New Microbiologica, 46, 1, 60-64, 2023, ISSN 1121-7138

SHORT COMMUNICATION

Virucidal efficacy of a novel silver-based disinfectant against SARS-CoV-2 Omicron BA.5

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SUMMARY

In this study we evaluated the antiviral activity of the Silver Barrier® disinfectant against SARS-CoV-2. Silver Barrier® showed time- and concentration-dependent antiviral activity against SARS-CoV-2. After 5 min contact time, Silver Barrier® at 0.002% showed a strong inhibitory effect ($p<0.001$), with a 2-fold reduction of viral genome copy numbers, and a robust suppression (94%) of SARS-CoV-2 infectivity. Considering the effects obtained in solution and within a very short time, Silver Barrier® stands as an excellent new candidate for the disinfection of work environments, especially at the healthcare level, where there are people at high risk of serious illnesses.

Received November 7, 2022 *Accepted* January 12, 2023

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a novel coronavirus responsible for coronavirus disease 2019 (COVID-19), which first emerged in December 2019 in Wuhan China (Lai *et al*., 2020). It has rapidly spread all over the world with devastating consequences, resulting in more than 600 million confirmed infections and around 6.5 milion deaths as of October 28, 2022 (WHO). SARS-CoV-2 is primarily transmitted via direct, indirect, or close contact with infected people through respiratory droplets (Meselson *et al*., 2020). The most common symptoms, at disease onset, are fever, cough and dyspnea (Harrison *et al*., 2020). In order to effectively prevent new infections and to control the spread of the virus, multiple strategies have been employed, including vaccines, immunotherapy and antiviral agents (Gavriatopoulou *et al*., 2021). In combination with these treatment strategies, contact tracing, use of surgical masks, hand hygiene and social distancing have also demonstrated high efficacy to prevent and control SARS-CoV-2 infection (Wang *et al*., 2020). Despite the global effort to contain the virus, it continues to spread across the world, allowing the generation of a large number of mutations which lead to distinctive SARS-CoV-2 variants (Bertelli *et al*., 2021). Since October 2020, numerous SARS-CoV-2 variants have arisen worldwide, promptly defined by the Centers for Disease Control and Prevention (CDC) as variants of concern (VOCs) (ECDC 2020). The first, namely Alpha variant, was initially detected in the United Kingdom (UK) (Challen *et al*., 2021) and soon after in South Africa, Brazil, and India; new circulating lineages were described and designated as Beta (Tegally *et al*., 2021), Gamma (Faria *et al*., 2021), and Delta (Dhar *et al*., 2021), respectively. At a genome level, all VOCs are characterized by a typical mutational pattern, especially in the S gene that is the most variable part of the coronavirus genome (Wu *et al*., 2020; Zhou *et al*., 2020), leading to human angiotensin-converting enzyme 2 (ACE2) increased affinity and, consequently, enhanced infectivity (Starr *et al*., 2020; Li *et al*., 2022). In November 2021, the last VOC, named Omicron, emerged in South Africa and has rapidly replaced all other variants worldwide and evolved into several sub-variants. The first dominant SARS-CoV-2 Omicron variant BA.1 harbors 35 mutations in its Spike protein and has been closely monitored due to its high infectivity rate (Viana *et al*., 2022). Currently, two new Omicron variants BA.4 and BA.5 are dominating the pandemic scenario, being more transmissible and resistant to immunity generated by previous SARS-CoV-2 infections (Shrestha *et al*., 2022). Moreover, it has been demonstrated that antibodies triggered by vaccination are less effective in preventing BA.4 and BA.5 infections compared to previous Omicron sub-variants, leaving vaccinated and boosted people vulnerable to multiple Omicron infections (Cao *et al*., 2022). Furthermore, the broadly neutralizing monoclonal antibodies, including those approved by the Food and Drug Administration (FDA) for therapeutic use against previous SARS-CoV-2 variants, are mostly ineffective against these Omicron sub-variants. In the Omicron era, effective

Key words:
SARS-CoV-2, Omicron BA.5 variant, disinfectants, Silver Barrier.

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disinfection of public and common areas plays a crucial role in containing the spread of COVID-19. It has been shown that SARS-CoV-2, under environmental conditions, remains viable in aerosols up to 3 h and on plastic and stainless steel up to 3 days, meaning that effective surface sanitizers can prevent indirect contact transmission (van Doremalen *et al.*, 2020). Enveloped viruses, such as Coronaviruses, are more susceptible to disinfectants due to the presence of essential lipids in their envelope (Sattar *et al.*, 2007), thus underlining the importance of such products in preventing direct contact transmission. The use of chemical disinfectants represents a widely accepted practice to prevent and control infections and to protect healthcare workers, patients, and people at a high risk of severe illness. Currently, the most commonly used broad-spectrum disinfectants are alcohol-based (ethanol and isopropanol) or chlorine-based (hypochlorite) products, hydrogen peroxide and ozone (Xiao *et al.*, 2022). However, the search for other products effective against SARS-CoV-2 still remains a global goal. Various metal-based nanoparticles (NPs) have been tested as delivery agents or surface modifying agents, but have never been evaluated for the ability to inactivate SARS-CoV-2. Silver-related nanomaterials (AgNMs) are one of the most effective NPs, thanks to their reported antibacterial properties and antiviral efficacy (Zhang *et al.*, 2016). The AgNMs antiviral activity involves the inhibition of viral entry step and the generation of radicals, such as

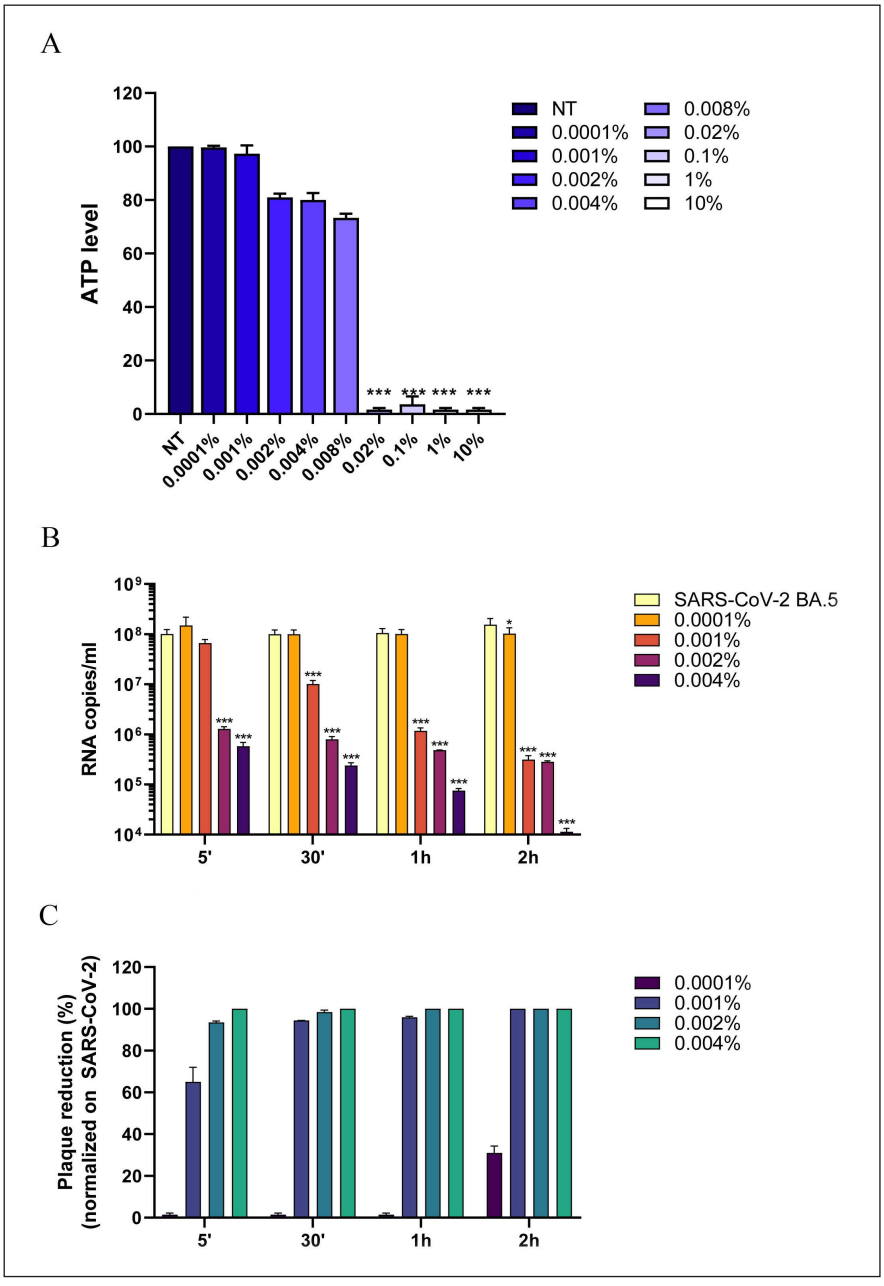

Figure 1 - *Cytotoxic effect of Silver Barrier® on Vero E6 cells and its virucidal activity against SARS-CoV-2 BA.5.*
Panel A reports the cytotoxicity of Silver Barrier® on Vero E6 cells at different concentrations.
Panel B shows the antiviral activity of Silver Barrier on the viral genome copy numbers.
Panel C highlights the inhibition of viral replication due to Silver Barrier treatment in terms of plaque reduction.
*Data are presented as the mean and the standard error of mean. NT, not treated cells. *p<0.05; ***p<0.001.*

reactive oxygen species, by interacting with biomolecules, causing cell membrane disruption and RNA damage (Das *et al.*, 2020). Here, we focused our attention on a patented product, namely Silver Barrier®, obtained from the association of stabilized silver ions with didecidylmethylammonium chloride (DDAC), a biocidal product known for its bactericidal activity against Gram-positive and Gram-negative and for its virucidal properties against envelope viruses such as hepatitis B and HIV.

The purpose of this study is to evaluate the effectiveness of Silver Barrier® in inhibiting SARS-CoV-2 infectivity and viability within a short time. All the experiments were carried out by using African green monkey kidney Vero E6 cells, obtained from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% Penicillin-Streptomycin. Three independent replicates were performed in all the experiments and the statistical significance was calculated using the two-way Anova test with the Bonferroni posttest correction. At first, the cytotoxicity of Silver Barrier® was evaluated. To this end, Vero E6 cells were seeded ($2x10^5$ cells/well) in 24-well plate for 24 h, and then treated with different dilutions of Silver Barrier® (range from 10% to 0.0001%) in DMEM without FBS. After incubation at 37°C for 1 h, the treatment was removed, cells were washed with warm phosphate saline buffer (PBS) (Gibco, Thermo Fisher Scientific) and incubated with fresh culture medium at 37°C for 24 h. Cell viability was assessed by quantitation of ATP levels using the bioluminescent reagent Cell Titer-Glo (Promega, Madison, WI, USA). ATP level was expressed as % of control (not-treated cells, NT), taken as 100%. As shown in *Figure 1A*, at 24 h post treatment, Silver Barrier® dilutions from 10% to 0.02% exhibited a significant ($p<0.001$) cytotoxic effect, almost completely abolishing ATP production, as compared to NT cells. Nevertheless, disinfectant toxicity was very low at the dilutions ranging from 0.008% to 0.002%, reaching an ATP production of about 80% as compared to NT cells; Silver Barrier® appeared to be completely free of toxicity at the concentrations of 0.001% and 0.0001%. After having established the cytotoxicity of the disinfectant, its antiviral activity against SARS-CoV-2 was evaluated. Infection experiments were carried out as previously described (Caccuri *et al.*, 2020; Caruso *et al.*, 2021) using the clinical SARS-CoV-2 BA.5 RMO58 isolate. The genomic sequence has been deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database (accession number: EPI_ISL_15082179). The virus was propagated in Vero E6 cells, and the viral titer was determined by a standard plaque assay. All the experiments were performed in a biosafety level-3 (BSL-3) laboratory using the virus at a multiplicity of infection (MOI) of 0.05. To evaluate Silver Barrier® virucidal efficacy, a suspension test was performed. Equal volumes of disinfectant, at different concentrations (0.004%, 0.002%, 0.001% and 0.0001%), and virus stock were mixed. Soon after the specific contact time (5 min, 30 min, 1 h, 2 h) at room temperature (RT), each virus-disinfectant mixture was place on ice to avoid extension of the incubation period. Two-hundred fifty µl of each virus-disinfectant mixture were used to infect the cells. Vero E6 cells, seeded the day before at a density of $2x10^5$ cells/well in 24-well plate, were infected in triplicates. Virus stock diluted in DMEM alone was use as positive control for each contact time. Cells were incubated at 37°C for 24 h and the supernatants were harvested for assessment of virus titer by quantitative real-time PCR (qRT-PCR). Viral RNA was extracted from clarified cell culture supernatants (16,000 g x 10 min) with the Nimbus automatic system (Arrow Diagnostics, Genoa, Italy), according to the manufacturer's instructions. The qRT-PCR was carried out following previously described procedures (Caccuri F. *et al*, 2021; Bugatti *et al.*, 2022). As shown in Figure 1B, Silver Barrier® antiviral activity against SARS-CoV-2 is time- and concentration-dependent. In particular, after 5 min of contact time, two concentrations (0.004% and 0.002%) of Silver Barrier® showed a strong inhibitory effect ($p<0.001$), with a 2-fold reduction of viral genome copy number as compared to virus stock. Lengthening of the contact time (30 min, 1 h and 2 h) resulted in highly anti-SARS-CoV-2 efficacy of Silver Barrier® ($p<0.05$) even at lower concentrations (0.0001%), thus showing time-dependent activity. At the same time, to determine the inhibition of viral replication in terms of infectious viral titer, the same supernatants were used to perform a plaque assay. Vero E6 cells were seeded at a density of $1x10^5$ cells/well in a 48-well plate and incubated at 37°C for 24 h. The virus-disinfectant mixtures were serially diluted 10-fold in DMEM without FBS and 250 µl of each dilution were added to the cells in triplicates. After incubation at 37°C for 1 h, the inoculum was removed, cells were washed with warm PBS and incubated with an overlay consisting of DMEM 2% FBS with 0.4% SeaPlaque (Lonza, Basel, Switzerland). The plate was cultured for 4 days at 37°C to allow plaque formation. Once plaques were established, cells were fixed with 10% formaldehyde for 3 h at RT to inactivate infectious virus. Formaldehyde was aspirated and the agarose overlay was removed. Cells were stained with 1% crystal violet and SARS-CoV-2 viral titer (Plaque Forming Unit, PFU/mL) was determined by counting the number of the plaques. As shown in Figure 1C, Silver Barrier® strongly inhibits the infectivity of SARS-CoV-2 (65%) within 5 min of contact time and at very low concentration

Table 1 - *Antiviral effect of Silver Barrier against SARS-CoV-2 BA.5 expressed in percentages. The table summarizes the inhibition percentages in terms of viral genome copies and infectious viral titer. SD, Standard deviation.*

	Viral genome copies inhibition (% ± SD)			
	Contact time period			
Concentrations tested	*5'*	*30'*	*1h*	*2h*
0.004 %	99.41 ± 0.15	99.75 ± 0.09	99.93 ± 0.03	99.99 ± 0.01
0.002 %	98.67 ± 0.38	99.18 ± 0.23	99.52 ± 0.12	99.80 ± 0.08
0.001 %	33.10 ± 11.43	89.43 ± 3.58	98.86 ± 0.25	99.78 ± 0.10
0.0001%	0 ± 0.00	0 ± 0.00	0 ± 0.00	28.33 ± 8.50

	Plaque reduction (% ± SD)			
	Contact time period			
Concentrations tested	*5'*	*30'*	*1h*	*2h*
0.004 %	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
0.002 %	93.50 ± 0.70	98.45 ± 0.92	100 ± 0.00	100 ± 0.00
0.001 %	65 ± 7.07	94.45 ± 0.07	96.00 ± 0.42	100 ± 0.00
0.0001%	0 ± 0.00	0 ± 0.00	0 ± 0.00	30.95 ± 3.32

(0.001%). *Table 1* summarizes the inhibition percentages in terms of viral genome copies and infectious viral titer, where the percentage of inhibition was calculated by comparing treated SARS-CoV-2 with the untreated virus. All together, these results show the strong virucidal capacity of Silver Barrier®, even after a short contact time, in a time-dependent manner. In agreement with previous studies, our data confirm the efficacy of silver nanoparticles in counteracting SARS-CoV-2 (He *et al.,* 2022; Jeremiah *et al.,* 2020; Talebian *et al.,* 2020). Silver particles exert their virucidal activity through two main mechanisms: they can bind to Spike proteins of enveloped viruses, inhibiting the attachment of these viruses to host cell receptors, or 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 (Nishihara *et al.,* 2021). For these reasons, the association of stabilized silver ions with DDAC in the Silver Barrier® allows the product to have a more effective antiviral action compared to common products used as disinfectants. In addition, Silver Barrier® may be used to sanitize much larger areas through the use of nebulization, allowing the compound to reach any surface, providing total coverage of spaces and eliminating any possible human variables due to poor cleaning or to the use of inappropriate tools. For all these reasons, although numerous disinfectants with antiviral properties are already in use, Silver Barrier® stands as an excellent new candidate for the disinfection of work environments, especially at the healthcare level, where the protection of patients and people at high risk of severe illness is a priority. Moreover, due to the recent increase of potential pathogens that can affect the world community, the availability of as many weapons as possible to counter the outbreak of epidemic or pandemic is even more important.

Acknowledgment

The Authors thank Teknofog S.r.l., Silver Barrier and CEO Dario Mattioli for the proficient collaboration.

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