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## Major Article

## Exploring inactivation of SARS-CoV-2, MERS-CoV, Ebola, Lassa, and Nipah viruses on N95 and KN95 respirator material using photoactivated methylene blue to enable reuse



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## Key Words:

Methylene blue  
N95 respirator  
Decontamination  
COVID-19  
Hemorrhagic fever virus  
Photochemical inactivation

**Background:** The COVID-19 pandemic resulted in a worldwide shortage of N95 respirators, prompting the development of decontamination methods to enable limited reuse. Countries lacking reliable supply chains would also benefit from the ability to safely reuse PPE. Methylene blue (MB) is a light-activated dye with demonstrated antimicrobial activity used to sterilize blood plasma. Decontamination of respirators using photoactivated MB requires no specialized equipment, making it attractive for use in the field during outbreaks.

**Methods:** We examined decontamination of N95 and KN95 respirators using photoactivated MB and 3 variants of SARS-CoV-2, the virus that causes COVID-19; and 4 World Health Organization priority pathogens: Ebola virus, Middle East respiratory syndrome coronavirus, Nipah virus, and Lassa virus. Virus inactivation by pretreating respirator material was also tested.

**Results:** Photoactivated MB inactivated all tested viruses on respirator material, albeit with varying efficiency. Virus applied to respirator material pre-treated with MB was also inactivated, thus MB pretreatment may potentially protect respirator wearers from virus exposure in real-time.

**Conclusions:** These results demonstrate that photoactivated MB represents a cost-effective, rapid, and widely deployable method to decontaminate N95 respirators for reuse during supply shortages.

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## BACKGROUND

The coronavirus disease 2019 (COVID-19) pandemic has placed a major burden on health care systems around the world. One major challenge that exacerbated this health crisis was scarcity of personal protective equipment (PPE), including N95 respirators. Health care workers sometimes resorted to reusing these single-use items due to supply shortages, which prompted the World Health Organization (WHO) to provide recommendations for rational use of PPE in health care settings and temporary strategies during acute supply shortages.<sup>1</sup> These WHO guidelines include the recommendation to decontaminate respirators

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before reuse. Various decontamination methods are listed for consideration, including ultraviolet (UV) irradiation, moist or dry heat sterilization, and vaporized hydrogen peroxide (VHP) treatment. Decontamination methods must be effective without adversely affecting N95 respirator integrity and fit, thus excluding decontamination methods using alcohol or intense heat. VHP treatment received US Food and Drug Administration (FDA) emergency use approval for decontaminating N95 respirators, but, like many of the aforementioned methods, it requires specialized equipment that is not available in many health care settings, especially in low resource settings.

Recently, photoactivated methylene blue (MB) was reported to decontaminate N95 respirators contaminated with various coronaviruses, including SARS-CoV-2.<sup>2</sup> Importantly, N95 integrity and fit were unaffected after 5 sequential applications of photoactivated MB decontamination, thus potentially enabling safe reuse of these respirators.<sup>2</sup> MB is a light-activated dye with demonstrated antimicrobial activity.<sup>3–9</sup> Photoactivated MB generates singlet oxygen, which damages viral nucleic acids and/or viral envelopes.<sup>10,11</sup> It is used to sterilize donor

plasma before transfusion and is approved by the FDA for the treatment of methemoglobinemia. Its efficacy has been demonstrated against a wide range of viruses in donor plasma.<sup>4,5,12</sup>

Photoactivated MB represents a promising novel decontamination method since it does not require specialized equipment, is inexpensive and commercially available, can be applied by spraying or immersing, and is effectively activated by exposure to bright or ambient light. Because ambient light can activate MB, electricity is not an absolute requirement for this decontamination technique, making it suitable for field settings.

In the last 2 decades, 3 coronaviruses, including the 1 causing the ongoing COVID-19 pandemic, have emerged and caused outbreaks with significant case fatality rates. In 2003, over 8,000 people were infected with severe acute respiratory syndrome coronavirus (SARS-CoV), and in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged, with cases reported in 27 countries in the subsequent years.<sup>13,14</sup> In addition, hemorrhagic fever viruses such as Ebola virus (EBOV), Lassa virus (LASV), and Nipah virus (NiV) give rise to regular outbreaks, primarily in Central and West Africa and parts of Asia.<sup>15–17</sup> The 2014–2016 EBOV outbreak in West Africa was the largest in history, with more than 28,600 cases and 11,325 deaths. Several smaller EBOV outbreaks have been reported since.<sup>18</sup> LASV causes an estimated 100,000–300,000 cases per year in parts of West Africa, with approximately 5,000 deaths.<sup>19</sup> NiV causes outbreaks almost annually in parts of Asia, including Singapore, Bangladesh, India, and Malaysia.<sup>15</sup> Many of these areas may not have access to reliable PPE supply lines, especially during outbreaks, and would benefit from a robust, simple, affordable decontamination method that does not require specialized equipment, enabling the safe reuse of PPE when facing supply shortages.

Here, we investigated the potential use of photoactivated MB as a decontamination method for N95 respirators in the field. In addition, we tested this method on KN95 respirator material, since this type of respirator is widely available and commonly used by the general public. We examined the ability of MB to inactivate 3 SARS-CoV-2 strains, including the Beta and Delta variants, as well as 4 other important human pathogens that pose a threat to public health: MERS-CoV, EBOV, LASV, and NiV. Photoactivated MB treatment efficiently inactivated all tested viruses on N95 and KN95 respirator materials, though EBOV, LASV, and NiV required higher MB concentrations and/or stronger light than SARS-CoV-2. Moreover, virus applied to respirator material pre-treated with MB was fully inactivated upon exposure to bright light. MB pre-treatment was especially effective against the tested coronaviruses.

## MATERIALS AND METHODS

### Biosafety statement

All experiments with SARS-CoV-2 and MERS-CoV were performed at biosafety level (BSL) 3 facilities at the George Washington University Milken Institute School of Public Health (Washington, DC, USA) or at BSL-4 facilities at the Centers for Disease Control and Prevention (Atlanta, GA, USA). BSL-3 facilities are sufficient for experiments with

SARS-CoV-2. All experiments with EBOV, LASV, and NiV were performed at BSL-4 facilities at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Experiments involving recombinant viruses were performed in accordance with approved Institutional Biosafety Committee protocols.

### Viruses and cells

SARS-CoV-2 isolates and MERS-CoV were obtained from BEI Resources: MERS-CoV isolate EMC/2012 (BEI NR-44260), SARS-CoV-2 isolate Italy-INMI1 (pre-Alpha; BEI NR-52284), SARS-CoV-2 isolate hCoV-19/South Africa/KRISP-EC-K005321/2020, lineage B.1.351 (Beta variant; BEI NR-54008), SARS-CoV-2 isolate hCoV-19/USA/PHC658/2021, lineage B.1.617.2 (Delta variant; BEI NR-55611) (Table 1). Viral titers were determined using plaque assays in Vero E6 cells (American Type Culture Collection (ATCC)). Vero E6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% GlutaMAX at 37°C and 5% CO<sub>2</sub>.

icSARS-CoV-2/mNeonGreen, rLASV/ZsG, rEBOV/GFP, and rNiV/ZsG were previously described.<sup>20–23</sup> icSARS-CoV-2/mNeonGreen was based on the virus strain 2019-nCoV/USA\_WA1/2020 isolated from the first reported SARS-CoV-2 case in the US (Table 1).<sup>24,25</sup> Viral titers were determined using Vero CCL-81 cells (ATCC) by tissue culture infectious dose 50 (TCID<sub>50</sub>) assays. Fluorescence was detected using an EVOS fluorescence microscope. Vero CCL-81 cells were cultured in DMEM containing 5% FBS, 1% NEAA, GlutaMAX, sodium pyruvate, and antibiotics at 37°C and 5% CO<sub>2</sub>.

### Photoactivated MB decontamination and pre-treatment

MB was obtained from Sigma-Aldrich and dissolved in ultrapure water. N95 (3M 1860) and KN95 (Chengde Technology CD9501B) respirators were cut into ~1 cm<sup>2</sup> coupons, which were placed in tissue culture plates during testing. For decontamination testing, coupons were inoculated with 10 μL virus stock, incubated for 20 min at room temperature, and treated with 20 μL MB and light for 30 min. MB was diluted in DMEM to the indicated concentration. Inoculated control coupons received 20 μL of DMEM only and served as a control for elution efficiency. Depending on the virus used, input titer ranged from ~1 × 10<sup>3</sup> to ~1 × 10<sup>5</sup> PFU or ~1 × 10<sup>5</sup> to ~1 × 10<sup>6</sup> TCID<sub>50</sub> per 10 μL virus stock. Coupons were exposed to bright or ambient light or protected from light (dark condition). Bright light (~50,000 lux) was provided by a lightbox developed at Colorado State University containing a 4000K Husky LED light. Ambient light (~700 lux) was provided by the biosafety cabinet lights. Light conditions were quantified using a light meter (Cooke cal-LIGHT 400).

For pre-treatment testing, masks were cut into strips, soaked in 10 or 100 μM MB, dried, and cut into coupons. Control coupons were left untreated. Coupons were inoculated with 10 μL virus stock and exposed to light for 30 min, or protected from light. Virus inoculum was eluted in DMEM and remaining infectious virus was quantified by TCID<sub>50</sub> or plaque assays.

**Table 1**  
Viruses

Virus name	Virus isolate/strain	BEI Identifier/reference
SARS-CoV-2 pre-Alpha	Italy-INMI1	NR-52284
SARS-CoV-2 Beta	hCoV-19/South Africa/KRISP-EC-K005321/2020 (lineage B.1.351)	NR-54008
SARS-CoV-2 Delta	hCoV-19/USA/PHC658/2021 (lineage B.1.617.2)	NR-55611
icSARS-CoV-2/mNeonGreen	2019-nCoV/USA_WA1/2020	Xie et al. <sup>20</sup>
MERS-CoV	EMC/2012	NR-44260
rLASV/ZsG	Lassa/Josiah, GenBank HQ688673.1, HQ688675.1	Welch et al. <sup>21</sup>
rEBOV/GFP	Ebola virus/H.sapiens-wt/LBR/2014/Makona-201403007, GenBank KP178538	Albarino et al. <sup>22</sup>
rNiV/ZsG	Nipah/Malaysia, GenBank AF212302	Lo et al. <sup>23</sup>

To investigate MB pre-treatment durability, masks were cut into strips, soaked in 10  $\mu\text{M}$  MB, dried, and cut into coupons. Control coupons were left untreated. Coupons were subsequently exposed to ambient light for the indicated time period (up to 3 weeks of 12 h dark/light cycles). Subsequently, coupons were inoculated with virus as above and further exposed to ambient light for 30 min. The inoculum was eluted in serum-free media (DMEM supplemented with 1% GlutaMAX) and quantified by plaque assays.

## RESULTS

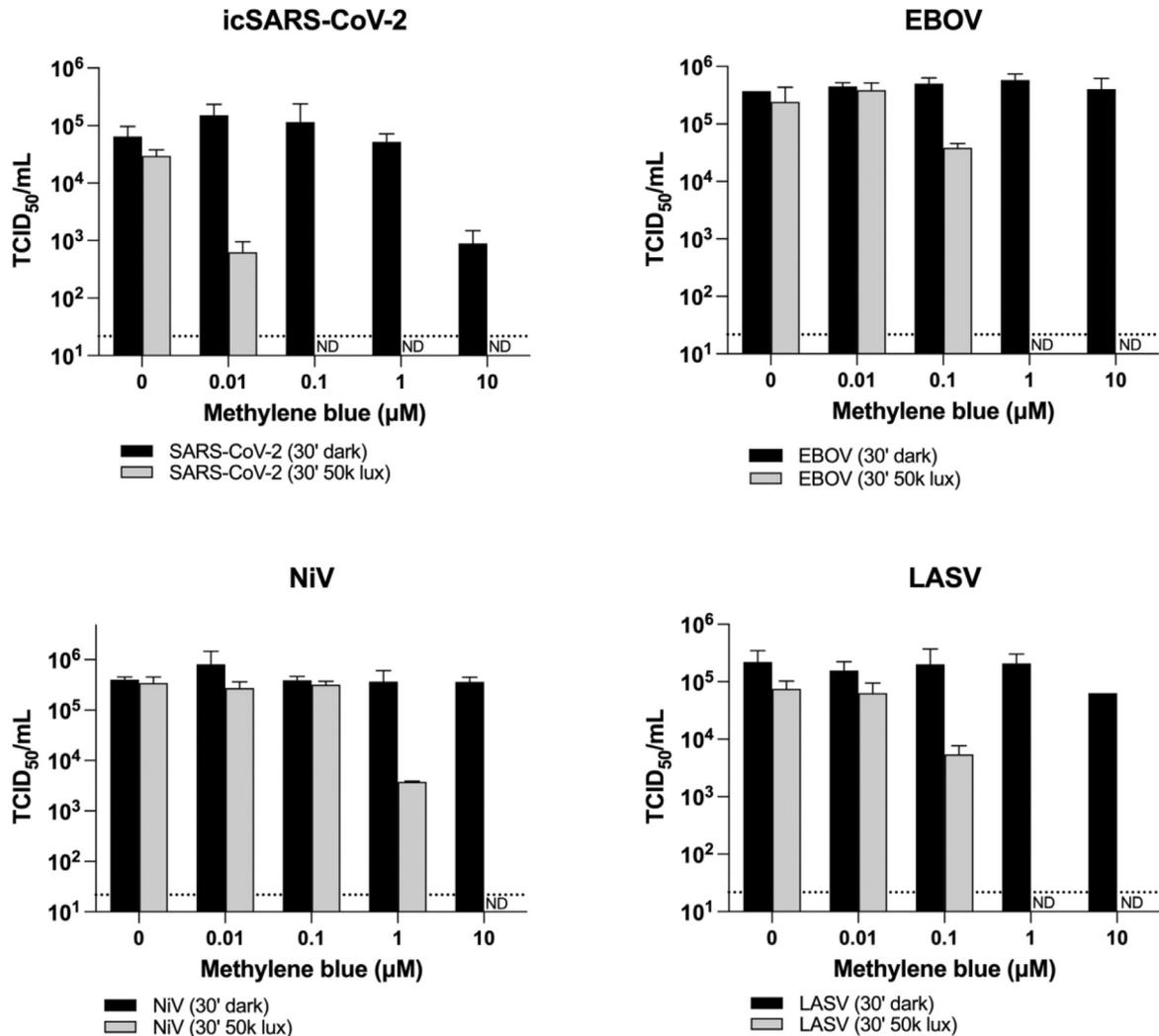
### Photoactivated MB inactivates SARS-CoV-2, EBOV, LASV, and NiV

Photoactivated MB has been demonstrated to inactivate various viruses in plasma, including SARS-CoV-2, EBOV, and NiV.<sup>4,5,8</sup> One of the aims of this study was to investigate whether photoactivated MB can be used as a method to decontaminate N95 respirators in the field during outbreaks of SARS-CoV-2 or other viral pathogens, such as EBOV, LASV, or NiV. First, to confirm that MB inactivates these pathogens and to define the required MB concentrations, serially diluted MB was combined with aliquots of different viruses. Virus-MB mixtures were placed

in empty tissue culture plates and exposed to bright white light (50,000 lux) for 30 min. As observed previously, SARS-CoV-2 is efficiently inactivated by 0.1  $\mu\text{M}$  MB under bright light.<sup>2</sup> EBOV and LASV were completely inactivated by 1  $\mu\text{M}$  MB under bright light, whereas NiV required 10  $\mu\text{M}$  MB and bright light for complete inactivation (Fig 1). In the absence of light, viral titers were largely unaffected by MB, though 10  $\mu\text{M}$  MB alone modestly reduced SARS-CoV-2 titers.

### Photoactivated MB inactivates virus on N95 respirator material

After establishing the MB concentration required to inactivate SARS-CoV-2, EBOV, LASV, and NiV in tissue culture plates, we examined the ability of photoactivated MB to decontaminate N95 and KN95 respirator material inoculated with these viruses. Decontamination of respirators with photoactivated MB would allow limited reuse of these single-use PPE items when supply lines are disrupted. A commonly used N95 respirator (3M 1860) was used as a representative model. In addition, we tested a KN95 respirator, since this type of respirator is currently easily available to the general public and therefore widely used. Contamination of mask material was mimicked by adding a 10  $\mu\text{L}$  droplet of virus to the outer layer. Next, MB was added to the



**Fig 1.** Methylene blue plus light inactivates SARS-CoV-2, EBOV, LASV, and NiV in tissue culture plates. To confirm that photoactivated methylene blue (MB) inactivates the tested viruses, serially diluted MB was combined with aliquots of the indicated viruses and exposed to bright light (50,000 lux) for 30 min. Control plates were stored in the dark. Titers of remaining infectious virus were determined by TCID<sub>50</sub>. Values represent means and standard deviations of triplicate samples. Dotted line represents the limit of detection. icSARS-CoV-2, SARS-CoV-2 derived from an infectious clone. ND, not detected. (Color version of figure is available online.)

contaminated surface, and the material was exposed to either ambient (~700 lux) or bright light (50,000 lux) for 30 min (Fig 2). A dose-dependent effect was observed for both MB concentration and light intensity, with similar results obtained for N95 and KN95 respirators. SARS-CoV-2 was again efficiently inactivated when 0.1  $\mu\text{M}$  MB was used under bright light or when 1  $\mu\text{M}$  MB was used with ambient light. EBOV, LASV, and NiV required a higher MB concentration and/or exposure to bright light to yield complete inactivation. As observed previously, MB treatment in the absence of light (dark condition) had less or no effect on viral titers, with the exception of SARS-CoV-2, which was inactivated by 10  $\mu\text{M}$  alone (Fig 2).

#### *Pre-treatment of N95 and KN95 respirators with MB inactivates SARS-CoV-2 variants, EBOV, LASV, and NiV*

Pre-treating masks with MB could potentially provide real-time protection against pathogens encountered whilst wearing. To test this, N95 and KN95 respirator material was soaked in MB and dried before use. First, we tested 3 different SARS-CoV-2 strains (pre-Alpha, Beta, and Delta variants) and MERS-CoV on material pre-treated with 10  $\mu\text{M}$  MB (Fig 3A). Exposure to ambient light (30 min) efficiently inactivated all 3 SARS-CoV-2 strains as well as MERS-CoV. In addition, MB alone (dark condition) was also able to inactivate SARS-CoV-2 and MERS-CoV. No difference was observed among SARS-CoV-2 strains or between respirator type (Fig 3A).

Next, we examined if hemorrhagic fever viruses are inactivated upon contact with MB-pre-treated respirator material. N95 and KN95 material was soaked in 10 or 100  $\mu\text{M}$  MB, dried, and inoculated with virus as above. Exposure to ambient light (30 min) did not reduce titers of NiV and EBOV on material pre-treated with 10  $\mu\text{M}$  MB, while a modest reduction in LASV titer was observed (Fig 3B). Pre-treating material with 100  $\mu\text{M}$  MB combined with exposure to ambient light also failed to reduce NiV titers, but resulted in a 1–3 log reduction in EBOV and LASV titers. In contrast, exposure to bright light (50,000 lux for 30 min) completely inactivated all tested viruses spotted onto material pre-treated with 100  $\mu\text{M}$  MB. LASV, but not EBOV or NiV, was completely inactivated on 10  $\mu\text{M}$ -pre-treated material exposed to bright light (Fig 3B).

#### *Exploring pre-treatment durability*

Next, we investigated if MB-pre-treated respirators lose their ability to inactivate virus after prolonged light exposure. N95 and KN95 material was pre-treated with 10  $\mu\text{M}$  MB and exposed to ambient light for up to 252 h (mimicking 7–21 days of 12 h dark and/or light cycles). Remaining MB virucidal activity was tested by inoculating the respirator material with SARS-CoV-2 (Delta variant) or MERS-CoV and further exposing it to ambient light for 30 min. Regardless of the duration of the pre-exposure period, SARS-CoV-2 and MERS-CoV were efficiently inactivated on all pre-treated respirator material (Fig. 4). This suggests that respirator pre-treatment with MB has the potential to provide robust and long-lasting protection against coronaviruses.

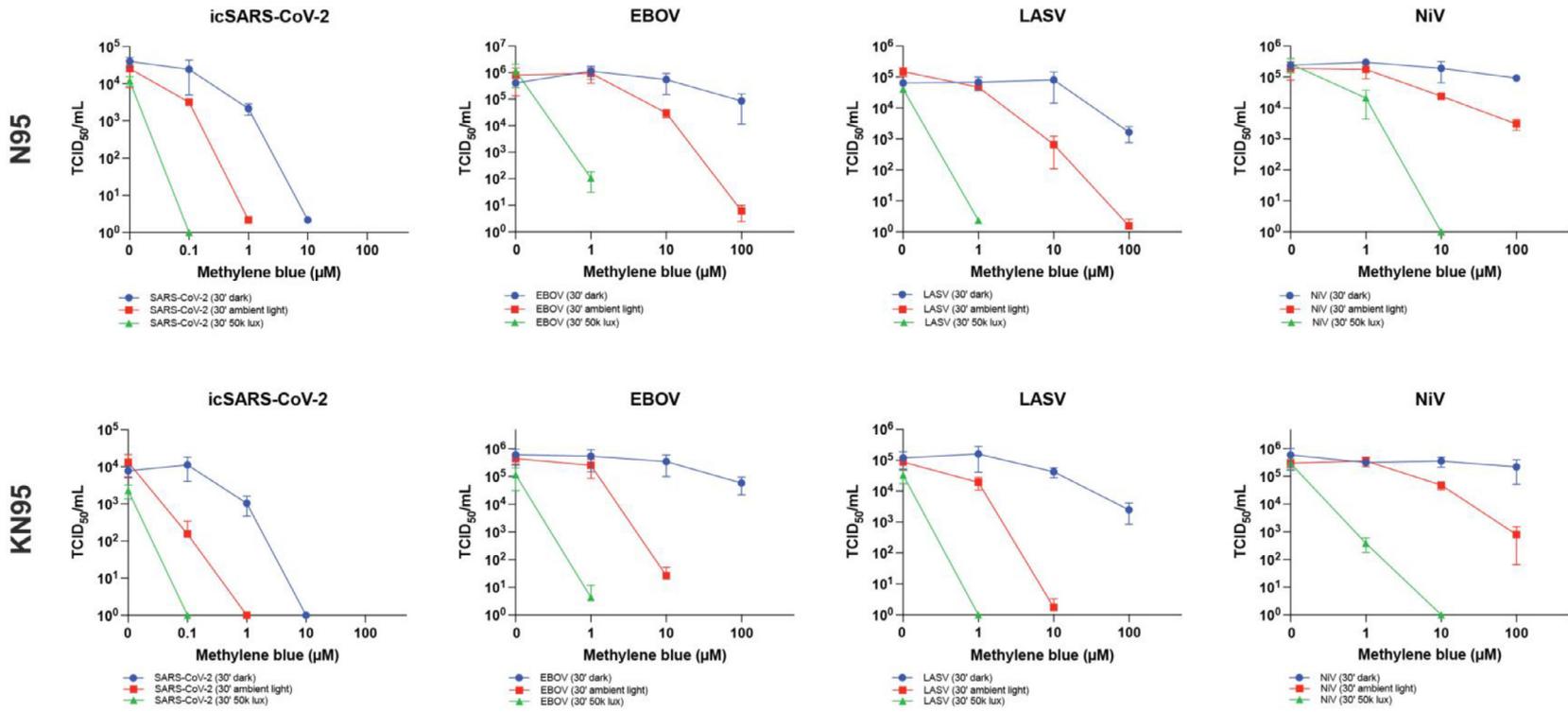
## DISCUSSION

The COVID-19 pandemic has demonstrated that emerging and reemerging coronaviruses are capable of causing worldwide infection events. This pandemic resulted in severe PPE shortages, underscoring the importance of establishing effective methods for PPE decontamination to allow safe reuse. Decontamination methods should not affect PPE integrity and be effective against a broad range of pathogens. These decontamination methods should be simple, efficient, and cost-effective, making them suitable for both high- and low-resource settings.

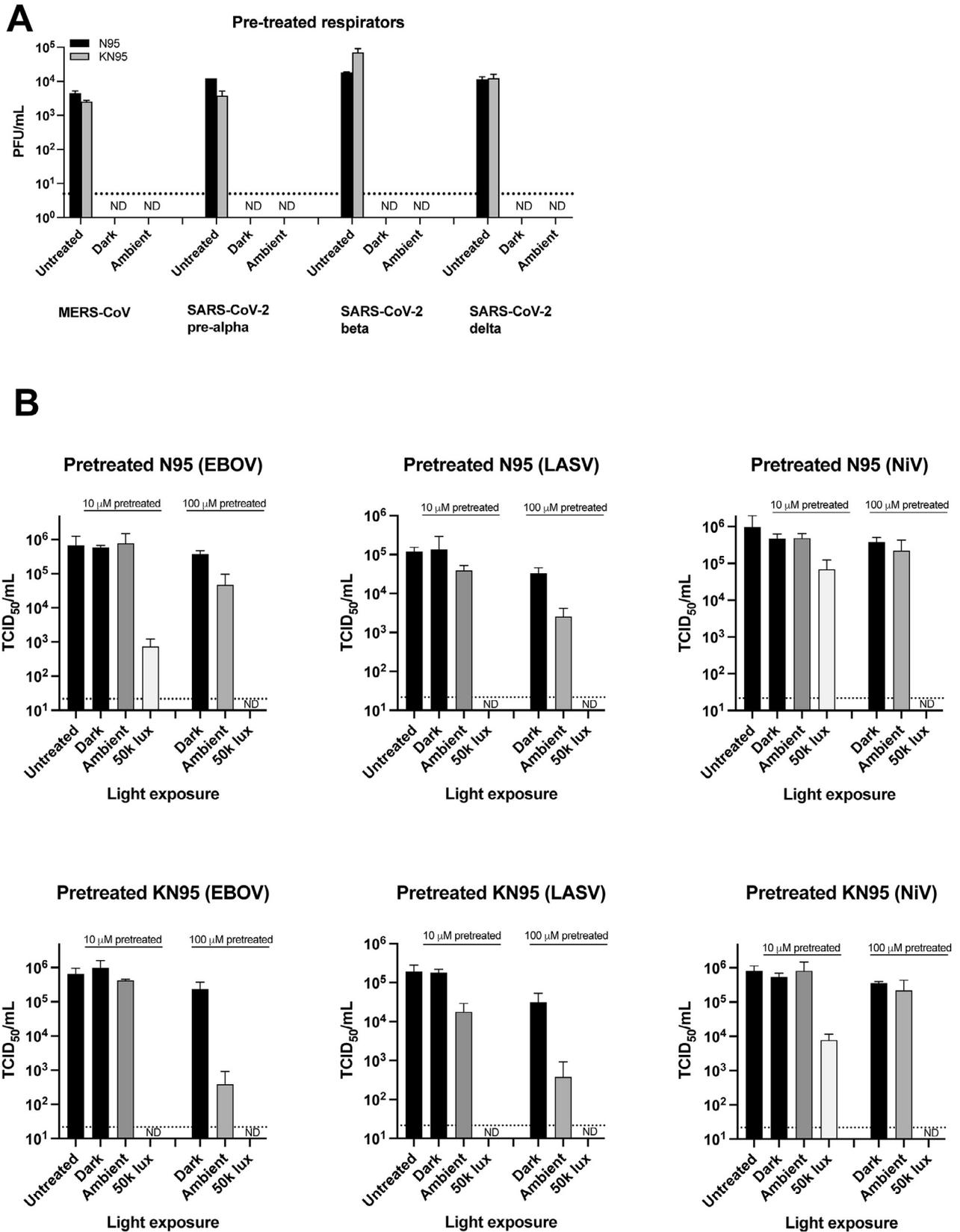
Recently, photoactivated MB was shown to inactivate coronaviruses on respirator and medical mask material, forming the basis for its use as a PPE decontamination method.<sup>2</sup> MB is approved by the US FDA and European Medicines Agency (EMA) to treat methemoglobinemia by intravenous injection. It is also used to sterilize blood products before transfusion in Europe. Here we demonstrate that photoactivated MB can be used to inactivate a collection of viruses that are pathogenic to humans and can cause major outbreaks. On N95 and KN95 respirator material, photoactivated MB efficiently inactivated SARS-CoV-2, MERS-CoV, EBOV, LASV, and NiV. Since this decontamination method does not require specialized equipment, it is particularly attractive for use in the field during outbreaks or in resource-poor settings when PPE supplies are insufficient. Importantly, respirator fit and integrity were not affected after 5 MB decontamination cycles.<sup>2</sup> MB is inexpensive, globally available, and effectively inactivated viruses in combination with bright light. The light source can be a simple bright LED light, ambient light, or direct sunlight, which has a broader spectrum of wavelengths and may activate MB more efficiently. Direct sunlight can provide ~32,000–100,000 lux, depending on geographic location, time of day, season, and cloud cover. Our data demonstrate that 10–100  $\mu\text{M}$  MB combined with 50,000 lux inactivates all tested viruses, including EBOV, LASV, and NiV, suggesting that MB can be used to inactivate these viruses under sunlight. We examined the ability of ambient light in an indoor setting (such as a clinic or in the field) to inactivate virus when combined with MB. For practical and biosafety purposes, we tested ambient light of ~700 lux generated by the lights of the biosafety cabinet. Combining 10–100  $\mu\text{M}$  MB with only ~700 lux (ambient light) readily inactivated EBOV, LASV, SARS-CoV-2, and MERS-CoV. Interestingly, NiV appeared more resistant to inactivation under similarly ambient conditions for reasons that remain to be explored. In this study, we tested multiple SARS-CoV-2 variants to address the question whether a variety of circulating variants would be inactivated by photoactivated MB. As expected, no difference in inactivation efficiency was detected, and only 1 variant was used for the subsequent experiments.

One of the main advantages of using photoactivated MB for virus inactivation is that MB activity is non-specific, and therefore the development of viral resistance is not expected. When combining MB with a light source, the energy is absorbed and transferred to molecular oxygen, resulting in the highly reactive singlet oxygen.<sup>26</sup> Singlet oxygen reacts with its cellular environment, leading to non-specific oxidative reactions. This results in damage to nucleic acids, proteins, and lipids.<sup>11,10</sup> Despite its non-specificity, not all viruses are equally sensitive to photoactivated MB (27, this study). This may be due to the type and size of the viral genome, type of viral envelope, and/or virion complexity. Though not tested in this study, non-enveloped viruses appear less sensitive to MB.<sup>27</sup> MB respirator decontamination or pretreatment could potentially be applied in hospital settings where it would encounter a wider range of pathogens. MB was demonstrated to be effective against other pathogens causing common hospital infections such as influenza, norovirus, *S. aureus*, and *E. coli*.<sup>8,28–32</sup> To inactivate harder pathogens such as norovirus, higher MB concentrations or longer exposure times may be required.

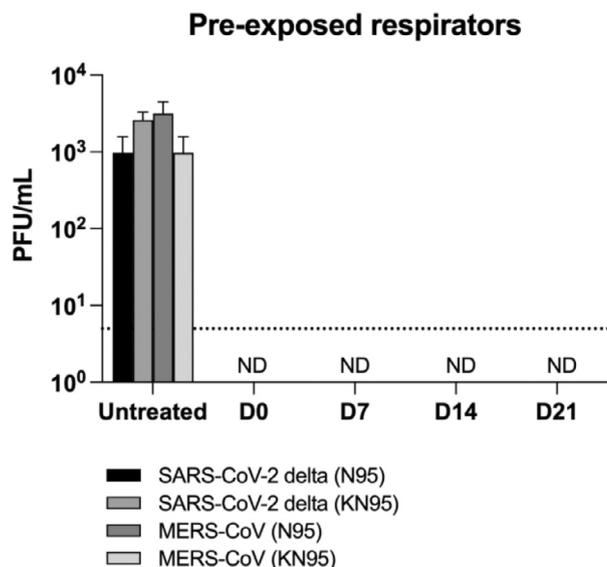
MB applied to respirator material retains potency after prolonged exposure to ambient light, demonstrating that such pre-treatment may represent a promising strategy for adding an extra layer of protection to this type of PPE. Additional studies are warranted to examine the durability of pre-treatment beyond the times tested here, as well as the effect of more intense light sources and natural sunlight. Since the outer surface of respirators is hydrophobic, MB could be



**Fig 2.** MB plus light inactivates SARS-CoV-2, EBOV, LASV, and NiV on N95 and KN95 respirator material. To investigate if photoactivated MB can decontaminate mask material, N95 or KN95 respirator coupons were inoculated with 10  $\mu$ L virus, incubated for 20–60 min, treated with 20  $\mu$ L of MB at the indicated concentrations, and exposed to ambient or bright (50,000 lux) light for 30 min. Control coupons were stored in the dark. Virus was eluted and quantified by TCID<sub>50</sub>. icSARS-CoV-2, SARS-CoV-2 derived from an infectious clone.



**Fig 3.** SARS-CoV-2, MERS-CoV, EBOV, LASV, and NiV are inactivated on respirators pre-treated with MB and exposed to light. To investigate if MB pre-treatment inactivates the tested viruses on respirators, pre-treated N95 and KN95 respirator material was inoculated with coronaviruses (A) or hemorrhagic fever viruses (B). N95 and KN95 respirator material was pre-treated with 10 or 100  $\mu$ M MB, dried, inoculated with 10  $\mu$ L virus, and exposed for 30 min to ambient light or bright light (50,000 lux). Control coupons were stored in the dark. Virus was eluted and quantified by plaque assay or TCID<sub>50</sub>. Dotted line represents the limit of detection. ND, not detected.



**Fig 4.** MB pretreatment of respirators is robust. To examine if MB pre-treatment of respirators loses potency after prolonged light exposure, material was treated with 10  $\mu$ M MB, dried, and exposed to ambient light for up to 252 h (mimicking 7–21 days of 12 h dark/light cycles). After pre-exposure, respirator material was inoculated with the indicated viruses and further exposed to ambient light for 30 min. Remaining virus was eluted and quantified by plaque assay. Dotted line represents the limit of detection. ND, not detected.

incorporated in the production process as an antimicrobial coating. This antimicrobial coating would begin to inactivate pathogens that come in contact with the mask surface in real-time, reducing the risk of exposure during doffing. Of note, antimicrobial wipes coated with another light-activated dye, rose bengal, have been produced. They efficiently inactivated influenza virus H1N1, but were less efficient against norovirus or human adenovirus type V (hAdV-5).<sup>33</sup>

In this study we examined virus inactivation on respirator material (coupons) rather than using intact masks. This allowed us to demonstrate the proof of principle that photoactivated MB can indeed inactivate the tested viruses on respirator material, but may not be fully representative for decontamination applications followed by reuse. MB has an excellent safety profile when used for injections, but its safety profile for potential inhalation after respirator decontamination or pre-treatment has not been fully assessed. This would need to be addressed before this method can be used in the field.

Taken together, our data demonstrate that photoactivated MB treatment inactivates various SARS-CoV-2 variants as well as hemorrhagic fever viruses on respirator material. This technique represents a low-cost decontamination method that is easily deployable and could help mitigate PPE shortages in all resource settings.

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