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Major Article

Evaluation of virucidal activity of residual quaternary ammonium-treated surfaces on SARS-CoV-2

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A B S T R A C T

Background: The COVID-19 pandemic has had an unprecedented impact on global health and the world's economies. Proliferation of virulent and deadly SARS-CoV-2 variants require effective transmission mitigation strategies. Under reasonable environmental conditions, culturable and infectious SARS-CoV-2 can survive on contaminated fomites from hours to months. In the present study we evaluated a surface-anchored polymeric quaternary ammonium antimicrobial to help reduce fomite transmission of SARS-CoV-2 from contaminated surfaces.

Methods: Two studies were performed on antimicrobial pre-treated metal disks in March 2020 by two independent Biosafety Level III (BSL-3) equipped laboratories in April 2020. These facilities were in Belgium (the Rega Medical Research Institute) and Australia (the Peter Doherty Institute) and independently applied quantitative carrier-based methodologies using the authentic SARS-CoV-2 isolates (hCoV-19/Australia/VIC01/2020, hCoV-19/Belgium/GHB-03021/2020).

Results: Residual dry tests were independently conducted at both facilities and demonstrated sustained virion destruction (108.23 TCID50/carrier GHB-03021 isolate, and 103.66 TCID50/carrier VIC01 isolate) 1 hour (drying) + 10 minutes after inoculation. Reductions are further supported by degradation of RNA on antimicrobial-treated surfaces using qRT-PCR.

Conclusions: Using a polymeric quaternary ammonium antimicrobial (EPA/PMRA registered) the results independently support a sustained antiviral effect via SARS-CoV-2 virion destruction and viral RNA degradation. This indicates that silane-anchored quaternary ammonium compound (SiQAC-18) treated surfaces could play an important role in mitigating the communicability and fomite transmission of SARS-CoV-2.

INTRODUCTION

Throughout the current pandemic, a severe respiratory tract infection in humans resulting from the SARS-CoV-2 coronavirus spread by aerosol droplets[1] from infected persons, and fomites on common touch surfaces have both been confirmed indirectly[2] as key vectors of transmission (Fig 1).[3] In the later instance, the persistence of the SARS-CoV-2 on inanimate surfaces has been documented up to three days, [4]and up to 20 days in cooler environments,[5] such as those found in meat packing plants.[6] The SARS-CoV-2 virus is a zoonotic virus, likely spread from bats to live wet markets in China, [7]and transmitted to humans.[8] While the projected infection mortality rate of this virus (0.4%-3.6%)[9] is lower than other more recent infectious

Abbreviations: ASTM, American Society for Testing and Materials; ATCC, American Type Culture Collection; BSL, biosafety level; C18, octadecane; CoV, coronavirus; DNA, deoxyribonucleic acid; MERS, middle ease respiritory syndrome; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA, ribonucleic acid; SARS, sudden acute respiritory syndrome; SiQAC, silane-anchored quaternary ammonium compound; $TCID_{50}$, median tissue culture infectious dose; UV, ultraviolet.

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Fig 1. Fomite transmission can occur from contact with surfaces contaminated with virion-containing water droplets, produced via coughing or sneezing by an infected individual.

viruses such as H5N1 influenza A virus (60%), MERS-V-CoV (34%-36%) and Ebola (41%), the more transmissible SARS-CoV-2 has been confirmed to have infected over 200 million individuals worldwide as of August 2021.[10-12] At this time, human-to-human SARS-CoV-2 infections caused by airborne or fomite transmission have exponentially risen with qRT-PCR cycle-threshold values (>33-34 for the N gene of SARS-CoV-2) known to produce a high rate of both false positive and false negative results, with over 3.1 million reported deaths.[12]

While the implementation of lockdowns, social distancing and mask mandates has stemmed viral spread in most jurisdictions, COVID-fatigue and economic decline have taken their toll. Hospitals, clinics, long-term/senior care facilities and institutions such as schools, nursing homes and prisons have been on the frontlines of the SARS-CoV-2 pandemic. Due to the aerosol nature of the virus, transmission from human to human via droplets or from contaminated surfaces, innovative mitigation and preventative approaches must be adopted. Current virus control has been carried out in part through the implementation of stringent cleaning protocols of all touch surfaces with traditional quaternary ammonium compounds,[13] bleach, stabilized chlorine dioxide, hydrogen peroxide, alcohols and other disinfectants as well as UV exposure.[14,15] While these biocidal chemicals and physical (UV, autoclave, etc.) disinfectants are effective at momentarily disinfecting surfaces of infectious virions, they do not render them antiviral after use.

Since surface-anchored, C18 quaternary ammonium antimicrobials (SiQAC-C18) are known to disrupt phospholipid membranes in bacteria and fungi, it has been previously theorized that a similar mechanism could work to de-protect viruses within the *Coronaviridae* family, ultimate leading to viral deactivation.[16-18] There is also additional evidence that quaternary ammonium antimicrobials are effective at deactivating other respiratory viruses.[19,20] The preventive role of fixed quaternary ammonium antimicrobials on hard or porous surfaces to kill and prevent the buildup of microbial pathogens and biofilms has been well established against both gram-positive and gram-negative bacteria in our research group.[21,22] Surface-anchored C18 quaternary ammonium cations to metals, plastics and glass materials have also been extensively studied and have been demonstrated to be effective in killing on contact.[23,24]

Antiviral agents for use on inanimate surfaces have been the subject of research and commercial activities since the 1950s, however only a few successful antiviral studies have been undertaken with coatings made with these materials on treated surfaces. Tsao et al first demonstrated that SiQAC-C18 polymerized onto alginate beads could remove viruses from pharmaceutical solutions and proposed a mechanism of inactivation.[16] A decade later a silane coated sand filter was shown to reduce non-enveloped viruses in water filtration applications.[25] Testing data also supports susceptibility of enveloped influenza virus organisms to the active ingredient in the commercial product tested on glass, [17]plastic [19]and cotton surfaces.[20] More recently, a continuously active antimicrobial coating has been effective at deactivating human coronavirus 229E, a related viral strain within the SARS-CoV-2 family.[26]

SARS-CoV-2 is an enveloped, non-segmented, positive sense RNA virus with a diameter of 65-125 nm, containing single strands of RNA and provided with crown-like spikes on the outer surface. The envelope is formed from the phospholipid membranes of host cells and serves to protect the virions from degradation.[27,28] We hypothesized that quaternary ammonium antimicrobial, an agent that is able to destabilize phospholipid membranes, could be used as a protective coating that would render SARS-CoV-2 non-infectious.

To investigate the efficacy of quaternary ammonium antimicrobials as antiviral coatings, a commercially available quaternary ammonium antimicrobial treatment, consisting of a coating of SiQAC-C18 that is cured to stainless steel carrier disks, and sent for evaluation against two distinct SARS-CoV-2 isolates. The results from these tests demonstrate evidence of an antiviral effect imparted from these coatings and potential use as a long-lasting antiviral surface treatment.

MATERIALS AND METHODS

Carrier surface preparation

Sample carriers consisted of treated and control stainless steel (2 cm, 2B finish) disks, kindly donated by Pegan Industries. At Ryerson University, Toronto, Canada carriers were pre-sorted, pre-cleaned per EN16777:2018,[29] and pre-dried by storing in an 80°C oven until use. All microbiological operations were performed using aseptic techniques. To reduce variation between carrier treatments, which were handled inside a type A2 biosafety cabinet, the individual carriers with the designated to be treated side up were placed into individual sterile Petri dishes lined with sterile filter paper to absorb over-spray and with the lids opened. The formulated organofunctional silane SiQAC-C18 product (0.5 w/v% active in water) was

applied by a commercial sprayer until thoroughly wetted: For samples provided to the Doherty Institute, the SiQAC-C18 product was applied using an air brush sprayer, distance of 20 cm at a 45° angle, 50 μL application volume per carrier, and the Rega Institute via an electrostatic sprayer, distance of 2 feet, 10 seconds spray time, 50 μL application volume). Surface carriers were either pre-treated with the antimicrobial test substance or left as untreated controls. Immediately after treatment, samples were transferred from Ryerson University to the Rega Institute at KU Leuven (S1) and the Doherty Institute at the University of Melbourne (S2, S3) for efficacy testing. Testing was performed 46 and 47 days respectively after the carrier were treated.

Cytotoxicity

Cytotoxicity of SiQAC-C18 active antimicrobial were performed on host monolayer cell lines (VERO cells; ATCC #CCL-18 for VIC01, ATCC #CRL-1586 for GHB-03021) without virus. This was accomplished by subjecting host monolayer cells with 50 μL of SiQAC-C18, following the $TCID_{50}$ protocol described above.

SARS-CoV-2 test isolates

The SARS-CoV-2 isolates used in this study were independently isolated and cultured by the Doherty Institute (Victoria, Australia)[30] and Rega Institute.[31] At the Doherty Institute, stocks of SARS-CoV-2 isolate hCoV-19/Australia/VIC01/2020 (VIC01)[3] was produced as previously described[4] and was within 2 passages of the original cultured patient isolate and sequence confirmed to match the publicly available genome data. Titres of the VIC01 isolate stocks was determined to be $10^{5.73}$ $TCID_{50}$/mL. The Rega Institute SARS-CoV-2 isolate hCoV-19/Belgium/GHB-03021/2020 (GHB-03021) was produced as described[31] and used within 42 passages and the stock titre determined to be $10^{8.24}$ $TCID_{50}$/mL.

Carrier surface efficacy test

A 50 μL viral suspension of SARS-CoV-2 patient isolate was inoculated (with soil load for GHB-03021) onto a 2 cm surface of control and SiQAC-C18 coated stainless steel carriers (VIC01 n = 4, GHB-03021 n = 3) and incubated at 20 ± 1°C for the contact time. The control and treated carriers were inoculated with 50 μL viral stocks of $10^{6.91}$ (GHB-03021) and $10^{4.00}$ (VIC01), respectively. After 10 minutes past each 1-hour drying period, antiviral material on the carrier samples was removed by rinsing with a select recovery media with vigorous pipetting to further recover remaining infectious viruses from the surface. Appropriate 1:10 dilutions of recovered control and test samples were plating onto monolayers prepared and performed as described.[4,31] The collected sample rinse media was assayed for infectious virus concentration by a cell culture-based viral infectivity assay ($TCID_{50}$ assay).[30,31] The amount of infectious virus was compared to that from the coated control carrier to determine the efficacy of the coating by the viral reduction by the treated carrier.

qRT-PCR test

One-forty microliters of sample of VIC01 virions were immediately added to AVL buffer for 10 minutes, then 100% ethanol to initiate RNA extraction for qRT-PCR analysis. The samples were removed from a BSL 3 facility and the RNA purification was completed using the QiaAmp Viral RNA mini kit in the BSL 2 facility. RNA was stored at -80°C until processed via qRT-PCR. To evaluate the amount of virus genome present in each sample, a quantitative reverse-transcription PCR (qRT-PCR) for detection of the SARS-CoV-2 envelope (E) gene was performed using the SuperScript III OneStep qRT-PCR System with Platinum_ Taq DNA Polymerase (Invitrogen, Carlsband, CA). The qRT-PCR assay was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the following conditions: a denaturation step at 55°C for 10 minute and 95°C for 3 minute, followed by 45 cycles of amplification (94°C for 15 seconds and 58°C for 30 seconds). A known amount of SARS-CoV-2 RNA (generated previously from virus stock cultures) diluted twofold was used to generate a standard curve. The Ct values from the standard curve were used to interpolate the amount of SARS-CoV-2 RNA in each sample.

RESULTS

To test the cytotoxicity of SiQAC-C18, we exposed undiluted solution to Vero and Vero E6 cells, which are the cell lines used for SARS-CoV-2 propagation and quantitation for infectious titre. We found that there was a negligible amount of cell death on the respective cell monolayers (S1, S2). As such, any leaching of SiQAC-18 during the washing and sample elution phase was determined to not induce detrimental effects on the cell monolayer. Thus, any cytopathic effects observed in sample eluates when quantitating the remaining infectious titre by conducting a 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay could be attributed to by presence of infectious virus and not cytotoxic effects of the SiQAC-C18 compound.

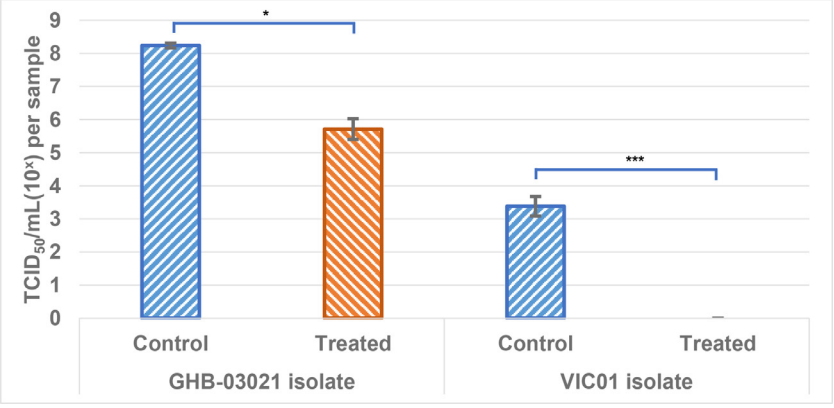

Fig 2. The carrier surface test was used to evaluate the antiviral activity against SARS-CoV-2 isolates GHB-03021 and VIC01. The treated surfaces for each isolate were prepared by two different methods. An electrostatic sprayer applied SiQAC-C18 to surfaces used for the GHB-03021 exposure, while product was applied using an air brush sprayer for samples exposed to VIC01. After drying and a further contact time of 10 min, surfaces were washed, eluate collected and remaining infectious virus titre determined by a $TCID_{50}$ assay.

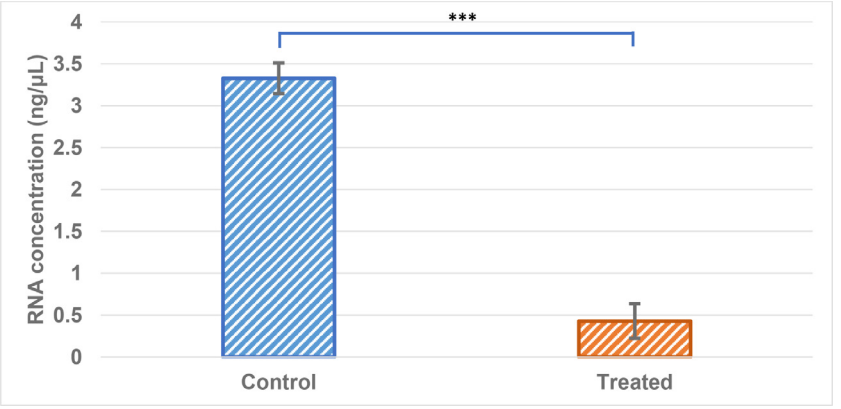

Fig 3. qRT-PCR data from the VIC01 isolate study. RNA was extracted from eluate samples, then assessed for intact E gene via quantitative RT-PCR. ***$P < .0001$ for unpaired t test across 4 replicates.

The ability for SiQAC-C18 to render SARS-CoV-2 non-infectious, was then examined by performing the carrier surface efficacy test. Combined data from studies performed on GHB-03021 and VIC01 isolates is shown in Figure 2. Data was collected and processed using the Reed-Muench method and was calculated to determine the viral load within the collection media. For the GHB-03021 isolate, a reduction of 102.93 was determined (*P = .0014 compared to control, unpaired t test, n = 3). While, for the VIC01 isolate there was no detectable infectious virus present, indicating a more than 103.38 reduction in infectious titre when samples were exposed to SiQAC-C18 (***$P < .0001$ compared to control, unpaired t test, n = 4 replicates).

We next evaluated whether exposure of SARS-CoV-2 to the SiQAC-C18 product could degrade virus genome in addition to reducing the infectious virus titre. To do this, at the same time as collecting samples from the SiQAC-C18 and untreated control exposed VIC01 isolate, an aliquot was processed to extract virus genome. We then performed a quantitative real-time PCR reaction to determine the amount of intact virus Envelope (E) gene present in the sample. Figure 3 reveals that for virus exposed to the SiQAC-C18 product, the genome was significantly degraded, with $>10^7$ less intact E gene detected compared to VIC01 exposed to the untreated disc (control). These results indicate that the viricidal activity of SiQAC-C18 disrupts the viral membrane and either the exposure of the viral RNA to the environment leads to natural degradation, or that the surface treatment can directly degrade the virus genome.

DISCUSSION

Our study reveals that SiQAC-C18 is highly viricidal against SARS-CoV-2. Importantly, as the carrier surface tests were conducted more than 40 days post-addition of the coating to the surface, then internationally transported to each institute for testing, the robustness of the findings of SiQAC-C18 viricidal activity against SARS-CoV-2 is significant. Further adding to the effectiveness of the SiQAC-C18 product, slightly different methodologies were employed and used two geographically distinct SARS-CoV-2 isolates. Analysis of the published genome sequence data for spike protein, which is the major virion surface glycoprotein involved with cellular attachment and infection, revealed only 1 amino acid difference between the VIC01 and GHB-03021 isolates, with the GHB-03021 isolate matching the original Wuhan sequence.[32] This genome sequence data reveals that the viruses used by our study, despite being isolated from infected humans in geographically distinct locations were closely related. However, the VIC01 SARS-CoV-2 was tested within 2 passages of the original cultured patient isolate, while the GHB-03021 had been passaged in cells more than 40 times and likely may have obtained evolutionary changes resulting in altered viral fitness. This postulation is supported by the fact that there was an $\sim 10^{2.5}$ increase in stock titre of the GHB-03021 virus compared to the VIC01 isolate. Thus, in addition to the coating techniques on the stainless steels surface, these potential differences in SARS-CoV-2 fitness, could have theoretically impacted the viricidal activity of the SiQAC-C18 surface coating. It is therefore noteworthy that both institutes independently revealed SiQAC-C18 exhibits viricidal activity against SARS-CoV-2. These findings are in agreement with previous data that demonstrated antiviral activity of SiQAC-C18 active is independent of the coating substrate (plastic, glass, and textiles), and the type of enveloped virus or variant.[19,20]

A viral load between $10^{4.8}$-$10^{6.3}$ per carrier (as indicated by the plate recovery control challenge) is the dose recommended for inoculation to determine antimicrobial activity of disinfectants against viruses dried on surfaces (ASTM E1053)[33] and on fingerpads cleaned with handrub agents (ASTM E1838).[34] Inoculums added to each carrier were slightly higher (GHB-03021 isolate), or slightly lower (VIC01 isolate) than recommended, but testing revealed $>10^3$ reduction compared to untreated controls, regardless of infectious dose added to the treated and untreated discs. We postulate that the

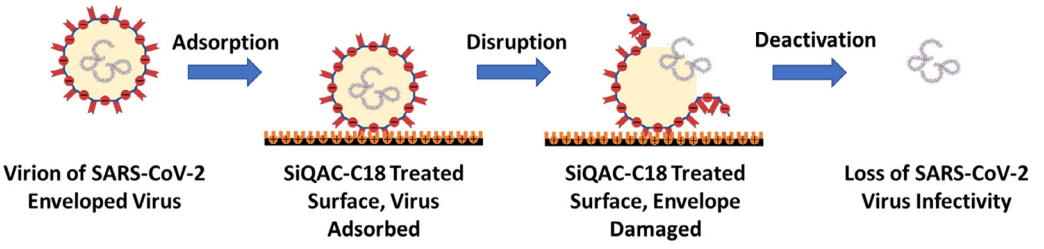

Fig 4. Proposed mechanism of enveloped virus deactivation by SiQAC-C18 treated surface demonstrating adsorption, disruption and deactivation of virion particles.

reduction in infectious titre indicates that the SiQAC-C18 product may have rendered SARS-CoV-2 non-infectious, either by altering the ability for the virion to attach and infect the cell by changing surface glycoprotein structures, or by causing virion membrane lysis and degradation. Given that the qRT-PCR analysis performed on the VIC01 isolate revealed degradation of viral genomic material when exposed to the antimicrobial coated surface, it is likely that structures integral to virion integrity, such as the viral envelope of SARS-CoV-2 are damaged following contact with a SiQAC-C18 functionalized surface (Fig 4). Following this, the SiQAC-C18 product either then continued act directly on the RNA genome, or environmental exposure induced degradation of the viral RNA.

In alignment with our current findings of SiQAC-C18 antiviral activity, antimicrobial organofunctional silane coatings have been demonstrated to reduce SARS-CoV-2 isolates on treated surfaces. While our study demonstrated the antiviral activity of SiQAC-C18 on non-porous metal coupons, we expect a similar efficacy to be achieved on a variety of porous and non-porous surfaces including textiles, glass, furniture, and plastics found in hospitals, transit, and other public settings, similar to the antimicrobial results observed quaternary ammonium treated surfaces.[21,35]

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Supplementary Material: Raw data reports from the Riga Institute (S1) and the Doherty Institute (S2, S3) are available here.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.ajic.2021.10.021.

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