]

- Viral-induced CPE must be distinguishable from the microbicide-induced cytotoxic effects (if any).
- Viruses must be recovered from the neutralizer effectiveness/virus interference control (not exhibiting cytotoxicity).
- The cell viability control (assay negative control) must not exhibit viral CPE.

Challenge virus	Log ₁₀ inactivation (log ₁₀ reduction in titer)		After contact time
	5 ppm Ag ⁺ solution	5 ppm Ag ⁺ + 26% (w/w) ethanol	(minutes)
Enveloped virus			
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; <i>Coronaviridae</i>)	0.70	≥3.72	1
	3.05	NT	360
Non-enveloped virus			
Feline calicivirus (FeCV; <i>Caliciviridae</i>)	NT	0.70	1
	NT	≥4.30	30

Ag⁺ = silver ion; NT = not tested; ppm = parts per million.

Table 1. Virucidal efficacy of Ag⁺ formulations against SARS-CoV-2 and FeCV evaluated in suspension inactivation studies.

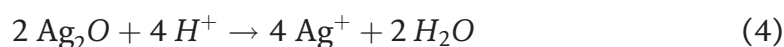
3.2 Results of inactivation studies

Virucidal activity studies were conducted according to a standardized quantitative suspension testing method ASTM E1052–20 [4]. Evaluation of virucidal activity against the enveloped coronavirus, SARS-CoV-2, demonstrated that 5 ppm Ag^+ formulated without ethanol caused minimal ($<1 \log_{10}$) inactivation within 1-min contact time, but $3.1 \log_{10}$ inactivations after 360 minutes (6 h) (Table 1). On the other hand, 5 ppm Ag^+ formulated with a low (26% w/w) concentration of alcohol caused $\geq 3.72 \log_{10}$ inactivations of SARS-CoV-2 within 1-min contact time (Table 1).

The 5 ppm Ag^+ formulated with 26% w/w ethanol also demonstrated efficacy ($\geq 4.3 \log_{10}$ inactivations) against the non-enveloped calicivirus, FeCV, within 30-min contact time, but only minimal ($<1 \log_{10}$) inactivation of FeCV within 1-min contact time (Table 1).

4. Silver ion (Ag^+) mechanisms of microbicidal activity

The broad antimicrobial activity of silver nanoparticles (AgNP) includes efficacy against over 650 microorganisms, including bacteria, fungi, and viruses. This activity is primarily due to the leaching of Ag^+ ions from the outer surface of the AgNP [9]. Xiu et al. [10] demonstrated that anaerobic conditions (i.e., in the absence of oxygen) prevent the Ag^0 oxidation and Ag^+ leaching from AgNP that is favored in acidic environments [Eqs. (3) and (4)].



Under anaerobic conditions, AgNP has no detectable effects on *E. coli* at concentrations 7665 times higher than the minimum lethal concentration of Ag^+ (0.025 mg/L) under the same exposure conditions. In addition, these authors found that the minimum lethal concentration for AgNP under anaerobic conditions was thousands of times higher than the minimum lethal concentration observed under aerobic conditions [10]. This discovery led the researchers to conclude that the antibacterial activity could be controlled by modulating the Ag^+ release (leaching) kinetics through modifications to the AgNP size, shape, and surface characteristics, including the presence of a coating [10].

There are four known antimicrobial actions of AgNP [11, 12]: 1) adhesion to the microbial cell membrane; 2) penetration of AgNP into the cell, causing disruption of biomolecules and intracellular damage; 3) induction of cellular toxicity mediated by reactive oxygen species (ROS), resulting in oxidative stress to the cell; and 4) disruption of signal transduction pathways of the cells.

When microbes are exposed to AgNP, the nanoparticles tend to stick or adhere to the cell wall or membrane due to the electrostatic attraction between the positive charge of Ag^+ generated during oxidation of AgNP and the negatively charged cell membrane of microorganisms (Figure 1). AgNP also displays a strong affinity for the sulfur-containing proteins in the microbial cell wall. The attachment of AgNP to the cell membrane causes irreversible morphological changes in the membrane structure. This can also cause a loss in the integrity of the lipid bilayer and changes in the permeability of the cell membrane. Alterations in such structures can cause increased permeability of the cell membrane, which, in turn, impacts the ability of the cell to regulate essential activities. For instance, the binding of AgNP and subsequent leaching of Ag^+ can alter transport and release of potassium ion (K^+),

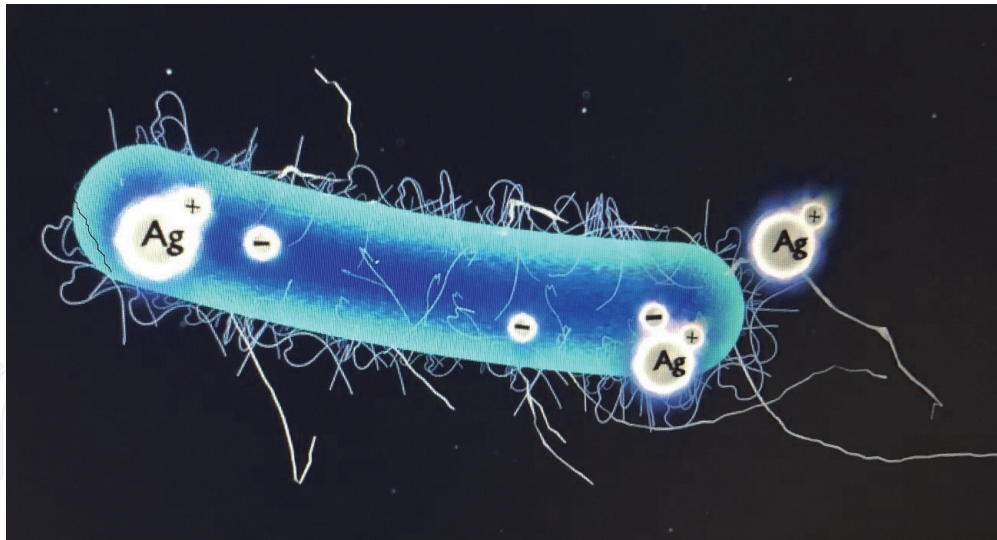


Figure 1.

Schematic depiction of interaction of extracellular Ag^+ with the bacterial cell membrane and subsequent entry of Ag^+ into the cell. Ag^+ and AgNP enter the bacterial cell membrane by porin proteins. Within the bacterial cell membrane, Ag^+ causes the formation of several highly oxidizing species, such as hydroxyl and superoxide free-radicals and hydrogen peroxide (H_2O_2), which quickly oxidizes DNA and RNA, and denature proteins. This results in cell membrane rupture and pore formation. Leakage of intracellular contents and cell lysis results [3].

thus affecting the transport activity of cells. An increase in cell membrane permeability may also cause loss or leakage of cellular contents such as cytoplasmic proteins, ions, and cellular energy reservoirs (adenosine triphosphate; ATP).

Following the adhesion of AgNP to the microbial membrane, the nanoparticles can penetrate the cell and impact important biomolecules and cellular activities. AgNP is able to enter Gram-negative bacteria, such as *E. coli*, through water-filled channels in the membrane called porins. After penetration of AgNP into the cells, these nanoparticles will start to bind with cellular structures and biomolecules, such as proteins, lipids, and DNA, thus damaging the internal structure of the bacteria. Any leached Ag^+ binds to negatively charged proteins, altering the proteins structurally and eventually resulting in denaturing of the proteins.

Another mechanism of action of AgNP is the production of ROS, which causes cellular oxidative stress in microbes. Reactive oxygen species is a general term for oxygenated compounds that are involved in various cellular biological events. These can include but are not limited to superoxide, hydrogen peroxide, and hydroxyl radicals. The antibacterial potential of AgNP is usually related to the ability of the nanoparticles to produce ROS and increase the oxidative stress in the cells. Production of intercellular ROS is thought to be the most important indicator of toxicity related to AgNP, as the ROS may induce lipid damage and leakage of cellular biomolecules, and may eventually lead to cell apoptosis [11, 12].

The virucidal efficacy of Ag^+ and AgNP is mediated by the following types of interactions: 1) the Ag^+ /AgNP bind to spike proteins of enveloped viruses, inhibiting the attachment of these viruses to host cell receptors (**Figure 2**); and 2) Ag^+ /AgNP bind to the genomic DNA or RNA of both enveloped and non-enveloped viruses, inhibiting the replication or propagation of the virus inside the host cells.

For example, in the case of the human immunodeficiency virus (HIV-1; family *Retroviridae*, enveloped), the AgNP binds to the sulfur groups of gp120 protein spikes on the viral envelope, thereby preventing infectivity due to the fusion of the viral envelope with the host cell membrane [13]. Similarly, the attachment and entry of herpes simplex virus type 1 (HSV-1; family *Herpesviridae*, enveloped) into cells involve interaction between viral envelope glycoproteins and cell surface heparan sulfate (HS). Viral entry can be prevented by AgNP capped with mercaptoethane sulfonate

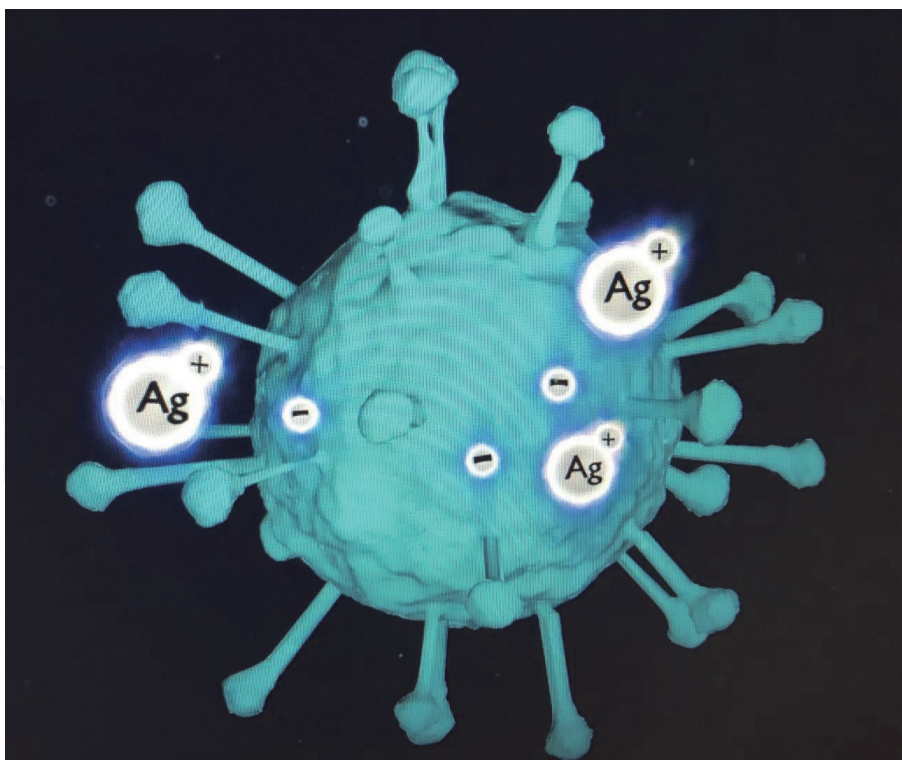


Figure 2.
 Schematic depiction of interaction of extracellular Ag^+ with enveloped viruses, such as SARS-CoV-2.

targeting the virus and competing for its binding to cellular HS through their sulfonate end groups [14]. The antiviral mechanism of inorganic metals such as copper and silver against influenza A viruses appears to be mediated through the inactivation of hemagglutinin (HA) and neuraminidase (NA) cell surface proteins [15].

It was demonstrated in experimental RSV infection studies by Morris et al. [16] that AgNP caused a reduction in RSV. In the mouse model, the antiviral activity appeared to be mediated to a large extent by neutrophils, which were recruited in higher numbers to the airways and activated *via* a neutrophil-specific program of cytokines. This was reported as the first *in vivo* study demonstrating antiviral activity of AgNP during RSV infection.

5. Discussion

Silver ion (Ag^+) has been used since ancient times for various purposes [1]. Silver plays a certain role in mythology and has found various usages as a metaphor. In folklore, silver was commonly thought to have mystic powers. For example, a bullet cast from silver was supposed in such folklore to be the only weapon effective against a werewolf, witch, or other monsters. From this mythology, the idiom of the silver bullet resulted in figuratively referring to any simple solution with very high effectiveness or almost miraculous results, as in the widely discussed software engineering paper “No Silver Bullet” [17]. Other mythic powers attributed to silver have included detection of poisons and facilitation of passage into the mythical realm of the fairies.

In medicine, silver has been incorporated into wound dressings and used as an antibiotic coating in medical devices. Wound dressings containing silver sulfadiazine or silver nanoparticles have been used to treat external infections. Silver has also been used in urinary catheters for reducing catheter-related urinary tract infections and in endotracheal breathing tubes for reducing ventilator-associated pneumonia [18, 19]. Silver ion is bioactive and, at sufficient concentration, readily kills bacteria *in vitro*. Silver and silver nanoparticles are used as antimicrobial ingredients in a variety of

industrial, health care, and domestic applications. For example, infusing clothing with AgNP allows the items to remain odorless longer [20].

Silver ion (Ag^+) displays broad-spectrum antimicrobial action, with efficacy against various bacteria, fungi, and viruses. Due to their versatility, AgNP is currently used as microbicides in wound dressings, medical devices, deodorant sprays, and fabrics. Studies have demonstrated the virucidal efficacy of AgNP against human pathogenic viruses, including enveloped viruses such as respiratory syncytial virus (RSV), influenza virus, hepatitis B virus (HBV), and human immunodeficiency virus (HIV), as well as non-enveloped viruses such as human norovirus [2]. In addition, Ag^+ has been shown to possess virucidal efficacy against severe acute respiratory syndrome coronavirus (SARS-CoV) [21] and SARS-CoV-2 [22, 23]. AgNP formulations have been proposed for cleaning inanimate surfaces to efficiently control the ongoing COVID-19 pandemic [23]. The hypothesis was based on the proposed mechanism of action of AgNP, involving binding to the spike glycoprotein of the virus, thereby inhibiting the binding of the virus to the host cells. Dissemination of respiratory pathogens such as SARS-CoV-2 from infected to susceptible individuals is believed to occur directly, *via* respiratory droplets and droplet nuclei/aerosols, and indirectly, through contaminated high-touch environmental surfaces (HITES) [24]. SARS-CoV-2 has been reported to remain infectious on contaminated HITES for hours to days [25–27].

The Ag^+ formulations discussed in this chapter are not considered silver zeolites or silver zirconium phosphate. These Ag^+ formulations are, therefore, not in scope for the European Union ban on the use of certain silver compounds in antimicrobial products [28].

Until the virucidal efficacy of microbicides is empirically demonstrated for SARS-CoV-2 specifically, the EPA has allowed agents to be used on the basis of their activity against other enveloped and non-enveloped viruses (**Box 1**). Virucidal efficacy of a selection of formulated microbicidal actives against SARS-CoV-2 has, to date, been assumed based on efficacy data obtained using other coronaviruses, or based on non-standardized methods of assessing viral inactivation (i.e., \log_{10} reduction in infectious titer) in suspension without details of the testing method used, including use of appropriate controls. To date, only limited virucidal testing against SARS-CoV-2 has been demonstrated definitively through testing conducted per standardized surface and suspension methodologies [27].

On March 5, 2020, the US Environmental Protection Agency (EPA) announced the release of a new list [29] of EPA-registered disinfectant products that were considered as qualified for use against SARS-CoV-2, the coronavirus that causes the disease COVID-19. Products on EPA's "List N: Disinfectants for Use Against SARS-CoV-2" are registered disinfectants qualified for use against SARS-CoV-2 through EPA's Emerging Viral Pathogen Program (EVPP) [30]. Currently, there are 85 products listed that are qualified for use against SARS-CoV-2. Of note, EPA states that if the directions for use for viruses/virucidal activity of the listed products provide different contact times or dilutions, the longest contact time or most concentrated solution should be used. The EPA initially issued guidance for the EVPP in 2016; the program was intended to "expedite the process for registrants to provide useful information to the public" regarding products that should be effective against emerging viral pathogens.

According to the EVPP, in the event of an outbreak, companies with pre-approved products can make off-label claims (e.g., technical literature distributed exclusively to healthcare facilities, physicians, nurses, and public health officials; 1–800 consumer information services; company websites (non-label related); and social media) for use of these products against the outbreak virus. These emerging pathogen statements do not appear on marketed (final print) product labels. Products that meet EPA's criteria for use against SARS-CoV-2 can be searched using the EPA database [29]. This database allows users to search by criteria such as EPA registration number, the active ingredient, use site, surface types, contact time, and keywords.

Box 1.

EPA-registered disinfectant products qualified for use against SARS-CoV-2.

6. Conclusions

In order to expand the known set of virucidal agents with efficacy for SARS-CoV-2, we conducted virucidal efficacy studies on Ag^+ with and without 26% (w/w) ethanol, according to the ASTM E1052 standardized suspension methodology [4]. The Ag^+ formulation with low concentrations of ethanol should be less flammable than 70% ethanol at the time of use or at the time of storage and during shipping. The formulation without ethanol proved effective for the enveloped SARS-CoV-2 virus, while efficacy for non-enveloped viruses such as FeCV required formulation with 26% ethanol.

To our knowledge, this is the first report of the virucidal efficacy of Ag^+ formulations, evaluated using standardized ASTM methodology, for inactivating SARS-CoV-2. From the viewpoint of infection prevention and control, effective surface disinfectants such as the Ag^+ ion formulations discussed in this chapter represent a possible intervention for interrupting the transmission of SARS-CoV-2.

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Conflict of interest

The authors declare no conflict of interest.

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