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Major Article

Of masks and methylene blue—The use of methylene blue photochemical treatment to decontaminate surgical masks contaminated with a tenacious small nonenveloped norovirus

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Key Words:
Personal protective equipment
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Photosensitiser
Light activation
Singlet oxygen
Large enveloped virus
Small nonenveloped virus

Background: In the context of the SARS-CoV-2 pandemic, reuse of personal protective equipment, specifically that of medical face coverings, has been recommended. The reuse of these typically single-use only items necessitates procedures to inactivate contaminating human respiratory and gastrointestinal pathogens. We previously demonstrated decontamination of surgical masks and respirators contaminated with infectious SARS-CoV-2 and various animal coronaviruses via low concentration- and short exposure methylene blue photochemical treatment (10 μM methylene blue, 30 minutes of 12,500-lux red light or 50,000 lux white light exposure).
Methods: Here, we describe the adaptation of this protocol to the decontamination of a more resistant, non-enveloped gastrointestinal virus and demonstrate efficient photodynamic inactivation of murine norovirus, a human norovirus surrogate.
Results: Methylene blue photochemical treatment (100 μM methylene blue, 30 minutes of 12,500-lux red light exposure) of murine norovirus-contaminated masks reduced infectious viral titers by over four orders of magnitude on surgical mask surfaces.
Discussion and Conclusions: Inactivation of a norovirus, the most difficult to inactivate of the respiratory and gastrointestinal human viruses, can predict the inactivation of any less resistant viral mask contaminant. The protocol developed here thus solidifies the position of methylene blue photochemical decontamination as an important tool in the package of practical pandemic preparedness.

In the context of the ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, the supply of personal protective equipment (PPE) remains under strain. Re-use of typically single-use only face coverings such as surgical face masks (SMs) and

Abbreviations: FFR, filtering facepiece respirator; SM, surgical mask; MNV, murine norovirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

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Conflicts of interest: T.S.L. is a cofounder of Singletto, Inc. All other authors report no conflicts of interest relevant to this article. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

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filtering facepiece respirators (FFRs) has been recommended.[1,2] Prior decontamination is paramount to safe PPE re-use and must guarantee the complete inactivation of SARS-CoV-2 as well as that of other contaminating respiratory or gastrointestinal human pathogens.[3] This is relevant in current circumstances (contamination with pathogens other than SARS-CoV-2 might easily occur, particularly in the context of widespread mask use and inexpert donning and doffing), but also plays a significant role in positioning the world for future pandemics.[4]

Human respiratory pathogens include other enveloped corona-, pneumo-, metapneumo-, paramyxo-, and orthomyxoviruses as well as nonenveloped coxsackie- and rhinoviruses; gastrointestinal pathogens include boca-, astro-, picorna-, rota-, and noroviruses (all nonenveloped).[5] Since the manipulation of enveloped SARS-CoV-2 poses obvious problems in terms of availability and equipping of BSL3 facilities, the use of conservative surrogates to test decontamination efficacy has been established in recent years; thus, porcine respiratory coronavirus (PRCV), a spike gene deletion mutant of transmissible gastroenteritis virus,[6,7] has previously been used by ourselves and other groups as a SARS-CoV-2 surrogate.[8–12] Both SARS-CoV-2 and PRCV are members of the *Coronaviridae* family and show sufficient physicochemical similarities for them to be expected to behave similarly outside their hosts. While lipid-enveloped viruses are susceptible to inactivating treatments, nonenveloped viruses are known to be significantly more resistant. Amongst them, the small, nonenveloped human norovirus (genus *Norovirus*, family *Caliciviridae*), recognized as the major global cause of viral gastroenteritis and well-known nosocomial pathogen,[13,14] is notorious for its tenacity in the face of decontamination and as such may be considered the gold standard for validating viral inactivation.[15,16] The genetically and structurally similar murine norovirus (MNV), which replicates efficiently *in vitro*, has been identified as an appropriate surrogate for modeling human norovirus inactivation.[17]

We previously demonstrated efficient inactivation of both a SARS-CoV-2 surrogate and an infectious animal norovirus via hydrogen peroxide-, ultraviolet germicidal irradiation-, and dry heat decontamination.[8,9,18] Particularly, the former 2 technologies, while easily deployable and extremely useful in high-resource settings, are not equitable as they remain less available in low-resource settings; accessible alternative decontamination methods are thus necessary to mitigate PPE shortages in restricted surroundings.

To address this issue, the Development and Methods for N95 Respirators and Mask Decontamination (DeMaND) study recently established a low-cost methylene blue (MB) photochemical treatment for the efficient decontamination of SMs and FFRs contaminated with infectious SARS-CoV-2 or surrogate animal coronaviruses.[10] Photosensitive MB dye, FDA-approved as an oxidation-reduction agent for the treatment of acquired methaemoglobinemia, has a long-standing history of use in pathogen inactivation.[19–22] For its application to photochemical PPE decontamination, contaminated materials were coated with MB and subsequently exposed to a visible light source triggering the generation of virucidal singlet oxygen. A 10 μM MB concentration and a 30-minute exposure to 12,500 lux (10.474 W/m^2) of red light or 50,000 lux (39 W/m^2) of white light reduced titers of SARS-CoV-2 and two surrogate viruses by more than three orders of magnitude on all tested materials.[10]

In the present investigation into decontamination of virus-inoculated SMs, we demonstrate inactivation of a highly resistant, small nonenveloped norovirus via MB photochemical treatment. Decontamination via a DeMaND-adapted protocol involving the spray-coating of SMs with a 100 μM MB solution followed by 30 minutes of 12,500-lux red light exposure, robustly reduced infectious norovirus titers by over 4 orders of magnitude on SM coupons, this in excess of the 3 orders of magnitude reduction outlined in the current FDA policy regarding face masks and respirators.[3] This study serves to future-proof MB photochemical treatment since inactivation of a norovirus, the most resistant of the respiratory and gastrointestinal human viruses, can predict the inactivation of any less resistant viral mask contaminant. The protocol developed here thus solidifies the position of MB decontamination as an important tool in the package of practical pandemic preparedness.

METHODS

Viruses and cells

Murine macrophage cell line RAW264.7 (ATCC TIB-71) was maintained in Dulbecco's modified Eagle's medium (Invitrogen) complemented with 10% heat inactivated fetal calf serum (FCS; BioWhittaker), 2% of an association of penicillin (5,000 SI units/mL) and streptomycin (5 mg/mL; PS, Invitrogen) and 1% 1 M HEPES buffer (pH 7.6; Invitrogen; DMEMc) at 37 °C with 5% CO_2. Stocks of MNV isolate MNV-1.CW1 were produced by infection of RAW264.7 cells as previously described.[9,18] Titers were determined via $TCID_{50}$ method; RAW 264.7 cells were seeded in 96-well plates, infected with 10-fold serial dilutions of MNV, incubated for three days at 37 °C with 5% CO_2, and finally stained with 0.2% crystal violet for 30 minutes. Titres, expressed as $TCID_{50}$/mL, were calculated according to the Reed and Muench transformation.[23] A virus stock with a titer of 7.06 $\log_{10}$ $TCID_{50}$/mL was used in subsequent steps.

The continuous swine testicle (ST) cell-line, grown from testicular fetal swine tissues as described by McClurkin and Norman (1966),[24] was maintained in MEM (GIBCO), supplemented with 5% fetal calf serum (FCS; Sigma), 1% sodium pyruvate 100x (GIBCO), and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.05 mg/mL gentamycin). Stocks of PRCV strain 91V44[25] were produced by infection of ST cells as previously described.[8,9] Titers were determined via the tissue culture infective dose ($TCID_{50}$) method; ST cells were seeded in 96-well plates and infected with 10-fold serial dilutions of PRCV and incubated for 4 days at 37 °C with 5% CO_2. Four days after inoculation, monolayers were analyzed for the presence of cytopathic effect by light microscopy. Titers, expressed as $TCID_{50}$/mL, were calculated according to the Reed and Muench transformation.[23] A PRCV stock with a titer of 7.80 $\log_{10}$ $TCID_{50}$/mL was used in subsequent steps.

Surgical masks

Type IIR-Class I 3-layer medical masks manufactured by Zarena AD, Bulgaria (LOT 0420; REF FMN99) were utilized in all assays. All masks, verified to be from the same manufacturing lot, were supplied by the Department of the Hospital Pharmacy, University Hospital Centre of Liege (Sart-Tilman).

Light box

The light box, designed by the Terra Research Centre, was previously used for MB activation and PRCV decontamination in the context of the DeMaND study.[10] Therein, Lendvay et al describe 2 light boxes producing either 50,000 lux of white light or 12,500 lux of red light; multiple exposures to either light source were shown not to adversely affect PPE integrity.[10] The light box utilized in the context of this study utilizes red light at the lower intensity of 12,500 lux (since red light contains a higher percentage of wavelengths that activate MB). It contains six 180 W horticultural LED lamps (Roleadro Culture Indoor IP65 LED Horticultural T5 Grow), which together emit 12,500 LUX (or 10.474 W/m^2) at 660 nm wavelength (luminescence verified using a light meter (DeltaOHM, Model HD2102.2)). The box interior is cooled by a ventilator equilibrating temperatures within the box (thus eliminating possible temperature effects on viral titers).

Exposure to the red light within the light box (LB) is henceforth referred to as "LB exposure". The design specifications of the light box have been made available in open source.

Validation of previously established methylene blue photochemical decontamination using a porcine coronavirus and evaluation of its efficacy in inactivating a murine norovirus

To validate previously published MB photochemical treatment conditions (10 μM MB and 30-minutes of LB exposure) and to set a "baseline" for further assays involving MNV inocula, the previously established DeMaND protocol was investigated on SMs experimentally inoculated with PRCV. The same protocol was then tested for decontamination of MNV-inoculated SMs. The general workflow followed previously described protocols for PRCV or MNV[8,9,18] inoculation, MB photochemical treatment,[10] and virus elution from SMs[8,9,18] and is also described in more detail below. Briefly, following injection of 100 μl undiluted PRCV or MNV suspension (7.80 and 7.06 $\log_{10}$ $TCID_{50}$/mL, respectively; verified via back-titration) under the first outer layer of designated SM coupons, inoculated SMs were comprehensively sprayed with 7-8 mL of a 10 μM MB solution (Sigma-Aldrich [M9140]; solution prepared in deionized water; total dose of 0.024 mg MB per SM), allowed to dry, and were then either kept in the dark or submitted to LB exposure for a duration of 30 minutes. Upon completion of the decontamination protocol, PRCV or MNV was eluted from the excised coupons and titers of recovered virus were determined via $TCID_{50}$ assay in ST (PRCV) or RAW264.6 (MNV) cells. Methylene blue only controls (cytotoxicity test) and virus only controls (positive control) accompanied these assays.

Establishment of concentration- and time-dependent virucidal kinetics of methylene blue photochemical treatment on murine norovirus

Virucidal methylene blue and light kinetics - microplate assay

To establish optimal treatment conditions and determine potential combined cytotoxic effects of higher concentrations of MB and light (MBL) on RAW264.7 cells, concentration- and LB exposure time-dependent virucidal MBL kinetics were investigated in a set of preliminary microplate-based assays. Ten-fold MB dilutions in deionized water (at final concentrations of 10 μM, 100 μM, and 1,000 μM) in a total volume of 500 μL DMEMc (additionally supplemented with 0.1% beta-mercaptoethanol) were added per well of a 48-well plate containing 10 μL MNV (7.80 $\log_{10}$ $TCID_{50}$/mL) (technical triplicates were performed utilizing separate 48-well plates). The plates were then either kept in the dark (0 minute LB exposure) or were subjected to LB exposure for 30 minutes, 60 minutes, 90 minutes, or 120 minutes. In a second step, 100 μM and 1,000 μM MB concentrations were tested against high-titer MNV (8.55 $\log_{10}$ $TCID_{50}$/mL) with LB exposure times of 1 hour, 2 hours, 3 hours, and 4 hours. Methylene blue only controls (cytotoxicity test) and virus only controls (positive control) accompanied each assay. Titers of infectious MNV recovered from individual wells were determined via $TCID_{50}$ assay. Back titrations of inoculum stocks were performed in parallel to each series of decontamination experiments.

Virucidal methylene blue kinetics - surgical mask assay

To establish optimal applied treatment conditions, LB exposure time- and concentration-dependent virucidal kinetics of MB were investigated on SMs experimentally inoculated with MNV.

Murine norovirus inoculation onto surgical masks. The workflow followed previously described protocols for SM inoculation with MNV.[8,9] Per SM, 100 μl of undiluted viral suspension were injected under the first outer layer at the centre of each of three square coupons (34 mm $\times$ 34 mm). The SMs were allowed to dry for 20 minutes at room temperature before decontamination via MB photochemical treatment.

Methylene blue photochemical treatment of surgical masks. Inoculated SMs were placed horizontally and were sprayed with a total volume of 7-8 mL of either a 100 μM or 1,000 μM MB solution (the final MB volume was determined based on 6 repetitive sprays into a graduated cylinder). In total, each SM was sprayed four times on the outer side and twice on the inner side (facing the wearer). Surgical masks were allowed to dry (absorption of the MB solution) for 30 minutes in a dark box and were then either kept in the dark (0-minute LB exposure) or were subjected to LB exposure for 120 minutes, 180 minutes, or 240 minutes. Methylene blue only controls (cytotoxicity test) and virus only controls (positive control) again accompanied each assay.

Murine norovirus elution from surgical masks. Upon completion of the decontamination protocol, downstream coupon excision and virus elution followed previously described protocols.[8,18] Briefly, MNV was eluted from three excised coupons per SM into 4 mL DMEMc via a 1-minute vortex at maximum speed (2,500 rounds per minute; VWR VX-2500 Multi-Tube Vortexer). Titers of infectious MNV recovered from individual coupons were determined via $TCID_{50}$ assay. Back titrations of inoculum stocks were performed in parallel to each series of decontamination experiments.

Validation of a virucidal photochemical methylene blue treatment to decontaminate murine norovirus – inoculated surgical masks

To verify a treatment protocol wherein MNV-inoculated SMs (7.5 $\log_{10}$ $TCID_{50}$/mL) were sprayed with 100 μM MB and subjected to LB exposure for 30 minutes, 4 biological and technical repeats were performed on 4 different days. Methylene blue only controls (cytotoxicity test) and virus only controls (positive control) accompanied these assays.

Testing shorter light box exposure (15 minutes) for photochemical methylene blue decontamination of murine norovirus – inoculated surgical masks

In an additional step, 100 μM MB concentrations were tested against MNV-inoculated SMs (7.30 $\log_{10}$ $TCID_{50}$/mL) in conjunction with a shorter LB exposure time of 15 minutes (biological and technical triplicates). Methylene blue only controls (cytotoxicity test) and virus only controls (positive control) again accompanied these assays.

Data analysis

Differences in infectious viral titers were computed and all graphs created using GraphPad Prism 7 (Graph-Pad Software). Statistical analyses of differences in infectious viral titers were performed using GraphPad Prism 7 (Graph-Pad Software) and *P*-values were computed by using a 2-sided independent sample t-test, where $^{****}P < .0001$, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$, and ns is $P \geq .05$.

RESULTS

Methylene blue photochemical treatment of surgical masks following previously established protocols for coronavirus inactivation reduces porcine respiratory coronavirus titers by over 5 orders of magnitude but does not inactivate murine norovirus

Photochemical treatment involving application of a 10 μM MB solution and a 30-minute LB exposure reduced infectious PRCV titers

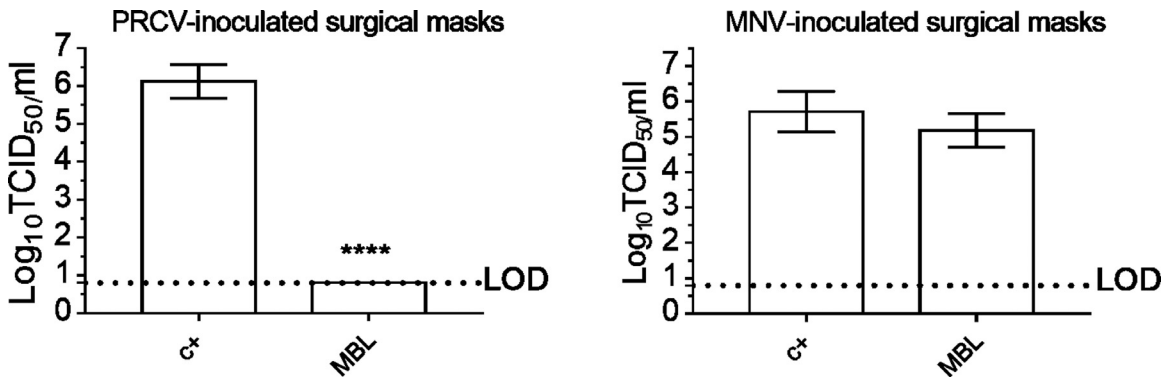

Fig 1. Validation of the previously established methylene blue (MB) photochemical treatment protocol using an enveloped animal coronavirus (left panel) and evaluation of its efficacy in inactivating a small non-enveloped norovirus (right panel). Porcine respiratory coronavirus (PRCV) - or murine norovirus (MNV) - inoculated surgical mask coupons remained untreated (c+) or were treated with a 10 μM MB solution and exposed to a 12,500-lux red light source (light box) for 30 min (MBL). The infectivity of PRCV recovered from surgical mask coupons (n = 3) was analyzed in swine testicular cells. The infectivity of MNV recovered from mask coupons (n = 9) was analyzed in RAW 264.7 cells. Values for positive controls (MB-untreated, but light box-exposed) ranged between 5.80 and 6.63 $\log_{10}$ $TCID_{50}$/mL (PRCV) 5.05 and 6.05 $\log_{10}TCID_{50}$/mL (MNV). The cell culture limit of detection (LOD) was 0.80 $\log_{10}$ $TCID_{50}$/mL for all analyses. Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. P-values were computed by using a two-sided independent sample t-test (where $^{****}P < 0.0001$).

to below the assay detection limit of 0.8 $\log_{10}$ $TCID_{50}$/mL on SM coupons (5.33 [±0.25] $\log_{10}$ $TCID_{50}$/mL reduction). Following the same treatment protocol, MNV titers dropped from 5.78 (±0.33) $\log_{10}$ $TCID_{50}$/mL to 5.19 (±0.16) $\log_{10}$ $TCID_{50}$/mL, resulting in a total reduction of 0.53 (±0.34) $TCID_{50}$/mL (Fig 1).

Murine norovirus inactivation in microplates via methylene blue photochemical treatment is concentration- and time-dependent

To establish optimal MNV treatment conditions, a preliminary microplate-based assay examined concentration- and LB exposure time-dependent virucidal kinetics of MB photochemical treatment. Again, treatment with 10 μM MB concentrations and 30 minutes of LB exposure (treatment conditions previously utilized to successfully inactivate SARS-CoV-2 and its surrogates by more than three orders of magnitude[26]) reduced viral titers by less than one order of magnitude (0.73 [±0.53] $\log_{10}$ $TCID_{50}$/mL). Protocols combining the same LB exposure time with 100 μM and 1,000 μM MB concentrations both reduced MNV titers by 0.93 (±0.35) $\log_{10}$ $TCID_{50}$/mL. With increasing exposure times, viral titers successively dropped, reaching absolute titer reductions of 1.73 (±0.53), 2.68 (±0.35), and 2.50 (±0.00) $\log_{10}$ $TCID_{50}$/mL after 120 minutes (2 hours) of LB exposure in conjunction with 10 μM, 100 μM, and 1000 μM MB concentrations, respectively. Ten-fold increases in MB concentrations coincided with higher MB cytotoxicity and correspondingly elevated assay detection limits (LODs) from 0.8 to 1.8, to 2.8 $\log_{10}$ $TCID_{50}$/mL (Fig 2).

In a second microplate-based assay, 100 μM and 1000 μM concentrations were tested against high-titer MNV in conjunction with longer LB exposure times of 1 hour, 2 hours, 3 hours, and 4 hours. Following 2 hours of LB exposure, virus titer reductions mirrored those observed in the first assay at this time point (2.69 [± 1.66] and 2.85 [± 1.58] $\log_{10}$ $TCID_{50}$/mL after treatment with 100 μM and 1000 μM MB solutions). Following treatment with a 100 μM MB solution and 3 hours of LB exposure, viral titers dropped by 4.80 (± 1.01) $\log_{10}$ $TCID_{50}$/mL; treatment with 100 μM MB and 4 hours of LB exposure as well as a 1000 μM MB followed by 3 and 4 hours of LB exposure lowered viral titers to below the LODs of each of the respective assays, thus yielding minimal titer reductions of 3.89 (± 0.90), 3.22 (± 1.45), and 2.89 (± 1.40) $\log_{10}$ $TCID_{50}$/mL, respectively (Fig 3).

Murine norovirus inactivation on surgical masks via methylene blue photochemical treatment is concentration- and time-dependent

To establish optimal applied treatment conditions on the actual PPE items themselves, LB exposure time- and concentration-dependent virucidal kinetics of MB were investigated on SMs experimentally inoculated with MNV. 100 μM and 1000 μM methylene blue solution were applied to inoculated SMs which were subsequently subjected to 2 hours, 3 hours, or 4 hours of LB exposure. Limits of detections of both series of experiments were respectively lowered by one order of magnitude in these assays (0.8 instead of 1.8 and 1.8 instead of 2.8 $\log_{10}$ $TCID_{50}$/mL for 100 μM and 1000 μM concentrations, respectively; effect attributed to a dilution of cytotoxic MB

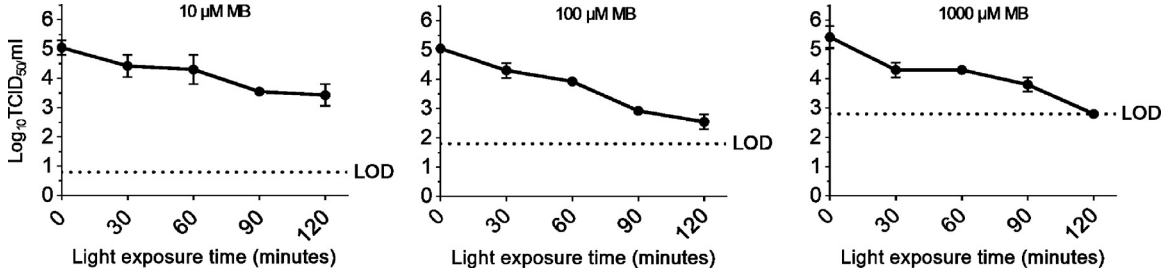

Fig 2. Evaluation of concentration- and time-dependent virucidal kinetics of methylene blue (MB) photochemical treatment on murine norovirus (MNV) *in vitro*. Murine norovirus was mixed with different MB concentrations in microplates and was exposed to a 12,500-lux red light source (light box) for varying amounts of time (0, 30, 60, 90, 120 minutes). The infectivity of surviving MNV recovered from microplate wells was then analysed in RAW 264.7 cells. The cell culture limit of detection (LOD) was 0.80, 1.8, and 2.8 $\log_{10}$ $TCID_{50}$/mL for 10 μM MB, 100 μM MB, and 1,000 μM MB concentrations, respectively. All assays were performed as technical duplicates (n = 2). Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. Values for positive controls (MB-untreated, but light box-exposed MNV) ranged between 4.80 and 5.30 $\log_{10}$ $TCID_{50}$/mL.

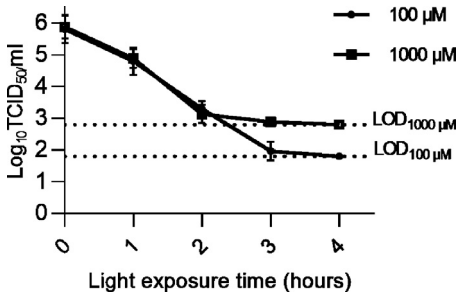

Fig 3. Evaluation of concentration- and longer exposure time-dependent virucidal kinetics of methylene blue (MB) photochemical treatment on murine norovirus (MNV) *in vitro*. Murine norovirus was mixed with different MB concentrations in microplates and was exposed to a 12,500-lux red light source (light box) for varying amounts of time (0, 1, 2, 3, 4 hours). The infectivity of surviving MNV recovered from microplate wells was then analysed in RAW 264.7 cells. The cell culture limit of detection (LOD) was 1.8, and 2.8 $\log_{10}$ $TCID_{50}$/mL for 100 μM MB and 1,000 μM MB concentrations, respectively. All assays were performed as biological triplicates and technical duplicates (n = 6). Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. Values for positive controls (MB-untreated, but light box-exposed MNV) ranged between 5.69 and 6.19 $\log_{10}$ $TCID_{50}$/mL.

during elution), allowing determination of virus titer reductions of close to or more than four orders of magnitude for 100 μM concentrations at all 3 exposure times (3.96 [± 0.29], 4.10 [± 0.00], and 4.13 [± 0.00] $\log_{10}$ $TCID_{50}$/mL) and over three orders of magnitude for 1000 μM concentrations (3.20 [± 0.00], 3.10 [± 0.00], 3.33 [± 0.14] $\log_{10}$ $TCID_{50}$/mL; Fig 4). At 1,000 μM concentrations, MB solutions were macroscopically seen to undergo a (subjective) color change from blue to violet (putatively indicative of aggregation) and to become viscous and agglomerate on SM surfaces, thus slowing the drying process.

Murine norovirus-inoculated surgical masks are reliably decontaminated via photochemical treatment involving 100 μM methylene blue coating of masks followed by 30 minutes of 12,500-lux red light exposure

To avoid issues associated with 1000 μM methylene blue concentrations (higher assay LOD, slow drying, putatively shifted absorption spectrum), further PPE-applied assays included only the 100 μM concentration. In view of potentially achieving faster decontamination turn-around, shorter exposure times (90 minutes, 60 minutes, 30 minutes) were tested in a small preliminary assay (results not

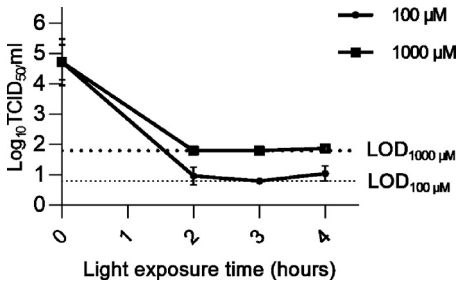

Fig 4. Evaluation of concentration- and time-dependent virucidal kinetics of methylene blue (MB) photochemical treatment on murine norovirus (MNV) – inoculated surgical masks. Murine norovirus-inoculated masks were treated with 100 μM or ,1000 μM MB concentrations and subsequently exposed to a 12,500-lux red light source (light box) for varying amounts of time (0, 2, 3, 4 hrs). The infectivity of surviving MNV recovered from surgical mask coupons was analyzed in RAW 264.7 cells. The cell culture limits of detection (LOD) were 0.8 and 1.8 $\log_{10}$ $TCID_{50}$/mL for 100 μM and 1,000 μM MB concentrations, respectively. All assays were performed as biological triplicates (n = 3). Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. Values for positive controls (MB-untreated, but light box-exposed MNV) ranged between 4.90 and 5.71 $\log_{10}$ $TCID_{50}$/mL.

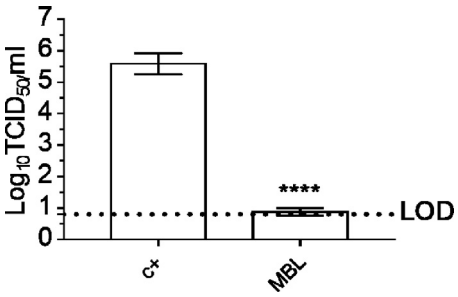

Fig 5. Evaluation of methylene blue (MB) photochemical treatment on murine norovirus (MNV) – inoculated surgical masks. The infectivity of MNV recovered from mask coupons treated with 100 μM MB and subsequently exposed to a 12,500-lux red light source (light box) for 30 minutes was analysed in RAW 264.7 cells. The cell culture limit of detection (LOD) was 0.8 log10 $TCID_{50}$/mL. All assays were performed as biological quadruplicates and technical triplicates (n = 12). Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. Values for positive controls (MB-untreated, but light box-exposed MNV) ranged between 5.80 and 6.05 $TCID_{50}$/mL. *P*-values were computed by using a two-sided independent sample t-test (where $^{****}P < 0.0001$).

shown). Since high viral titer reductions were already observed following a 30-minute exposure in this preliminary assay, the 30-minute exposure was then tested and validated in four individual biological repeats (technical triplicates; Fig 5). In addition, 15-minute LB exposures were assayed as biological and technical triplicates (Fig 6). While both the 30-minute- and the 15-minute protocol reduced mean infectious MNV titers by more than 4 orders of magnitude (4.71 [±0.10] $\log_{10}$ $TCID_{50}$/mL and 4.53 [± 0.23] $\log_{10}$ $TCID_{50}$/mL, respectively), the shorter LB exposure led to higher inter-SM (and inter-coupon) variability (Fig 6) and did not consistently reduce virus titers to below the assay LOD.

DISCUSSION AND CONCLUSIONS

Supply issues at the beginning of the COVID-19 pandemic impressively illustrated that the world at large needs to be better positioned to deal quickly with prospective, potentially unknown, disease-causing agents and sanitary crises. Decontamination methods developed now, and perforce primarily targeting SARS-CoV-2, should thus already be future-proofed at this time by testing them against hardier pathogens. Here, we adapted an inexpensive and universally accessible photochemical decontamination protocol, recently developed against SARS-CoV-2 and other coronaviruses (DeMaND study),[10] to the treatment of norovirus-inoculated surgical masks.

To validate previously published MB photochemical treatment conditions[10] and to set a "baseline" for further development, the DeMaND MBL protocol involving 10 μM MB and 30-minutes of LB exposure was first investigated on either PRCV- or MNV-inoculated SMs. While this treatment reduced PRCV titers by over 5 orders of magnitude, it did not lead to significant inactivation of MNV.

Ten- and hundred-fold higher MB concentrations were subsequently tested in combination with longer LB exposures, in a series of microplate-based assays and on MNV-inoculated SMs. In line with previous assays reporting oxygen-dependent laser inactivation of MNV (in solution) after long LB exposure times,[27] inactivation of MNV proved both concentration- and time dependent in both matrices. In microplates, 100 μM MB concentrations markedly improved infectious titer reductions; 1,000 μM MB solutions, however, did not further visibly enhance infectivity losses, this likely attributable to a saturation effect and the fact that the assay LOD using 1,000 μM concentrations was ten-fold elevated as compared to that of assays performed using 100 μM concentrations. A minimum 4.80 (±1.01) log10 $TCID_{50}$/mL titer reduction was attained with 100 μM MB after 3 hours of LB exposure).

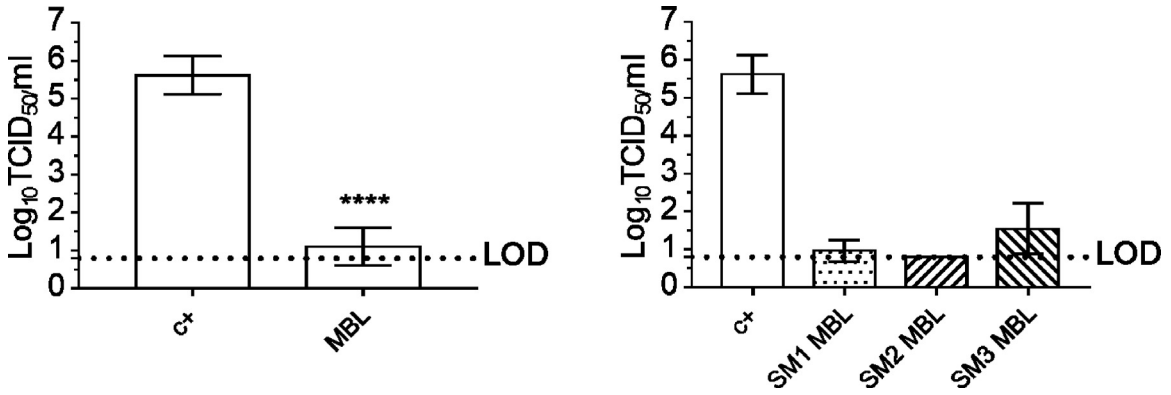

Fig 6. Evaluation of methylene blue (MB) photochemical treatment on murine norovirus (MNV) – inoculated surgical masks. The infectivity of MNV recovered from mask coupons treated with 100 μM MB and subsequently exposed for 15 min to a 12,500-lux red light source (light box) was analysed in RAW 264.7 cells. The cell culture limit of detection (LOD) was 0.8 $\log_{10}$ $TCID_{50}$/mL. All assays were performed as biological and technical triplicates (n = 9). The left panel represents a summary analysis of all obtained values. The right panel shows results for individual masks (n = 3) and illustrates the varying decontamination efficacies of this protocol. Means $\log_{10}$ $TCID_{50}$/mL and standard deviations are represented. Values for positive controls (MB-untreated, but light box-exposed MNV) ranged between 5.05 and 6.55 $TCID_{50}$/mL. P-values were computed by using a 2-sided independent sample t-test (where $^{****}P < 0.0001$).

Interestingly, MNV inactivation proved to be significantly more efficacious on SMs than in microplates and titer reductions of close to four orders of magnitude were already reached following 2 hours of LB exposure in initial SM assays. The differences between MNV inactivation in microplates and SMs may be attributable to 2 not mutually exclusive effects. Thus, the LB-emitted light may not have been sufficiently powerful as to penetrate a highly concentrated 100 μM solution in deep microplate wells, whereas MB sprayed as a thin surface layer onto SMs was easily accessible to photons, presenting better opportunities for excitation and singlet oxygen production. Equally, since the concentration of oxygen in air is one order of magnitude greater than in water,[28] a thin liquid-air interface (MB spray on SMs) could have positively impacted singlet oxygen production, whereas a larger-volume liquid phase (MB solution in microplate wells) may have had a negative impact.

Consolidated tests demonstrated that a MBL protocol involving 100 μM MB and a 30-minute LB exposure reliably reduced infectious MNV titers by more than 4 orders of magnitude. Shorter LB exposure led to higher inter-SM (and inter-coupon) variability and did not as consistently reduce MNV titers to below the assay LOD. A protocol involving shorter LB exposure times may be achievable via optimization of MB dispersion (greater homogeneity via standardized nebulizer may improve virus inactivation); meanwhile, the 30-minute LB exposure in conjunction with simple MB application via spray bottle presents a low-cost and low-tech protocol and is recommended at this time for the inactivation of small nonenveloped viruses. The light box utilized in this study is simply constructed and uses commercially available horticultural LED lamps; further simplification of the method may be achieved by eliminating the need for light boxes entirely – the possibility of leveraging solar irradiation for MB activation is currently under investigation by other teams within the DeMaND consortium.

The precise mode of action of MB is yet to be to determined,[19] while the vastly different sensitivities between PRCV and MNV may implicate the viral envelope as being one of the targets of MBL treatment, varying densities of viral proteinaceous capsids as well as differences in viral genome size and susceptibility to nucleic acid strand breakage (at 7.4 kb MNV is roughly four times smaller than PRCV) may also play a role. Further studies are indicated to pinpoint the definitive virucidal effect(s) of photochemical decontamination.

Methylene blue photochemical treatment is easily adaptable to other SM or FFR types.[10] Owing to the variation in SM and particularly FFR models the CDC recommends that the effectiveness of decontamination be evaluated for specific FFRs in collaboration with the manufacturer, and if needed, a third-party laboratory. This holds true for inactivation of all contaminants and should ideally be performed for the norovirus-inactivating conditions tested in our study. While the decontamination of a small non-enveloped virus can be considered a reliable indicator for inactivation of any other less robust virus or indeed any pathogen ranking lower in the hierarchy of pathogen resistance, a limitation of this work pertains to the fact that the inactivation of other tenacious pathogens (prions, bacterial spores, protozoal oocysts, helminth eggs, and mycobacteria) via MBL was not investigated. This does not pose a caveat to MBL decontamination of PPE in the current scenario of universal masking. However, in healthcare settings likely to be highly contaminated with a variety of nosocomial pathogens, photochemical treatment should ideally be combined with other protective measures until future studies can confirm whether MBL may be used for "wide-spectrum" inactivation.

Methylene blue concentrations necessary for norovirus inactivation (the total dose of MB per SM is 0.24 mg), are 10-fold higher than those needed for coronavirus decontamination. Since there is considerable clinical experience to support that MB exhibits good safety[29,30] and MB concentrations used here were below those administered clinically, the use of this higher dose is unlikely to pose safety concerns. Methylene blue is routinely applied directly to mucous membranes in the treatment of maxillary sinusitis (lavage using 1.5 ml aqueous 0.1% MB solution),[22] and has further been used as an efficient treatment of infected wounds both in human (up to three applications of a 20 mg MB dose)[31] and veterinary medicine.[32] In addition, ultraviolet spectroscopy analyses testing the amount of MB that may leach off SMs and be inhaled by a wearer during the course of a 10-hour healthcare provider work shift, have shown that MB does not leach off SM (or other PPE) materials (unpublished data; ongoing project with the WHO). Briefly, a panel of SMs, FFRs, and a cloth community mask were subjected to a total of five 1,000 μM MB treatment cycles (1,000 μM MB solution; total volume: 35-40 mL MB applied). Treated and excised PPE coupons were then subjected to full-mask equivalent airflow rates of 120 L/minute with a total airflow of 43,200 L/coupon. With a level of detection of 0.004 mg/m^3, no MB was observed within the spectroscopy parameters for all tested PPE items.

This is the first description of stable MB photochemical decontamination of SMs contaminated with an infectious norovirus. We describe successful validation of MB photochemical treatment for inactivation of small non-enveloped viruses that exceeds current FDA policy recommendations.[3] The highly resistant MNV surrogate supplements existing data regarding photochemical decontamination of SMs. It

serves to future-proof this method against viral mask (and other PPE) contaminants, thus solidifying the position of MBL PPE treatment both to combat PPE shortages in austere or low resource environments and as an important tool in the global package of practical pandemic preparedness.

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