Methods and Materials

Cell Culture and Virus

African green monkey Vero E6 cells (ATCC CRL 1586; American Type Culture Collection, Manassas, VA, United States) were maintained at 37°C+5% CO_2 in Cell Culture Medium (CCM) consisting of Dulbecco's modified Eagle cell culture medium (DMEM; Hyclone SH3024302) supplemented with 10% Fetal Bovine serum (FBS; Gibco Grand Island, NY, United States) and 10 units per ml of Penicillin/Streptomycin (Gibco Grand Island, NY, United States). Medium utilized for experimental infections, denoted as virus culture medium (VCM), consisted of DMEM supplemented with 2% Fetal Bovine serum and 10 units per ml of Penicillin/Streptomycin. Low passage P2 SARS CoV-2 (*hCoV-19/Canada/ON-VIDO-01/2020, GISAID accession# EPI_ISL_425177*) was used to prepare working stocks and concentrated stock collections.

Stock Virus Preparation

Low passage SARS-CoV-2 (P3) was produced by infection of Vero E6 cells at a multiplicity of infection 0.01. By 3-4 days post infection, cytopathic effect (CPE) became evident where over 90% of the cell monolayer had lifted in infected flasks. At this point, supernatants from infected flasks were aspirated, pooled, and clarified by low-speed centrifugation (4500 xg) for 10 minutes. Resulting clarified supernatants were aliquoted and stored at -70°C. Fresh vials of stock virus were utilized for each experimental replicate to avoid potential effects of freeze-thaw cycles.

Disinfectant Preparation

Povidone-iodine (PVI) 5% w/v (Bausch & Lomb, INNOVA Medical Ophthalmics, SKU: DR2CB10944CA) and chlorhexidine gluconate (CHX) 0.1% and 0.05% w/v (Aved Compounded Products, Lot: 10082020@6) were used at full concentrations as provided by manufacturer and hereby referred to as neat dilution. Commercial products requiring further dilution for efficacy testing were two-fold serially diluted in phosphate buffered saline (PBS) to achieve testing dilutions of 1:4, and 1:16 in addition to the neat preparation. Diluted products were prepared fresh on day of assay and used with 10 minutes of preparation.

Biocide Neutralization Assay

Prior to determining the efficacy of the biocides against SARS-CoV-2, a neutralization assay, as described by Cutts et al 2019[1], was conducted to determine the impact of the reagents on the virus and the health of the indicator host reporter cell line.

To test the effect of the neutralized biocides on the health of the cells, 50µl of the neat concretions of PVI and CHX were added to 950µl neutralizer (VCM). Solutions were mixed by pipetting and ten-fold serially diluted. In replicates of five per dilution series, 50µl of diluted solution was added to Vero E6 cells at 80% confluence overlaid containing 150µl of fresh VCM.

To test the effect of the neutralized biocides on the viability of the virus, 50µl of the neat concretions of PVI and CHX were added to 940µl VCM and mixed. Ten microliters of stock virus were then added to the neutralized solution, mixed well by pipetting and subsequently ten-fold serially diluted.

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A corresponding positive control, consisting of 10µl of stock virus added to 990µl VCM, was included for comparison. Negative controls, consisting of cells overlaid with VCM only, were additionally included.

In replicates of five per dilution series, 50µl of the diluted test solutions were overlaid onto Vero E6 cells at 80% confluence overlaid containing 150µl of fresh VCM. Cells were monitored daily for evidence of cytotoxicity and/or cytopathic effect as appropriate for a period of 5 days.

Efficacy Testing

ASTM 1052[2] was used as a guide for this procedure with the only difference amounting to the amount of biocide being applied to the virus. ASTM 1052 indicates that a 10µl of virus should be used to 90µl of biocide, we used 50µl of biocide to 10µl of virus to increase the stringency of the assay and reflect real world condition and applications.

Inoculum preparation consisted of mixing the stock virus with a tripartite 'soil load', used to increase the bioburden against biocides and provide an additional layer of challenge to surfaces and materials needing to be decontaminated. Briefly, a tripartite soil load (12.5µl of 5% BSA, 17.5µl 5% Tryptone, and 0.4% Mucin) was added to 170µl of stock virus as per ASTM 1052[2]. Using a positive displacement pipette, 10µl of virus-soil load mixture was deposited on the bottom of a sterile Sarstedt cryotube. Prepared disinfectants in 50µl aliquots were added to prepared virus, mixed twice and left for the incubation times of 60 seconds, 5 minutes, and 10 minutes, with an additional 30-minute incubation time used solely for the CHX biocide assay. After set time was reached, virus-biocide mixtures were neutralized with 940µl VCM, mixed by pipetting and immediately assessed for viable virus by quantitative (50% tissue culture infective dose ($TCID_{50}$) assay) and qualitative (safety flask) means. Three independent experiments consisting of three biological replicates per contact time were conducted for each of the PVI and CHX test solutions, with the exception of 0.1% CHX, which was assessed in a single experiment.

Quantification of residual infectivity

Vero E6 cells were seeded 96-well tissue culture plates to attain 80% confluence on the day of the assay. Quantification of viable virus from neutralized biocidal test solutions and positive controls was achieved through ten-fold dilution series in VCM. Cell culture medium was aspirated from each plate, replaced with 150µl of fresh VCM per well, and a volume of 50µl of neutralized virus-biocide solution, in replicates of five per dilution series, was added to each well and incubated for 5 days and monitored for CPE. Remaining virus titers were determined by the Reed Muench $TCID_{50}$ procedure.[3] Residual volumes of neutralized solutions were added to a single well of a six well plate of Vero E6 cells. This served as a safety flask test to qualitatively determine if any amount of virus was present that could have been missed by the smaller amounts used in the $TCID_{50}$ procedure.

Statistical Analysis

Means for continuous variables were compared using independent-group t-test. A 2-sided α of less than 0.01 was considered statistically significant. All statistical analysis was performed using SPSS v24.0.

References

1. Cutts TA, Ijaz MK, Nims RW, Rubino JR, Theriault SS. Effectiveness of Dettol Antiseptic Liquid for Inactivation of Ebola Virus in Suspension. *Sci Rep*. 2019;9(1):1-8. doi:10.1038/s41598-019-42386-5
2. ASTM E1052-20. *Standard Practice to Assess the Activity of Microbicides against Viruses in Suspension*.; 2020.
3. Reed L, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg*. 1938;27:493–497.