

In vitro efficacy of an automatic UVC-LED device for disinfection of the stethoscope diaphragm against bacteria, fungi and enveloped respiratory viruses

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Abstract

The stethoscope moves from patient to patient far more often than it is cleaned, and chemical disinfection is applied inconsistently because it depends on the operator remembering and on a wipe being within reach. We tested a UVC-LED device emitting at 275 nm that runs a single automatic 150-second cycle on the stethoscope diaphragm, against nine reference bacterial and fungal strains and four enveloped respiratory viruses (influenza A and B, human parainfluenza virus 3 and human metapneumovirus) placed onto a quartz carrier. One cycle drove viable counts to at or below the limit of detection across the whole panel, a reduction of roughly $5 \log_{10}$ that held for the multidrug-resistant yeast *Candida auris*, the UV-resistant conidia of *Aspergillus brasiliensis* and the rapidly growing *Mycobacterium abscessus*; because the treated samples fell below detection, these are conservative lower bounds set by the inoculum. All four viruses lost their ability to infect cells down to the lowest concentration that could be measured. For influenza viruses we traced that loss to the genome itself: a long-range PA-gene product disappeared, and sequencing coverage collapsed first on the longest segments, while a short diagnostic RT-PCR target barely moved, below $1 \log_{10}$. Because damage builds up with the length of the target, short-amplicon assays overstate the residual infectious load. A single operator-independent cycle cleared a deliberately broad panel, drug-resistant and UV-resistant organisms included, and abolished viral infectivity through real loss of genome integrity, not surviving nucleic acid. Confirmation under soiled conditions and in clinical use would carry it into routine practice.

Introduction

The stethoscope is among the most frequently used diagnostic instruments, and its diaphragm and earpieces come into repeated contact with the skin and clothing of many patients over a working day [1, 2]. Contamination of these surfaces has been documented for decades: reviews report bacteria on about 85% of examined devices [3]. In some studies, it has been reported that these contamination rates reach 100% of the examined devices [3, 4]. The load recovered from the diaphragm is comparable to that on the physician's dominant hand after examination, and molecular analyses have identified stable bacterial communities on the instrument [4, 5].

Coagulase-negative staphylococci, usually commensals, predominate among the isolates, but recognised pathogens are recovered regularly, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, vancomycin-resistant enterococci (VRE), *Clostridioides difficile* and other multidrug-resistant organisms [5]. Bacterial transfer between a patient's skin and the diaphragm has been shown to run in both directions, so a contribution of stethoscopes to healthcare-associated infections (HAIs) is biologically plausible, even though a causal link to specific infections has not been formally established [4].

Guidelines therefore recommend disinfecting the instrument between patients, but adherence is poor [6]. In an observational study of 400 encounters, disinfection consistent with the Centers for Disease Control and Prevention recommendations was performed in fewer than 4% of cases, and the instrument was not

disinfected at all in 82% [7]; comparable figures recur across the literature, with pre- and post-contact rates between 0 and 24% [4]. The chemical options in use, ethyl and isopropyl alcohol, chlorhexidine and quaternary ammonium compounds, reduce the bacterial load effectively but only when applied consistently, and their use is limited by the time required, the availability of materials at the point of care, human error, the risk of damaging surfaces and the lack of a systematic reminder [8, 9]. Educational and visual-reminder interventions give variable results and have prompted calls to revise current recommendations [10]. The gap between recommended and actual practice has persisted, and it is unlikely to close through measures that depend on operator behaviour [11].

Ultraviolet radiation in the C band (UVC, 200–280 nm) offers an alternative that works once initiated, without operator intervention. UVC photons are absorbed by nucleic acids, with peak germicidal effect near 260–265 nm, and generate pyrimidine dimers that block replication; the process leaves no chemical residue and selects for no resistance [12–14]. In hospitals, UVC disinfection of surfaces and environments has reduced HAI-associated pathogens, including MRSA, VRE and *C. difficile* [12, 15].

The traditional UVC source is the mercury-vapour lamp, emitting mainly at 254 nm. Semiconductor UVC light-emitting diodes (LEDs) are the more recent option [16] and suit a dedicated point-of-care device well: they contain no mercury (which simplifies safety and disposal), switch on instantly, are small and mechanically sturdy, draw little power and can be built to emit close to the DNA absorption peak [13, 14]. What matters is the emission of the source and the fluence at the treated surface; the housing only decides what can be placed in front of it. On this basis, a UVC-LED device for automatic disinfection of the stethoscope diaphragm was developed and tested; under experimental conditions a single minute of exposure reduced the bacterial load of every species tested (*S. aureus*, *Escherichia coli*, *P. aeruginosa* and *Enterococcus faecalis*) by roughly 85% to 95% [17], with performance retained after simulated prolonged use and an ergonomics compatible with routine clinical work [18, 19].

Contamination of clinical surfaces and shared devices is not limited to common nosocomial bacteria; it includes organisms with different structures and resistance profiles, so a disinfection method meant for routine use must act across a broad range, environmental species included. Standardised efficacy testing of surface disinfectants relies on a panel combining a Gram-positive bacterium, a Gram-negative bacterium, a yeast and a filamentous fungus, usually *S. aureus*, *P. aeruginosa*, *E. coli*, *Candida albicans* and *Aspergillus brasiliensis* [20–23], so that activity can be claimed across the main microbial groups. Of these, the pigmented conidia of *A. brasiliensis* are markedly UV-resistant and serve as the reference target in UV-C bio-validation of packaging and surfaces [24, 25], which makes the mould the demanding member of that panel. Non-tuberculous mycobacteria (NTM), including *Mycobacterium abscessus*, are environmental organisms that form biofilms and resist many chemical disinfectants, and they are increasingly implicated in device- and water-associated HAIs [26]. *Candida auris* adds to these concerns: it survives on dry surfaces for weeks, resists several antifungals and common disinfectants, and spreads readily through contaminated surfaces and equipment, which has drawn attention to non-chemical options such as UVC [27].

During cardiac and respiratory examinations, the stethoscope touches the chest directly, often in settings where respiratory viruses circulate. Influenza A (IAV) and B (IBV) viruses remain infectious on hard, non-porous surfaces for 24 to 48 hours and can transfer to the hands from them, which places fomites within the transmission chain [28, 29]. Human parainfluenza virus type 3 (HPIV3) and human metapneumovirus (hMPV) are enveloped respiratory viruses of clinical importance, causing upper- and lower-respiratory infection especially in children, older adults and immunocompromised patients [30, 31], and they too retain infectivity on surfaces for several hours, which justifies their inclusion in decontamination testing [32, 33]. As enveloped viruses they are susceptible to UVC, whose photons are absorbed by nucleic acids and abolish replication [34, 35]. Because nucleic acid amplification alone cannot separate infectious from inactivated particles, the loss of infectivity, which arises mainly from genomic strand breaks, can be probed with a long-range RT-PCR assay [36].

This study aims to evaluate the *in-vitro* antimicrobial efficacy of a recent UVC-LED device for automatic disinfection of stethoscope diaphragms, against a deliberately broad panel: Gram-positive and Gram-negative bacteria (including an MRSA strain and the environmental *Bacillus subtilis* in vegetative form), a rapidly growing NTM, two yeasts (including the multidrug-resistant *C. auris*), a filamentous mould, and the enveloped respiratory viruses IAV, IBV, HPIV3 and hMPV. Using a long-range RT-PCR assay, we also evaluated whether the loss of viral infectivity reflected genuine genomic damage. The aim was an operator-independent disinfection step fast enough to fit routine practice.

Materials and Methods

UVC-LED device and experimental set-up

The test device is a UVC-LED system designed for the automatic disinfection of stethoscope diaphragms. The unit houses three fixed-emission UVC LEDs (OSRAM SU CULBN2.VC; OSRAM Licht, Munich, Germany) with the following specifications:

- Peak emission wavelength: 275 nm;
- Total radiant power: 6.9 mW (≈ 2.3 mW for each LED);
- Irradiance at the centre of the device: $650 \mu\text{W}/\text{cm}^2$ (Fig. 1);
- Irradiance at the lateral point where the bacterial or viral inoculum droplets were placed (approximately 2 cm from the centre; Fig. 1): $85 \mu\text{W}/\text{cm}^2$;
- Dose delivered per cycle at the lateral point: $12.8 \text{ mJ}/\text{cm}^2$.

The device performs a single automatic disinfection cycle lasting 150 s, signalled by a status light. These quantities, and not the model of the unit, define the exposure.

The spectral output and irradiance of the source were characterised using a calibrated fibre-optic spectrophotometer (AvaSpec-ULS2048CL-EVO-USB3UV/VIS/NIR; Avantes BV, Apeldoorn, the Netherlands). A photometric simulation was performed using Ansys Speos 2024 software (Ansys Inc.,

Canonsburg, PA, USA) to model the spatial distribution of UVC LED irradiance on the plane. This considered the characteristics of the LEDs and the empirical measurements obtained (Fig. 2). A circular fused-silica (quartz) disc transparent to UVC (Hellma GmbH & Co. KG, Müllheim, Germany) was placed inside the device in the position normally occupied by the stethoscope membrane. This disc, with a light transmittance of 89%, was used as the test carrier to simulate the diaphragm surface. All exposures were carried out at room temperature (RT; 22°C and between 30–40% relative humidity). Before each experiment the temperature and relative humidity at the carrier were checked with a thermo-hygrometer (Testo SE & Co. KGaA, Titisee-Neustadt, Germany) to exclude any thermal contribution to inactivation.

Microbial strains, media and culture conditions

The antimicrobial spectrum of the device was assessed against nine reference strains, which were selected to represent the main microbial groups relevant to contaminated healthcare surfaces. These reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Japan Collection of Microorganisms (JCM, Tsukuba, Japan). The bacterial panel comprised the Gram-positive MRSA (ATCC 43300), the Gram-negative *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 27853), the enteric Gram-negative *Salmonella enterica* ser. *Choleraesuis* (ATCC 10708), the Gram-positive *B. subtilis* (ATCC 11774), tested as vegetative cells, and the rapidly growing NTM *M. abscessus* subsp. *bolletii* (JCM 15297). The fungal panel comprised two yeasts, *C. albicans* (ATCC 10231) and *C. auris* (ATCC 12372), and the filamentous mould *A. brasiliensis* (ATCC 16404).

Working suspensions were prepared in phosphate-buffered saline (PBS, pH 7.4; Euroclone S.p.A., Pero, Italy). The turbidity of the starting inoculum was adjusted with a densitometer DEN-1B (Biosan SIA, Riga, Latvia) to 0.5 McFarland, corresponding to $\approx 1.5 \times 10^8$ CFU/mL for bacteria and $\approx 1 \times 10^6$ CFU/mL for yeasts; *A. brasiliensis* was adjusted to $\approx 1.5 \times 10^6$ CFU/mL; for *M. abscessus* subsp. *bolletii* a 1 McFarland suspension was found to correspond to $\approx 3 \times 10^7$ CFU/mL. Suspensions were then serially diluted to the final test concentration of $\approx 1.5 \times 10^5$ CFU/mL ($\approx 10^5$ CFU/mL).

Each microorganism was enumerated on its elective solid medium. *S. aureus*, *E. coli* and *P. aeruginosa* were plated on Plate Count Agar (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) and incubated at 37°C for 48 h; *S. enterica* ser. *Choleraesuis* and *B. subtilis* were plated on Tryptic Soy Agar (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) and incubated at 36°C for 24–48 h; *C. albicans* and *C. auris* were plated on Sabouraud Dextrose Agar (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) and incubated at 36°C for 48 h; and *A. brasiliensis* were plated on Potato Dextrose Agar (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) and incubated at 26°C for 3–4 days. *M. abscessus* subsp. *bolletii* was grown on Middlebrook 7H10 agar at 36°C for 72 h; the medium was reconstituted in ultrapure water (Milli-Q; Merck Millipore, Burlington, MA, USA) and supplemented with OADC growth supplement (HiMedia Laboratories, Thane, India) and 2.5 mL/L glycerol (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA).

Exposure of bacteria and fungi and viable counts

For each test, 50- μ L droplets of the final microbial suspension were dispensed onto the quartz disc inside the device with a micropipette (Gilson Inc., Middleton, WI, USA); six droplets were arranged in a fixed pattern on the disc per run (Fig. 1). PBS does not absorb appreciably at 275 nm and the droplet forms a cap about a millimetre deep, so the meniscus was taken as negligible for the dose received. The device was then run for a single 150 s disinfection cycle. Following exposure, each droplet was recovered, in clockwise order, and plated onto 90-mm Petri dishes (VWR, Radnor, PA, USA) containing the designated medium. Plates were incubated (BINDER GmbH, Tuttlingen, Germany) under the conditions described above, and colonies were enumerated manually with a SCAN 100 manual colony counter (Interscience, Saint-Nom-la-Bretèche, France). Each microorganism was tested in triplicate, and non-irradiated droplets that had been handled under identical conditions served as positive controls. The antimicrobial efficacy of the samples was expressed as the $\log_{10}$ reduction in CFU/mL between the treated samples and the corresponding non-irradiated control samples.

Viruses and cell cultures

Two influenza strains were used in the study: A/Wisconsin/588/2019 (H1N1)pdm09, representative of IAV, and B/Austria/1359417/2021 (B/Victoria lineage), representative of IBV. Both viruses were propagated in Madin–Darby canine kidney (MDCK) cells (ATCC CCL-34), in accordance with the methods previously outlined by Amodeo et al. [34]. Briefly, MDCK cells were cultured in Eagle's minimum essential medium (EMEM; Lonza, Milan, Italy) supplemented with 2 mM L-glutamine (Lonza, Milan, Italy), 1% non-essential amino acid solution (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA), 100 U/mL penicillin/streptomycin (Lonza, Milan, Italy) and 10% foetal bovine serum (FBS; Euroclone S.p.A., Pero, Italy). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Viral propagations were performed in serum-free UltraMDCK medium (Lonza, Milan, Italy) supplemented with 100 U/mL penicillin/streptomycin and 1 μ g/mL TPCK-treated trypsin (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA).

HPIV3 strain C243 was propagated in VERO cells (ATCC CCL-81). VERO cells were cultured in EMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% FBS and maintained at 37°C in a humidified atmosphere with 5% CO₂. Viral propagations were carried out in EMEM containing 2% FBS and 11 μ g/mL of acetylated trypsin (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA).

hMPV strain NL/1/00 (subgroup A1) was propagated in VERO E6 cells (ATCC CRL-1586), as previously reported [37]. VERO E6 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Euroclone S.p.A., Pero, Italy) supplemented with 2mM L-glutamine, 100 U/mL penicillin/streptomycin and 10% FBS and maintained at 37°C in a humidified atmosphere with 5% CO₂. Viral propagation was performed in DMEM containing 2% FBS and 5 μ g/mL TPCK-treated trypsin at 35°C in a humidified atmosphere with 5% CO₂.

Exposure of viruses to UVC and virus titration

The viral suspensions were exposed to the test device using the same setup adopted for bacteria and fungi. For each test, six 50- μ L droplets of the viral suspension were placed on the quartz disc in the same fixed pattern used for bacteria and fungi (Fig. 1) and exposed to one complete 150 s UVC cycle. Non-irradiated suspensions were handled in the same way for use as controls. After exposure, the six droplets were divided into two mirrored sets of three and infectious titres were determined by endpoint titration on MDCK, VERO and VERO E6 cells for IAV/IBV, HPIV3 and hMPV-A1, respectively. Irradiated and control suspensions were serially diluted and used to infect pre-seeded cell monolayers in 96-well tissue culture plates (VWR, Radnor, PA, USA). Plates were incubated at 35°C for 24 h for hMPV-A1, or at 37°C for 48 h for HPIV3 and 72 h for IAV and IBV.

At the end of incubation, plates were washed twice with Dulbecco's phosphate-buffered saline 1X (DPBS; Euroclone S.p.A., Pero, Italy). Cells were fixed with 100 μ L per well of cold 80% v/v acetone (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) in DPBS 1X for 10 min at RT. Plates were then emptied, air-dried and processed for ELISA read-out. Plates were washed three times with 300 μ L per well of wash buffer, consisting of DPBS 1X containing 0.3% Tween-20 (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA), using an automatic plate washer.

Primary antibodies were added at 100 μ L per well and incubated for 1 h at RT. Anti-prefusion viral F protein DS7 antibody (Absolute Antibody Ltd., Redcar, Cleveland, UK) was used for hMPV-A1; anti-Fusion Glycoprotein of HPIV3 antibody (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) was used for HPIV3; anti-Influenza A Nucleoprotein mouse mAb (Millipore, Sigma, Burlington, MA, USA) and anti-Influenza B NP antibody (Santa Cruz Biotechnology, Dallas, TX, USA) were used for IAV and IBV, respectively. Plates were then washed three times, and Goat Anti-Mouse IgG (H + L)-HRP conjugate (Bio-Rad Laboratories, Hercules, CA, USA) was added at 100 μ L per well for 1 h at RT in the dark. Both primary and secondary antibodies were diluted in antibody diluent, consisting of 5% non-fat dried milk (AppliChem GmbH, Darmstadt, Germany) in wash buffer.

Plates were subsequently washed six times with 300 μ L per well of wash buffer, and 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) was added to each well. After 20 min of incubation at RT in the dark, the reaction was stopped by adding 100 μ L per well of 0.5 M hydrochloric acid (Fisher Scientific, Thermo Fisher Scientific, Waltham, MA, USA). Optical density was read at 450 nm using a VICTOR Nivo Multimode Plate Reader (Revvity, Waltham, MA, USA).

Viral titres were calculated using the Reed–Muench method and expressed as TCID₅₀/mL. Viral inactivation was expressed as the log₁₀ reduction in TCID₅₀/mL compared with the corresponding non-irradiated control. All experiments were performed in triplicate.

Real time RT-PCR quantification of influenza genome copies

Genome copies of the influenza viruses were quantified before and after treatment by real-time reverse-transcription PCR (RT-PCR) using a commercial multiplex assay (1copy[™] COVID-19/FluA/FluB/RSV PCR Kit, 1drop Inc., Seongnam, Republic of Korea), which targets short, conserved regions of IAV and IBV genomes detected in the FAM and VIC channels, respectively. Each 20 µL reaction contained 10 µL of master mix, 1 µL of primer–probe mix and 4 µL of nuclease-free water, to which 5 µL of extracted RNA was added. Reactions were run on a CFX96 Opus system (Bio-Rad Laboratories, Hercules, CA, USA) with reverse transcription at 55°C for 5 min, initial denaturation at 95°C for 3 min, and 40 cycles of 95°C for 5 s and 62°C for 12 s, with fluorescence acquired at 62°C against a fixed threshold of 500 relative fluorescence units. Quantification-cycle (Cq) values of paired control and treated aliquots processed in parallel were compared, and the difference (ΔCq) was expressed as a relative reduction in amplifiable copies assuming a doubling of product per cycle.

Long-range RT-PCR and next-generation sequencing for assessment of influenza genomic integrity

To determine whether the loss of infectivity induced by UVC irradiation was accompanied by genomic damage, the integrity of the influenza genome was analysed using a long-range RT-PCR approach adapted from Nakaya et al. [36]. Viral RNA was extracted from both irradiated and non-irradiated control suspensions using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). One-step reverse transcription and PCR amplification (long-range RT-PCR) were performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and virus-specific primers (synthesized by Metabion and supplied by Carlo Erba, Cornaredo, Italy). Two distinct viral targets spanning parts of the acidic polymerase (PA) gene of IAV and IBV were analysed: Wisconsin_H1N1_PA (derived from the A/Wisconsin/588/2019 (H1N1)pdm09 strain) and Austria_FluB_PA (derived from the B/Austria/1359417/2021 strain). Both targets had an expected product size ranging between 1000 and 1500 bp. The primers for Wisconsin_H1N1_PA were 5'-TTCCTTGAGGACGACACCACG-3' (forward) and 5'-CCCTCACCTCCTTCGAGAT-3' (reverse); those for Austria_FluB_PA were 5'-GGAAGGGAAAGGGAGAGTGC-3' (forward) and 5'-CCCACCTGAGTCCTTTCACC-3' (reverse).

The RT-PCR master mix was prepared in a 1.5 mL Eppendorf tube. For each reaction, the mix comprised 25 µL of 2X Reaction Mix, 1 µL of the forward primer (10 µM), 1 µL of the reverse primer (10 µM), 2 µL of the SuperScript III RT/Platinum Taq Mix, and 16 µL of nuclease-free distilled water. Subsequently, 45 µL of this master mix was dispensed into each tube of a PCR strip, followed by the addition of 5 µL of template RNA to yield a final volume of 50 µL. Thermal cycling was carried out on a CFX96 Opus Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: an initial denaturation and enzyme activation step at 50°C for 20 min, 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension 68°C for 1 min.

Standard curves were prepared using gel-purified amplicons. To resolve the PCR products, a 1% (w/v) agarose gel was prepared by dissolving 1 g of agarose (Sigma-Aldrich, St. Louis, MO, USA) in 50 mL of 1X Tris-Borate-EDTA (TBE) buffer, prepared by diluting a 10X TBE stock solution (Sigma-Aldrich, St. Louis, MO, USA) in distilled water. The mixture was brought to a boil under magnetic stirring, and 35 mL of the solution was cast into an electrophoresis tray. Once the gel had solidified, the comb was removed, and the gel was submerged in a Sub-Cell GT Mini electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA) containing the remaining 1X TBE buffer. For each sample, 5 μ L of the PCR product was mixed with 6 μ L of gel loading buffer and loaded into the wells starting from the second lane; 10 μ L of a molecular weight marker was loaded into the first lane. Electrophoresis was performed at 100 V for 70 min using a PowerPac 300 power supply (Bio-Rad Laboratories, Hercules, CA, USA). The gel was subsequently stained for 20 min in ethidium bromide, and the DNA bands were visualized on a UV Transilluminator 2000 (Bio-Rad Laboratories, Hercules, CA, USA) and photographed under a UV filter.

The next-generation sequencing (NGS) analysis was carried out using the MiSeq i100 system (Illumina, San Diego, CA, USA). The UVC-exposed and the control virus supernatant-extracted RNAs (QIAamp Viral RNA mini kit; Qiagen, Hilden, Germany) were used to prepare the library for IAV and IBV whole-genome sequencing by using the microbial amplicon prep Assay (Illumina, San Diego, CA, USA) and Influenza A/B primer pool, following the manufacturer's instructions. Influenza sequences were assembled with the DRAGEN microbial amplicon software (version 1.1.0). The software assigns each sample to the closest genome in its reference panel. Both the control and the irradiated IAV samples were assigned to A/Human/New York City/PV34639/2021 (H1N1)pdm09 (GenBank ON531356-ON531362 and ON620930). The two IBV samples were assigned to different references: the control to B/Brisbane/60/2008 (GenBank FJ766839, KC866602-KC866607 and KX058884) and the irradiated sample to B/Florida/29/2016 (GenBank KX920661 and KX920663-KX920666). The coverage of the assigned reference was compared segment by segment.

Statistical analysis

Data were organised in Stata v.17 (StataCorp LLC, College Station, TX, USA) and plotted in GraphPad Prism v.9 (GraphPad Software, La Jolla, CA, USA). For bacteria and fungi, colony counts from the eighteen droplets of each organism were aggregated and the $\log_{10}$ reduction relative to the pooled control was calculated on the combined volume, with 95% confidence intervals derived from the Poisson distribution; where no colonies were recovered after treatment, the reduction is reported as a lower bound defined by the control count and the limit of detection. For viruses, each run yielded two titres from mirrored droplet sets, and results are expressed as the mean $\log_{10}$ reduction in TCID₅₀/mL with standard deviation across the three independent runs; control titres that exceeded the quantifiable range of the endpoint titration are reported as censored values and the corresponding reductions as conservative estimates. With three independent replicates per condition, effect sizes are reported with their intervals, and no null-hypothesis significance testing was applied.

Results

A single 150 s cycle reduced viable counts below the limit of detection for every strain except *M. abscessus*, from which a small number of colonies was recovered: the vegetative bacteria, including *B. subtilis*, the yeasts *C. albicans* and *C. auris* and the mould *A. brasiliensis* were all reduced below detection. Because the treated counts were at or below the detection limit for these strains, the reductions in Fig. 3 are lower bounds set by the control count and the limit of detection rather than point estimates of survival, and their similarity across the panel (between 4.79 and 5.47 log₁₀) reflects the shared dynamic range of the assay and not a difference in susceptibility. *M. abscessus* was reduced by 5.24 log₁₀, with sporadic residual growth (one to two colonies in three of eighteen droplets) consistent with the tendency of the species to aggregate. Counts from the six droplets of each run were comparable, in agreement with the modelled irradiance distribution (Fig. 2), and were aggregated for analysis.

All four enveloped respiratory viruses lost infectious titre after a single 150 s cycle (Fig. 4), and residual titres from the two mirrored droplet sets were comparable in every run. The control titres of IAV, IBV and HPIV3 remained positive at every dilution tested and so exceeded the upper quantifiable range of the endpoint titration; they are reported as censored values, and the corresponding reductions as conservative estimates bounded by the dynamic range of the assay. Residual titres were 2.82 ± 0.25 log₁₀ TCID₅₀/mL for IAV, 1.67 ± 0.19 for IBV and 2.38 ± 0.06 for HPIV3, at or close to the floor of the titration, and for the influenza viruses this loss was corroborated at the genomic level. hMPV was the only virus whose control titre fell within the quantifiable range in every run; it started from 4.92 ± 0.62 log₁₀ TCID₅₀/mL and fell to 2.02 ± 0.45 log₁₀ TCID₅₀/mL, a reduction of 2.90 ± 0.48 log₁₀. With a starting titre near 5 log₁₀ and an assay floor at 1.50 log₁₀, the resolvable reduction for hMPV was about 3 log₁₀, so its lower value reflects the available range and not a lower susceptibility.

By real-time RT-PCR on the short diagnostic amplicon, treated samples crossed the threshold 2.70 cycles later than the paired control for IAV (Cq 18.57 against 15.87) and 3.04 cycles later for IBV (Cq 17.86 against 14.82), corresponding to reductions in amplifiable genome copies of about 0.8 and 0.9 log₁₀.

The integrity of the influenza genome following irradiation was examined by resolving the long-range PA-gene products on an agarose gel (Fig. 5). In the non-irradiated controls, both targets yielded the anticipated product of approximately 1000–1500 bp, migrating slightly above the 1000 bp reference threshold. A smear trailed below the primary band, a trend that was more pronounced for IBV. Following a 150 s exposure to UVC, neither amplicon was successfully recovered, with no band detectable in either irradiated lane.

Further examination of genome integrity was conducted using amplicon-based NGS of the same four preparations, with reads mapped to influenza reference genomes. In the non-irradiated controls, the majority of reads mapped to the reference genome (99.5% for IAV and 99.9% for IBV). The median depth of coverage across the genome was 109x for IAV and 69x for IBV, with 71% and 61% of the genome covered at 10x or greater. Irradiation altered both samples. The proportion of reads mapping to influenza decreased to 57.8% for IAV and 39.4% for IBV; the median depth decreased to 1x and 0x; and the

proportion covered at 10x or more decreased to 41% and 26%, respectively (Fig. 6). The loss was uneven along the genome. In the irradiated IAV sample, coverage remained high on the two shortest segments, M and NS, which remained close to their control breadth at approximately 97%. In contrast, every segment exceeding 1.5 kb dropped to a median depth of at most one read, and the NA segment was not recovered (Fig. 6a). In the irradiated IBV sample, the loss affected every segment, including the short ones. M, NS and PB2 were not recovered and no segment maintained a median depth above one read (Fig. 6b). IBV showed a greater reduction than IAV in terms of mapping rate, breadth, median depth and the number of segments recovered.

Discussion

For a shared instrument such as the stethoscope, the value of an automatic UVC-LED cycle rests on two conditions that this study examined: that inactivation extends across the organisms deposited on the diaphragm, including structurally protected and drug-resistant species that chemical disinfection handles poorly, and that the reduction in recoverable microorganisms reflects genuine inactivation rather than a fall below the infectivity threshold. A single 150 s cycle reduced viable counts below the limit of detection for all nine bacterial and fungal strains and lowered the infectious titre of the four enveloped respiratory viruses. For the influenza viruses, the reduction was resolved at the level of the genome by real-time and long-range RT-PCR and by amplicon sequencing, which separated genuine inactivation from the persistence of amplifiable but non-infectious nucleic acid. Because UVC acts through the photochemical modification of nucleic acids and proceeds without operator intervention once initiated, these results are interpreted below in terms of the organisms inactivated and the molecular signature of that inactivation.

The reductions were of similar magnitude across the panel, at or below the detection limit set by the inoculum, so the reported values are conservative lower bounds and the differences among organisms reflect the starting count of each and not a difference in susceptibility. Read against the fluence–response behaviour at 275 nm, the panel spans the susceptibility range, from vegetative Gram-negative bacteria inactivated at the lowest fluences [38] to two markedly more resistant members: the pigmented conidia of *A. brasiliensis*, the reference organism in UV-C bio-validation [25] and resistant enough to need several hundred mJ/cm² for a high-log reduction at 280 nm [24], whose aggregation can further shield part of the population [39], and the mycolic-acid-rich *M. abscessus* [40]. Both were reduced below detection, which places the fluence delivered at the droplet positions well above the peripheral value and within the range these organisms require, consistent with the higher irradiance modelled at the central positions where the bacterial and fungal droplets were placed (Fig. 2). A 280 nm UVC-LED device has previously inactivated *E. coli*, *S. aureus*, a mycobacterium and spore-forming bacteria on surfaces [41], and the present panel extends that range to a rapidly growing NTM and a multidrug-resistant yeast. Against the earlier point-of-care prototype, which reduced four species by about 1 log₁₀ over a 60-second cycle [19], the present configuration covers a wider panel at a longer cycle that still fits the interval during which a stethoscope is idle.

Two organisms merit specific comment, since point-of-care UVC data for them are scarce. *M. abscessus*, among the least UV-susceptible pathogens of clinical concern and more resistant than *P. aeruginosa* and *L. pneumophila* [40], was reduced by more than 5 log₁₀; any residual colonies are consistent with the hydrophobic, mycolic-acid-rich envelope and with the tendency of the species to aggregate, which shields a fraction of cells from the incident fluence. For *C. auris*, UVC operates through a physical mechanism that exerts no selective pressure for resistance, an advantage against a species already tolerant of several disinfectants in routine use; inactivation of *C. auris* by a UVC source has been reported previously [27]. Susceptibility is clade-dependent and lower in aggregating isolates, and the germicidal action spectrum peaks near 267 to 270 nm, close to the 275 nm emission used here [42]. Testing was confined to a single clade, which bounds the generalisation that can be drawn.

All four enveloped respiratory viruses lost infectious titre after a single cycle, consistent with the susceptibility of single-stranded RNA genomes to direct UVC absorption [43–45] and with previous reports of influenza inactivation by UV-LED sources [35]. Each virus was assessed against its own control, and the absolute reductions are not expected to coincide, since UVC susceptibility scales with the size of the target genome and with its pyrimidine-dinucleotide content [43, 46]. The control titres of IAV, IBV and HPIV3 lay beyond the upper quantifiable range of the titration assay, so their reductions are reported as conservative estimates bounded by the dynamic range; the concurrent genome-level damage documented for the influenza viruses confirms that the loss of titre reflects true inactivation. For hMPV, the only virus with a control titre within the quantifiable range, the resolvable reduction was limited to about 3 log₁₀ by the starting titre, which does not indicate lower susceptibility. For LED sources the primary determinant of inactivation is genomic damage, with the structural proteins largely spared, and enveloped RNA viruses are more susceptible than non-enveloped ones [44]. The effect observed here is therefore referable to the viral genome and not to the lipid envelope that many chemical disinfectants target. Inactivation of an enveloped respiratory virus at 405 nm, by contrast, is mediated by endogenous photosensitisers and reactive oxygen species [34]. Non-enveloped viruses, which were not tested, are generally more UVC-resistant [43].

The pattern of coverage loss across the influenza genome is consistent with UVC lesions accumulating in proportion to the length of the target sequence. At 275 nm, absorption by adjacent pyrimidines generates cyclobutane pyrimidine dimers, (6–4) photoproducts and, in RNA, pyrimidine hydrates [45], and the probability that a region carries at least one lesion increases with the number of dipyrimidine sites it contains; for RNA viruses the inactivation rate is approximately proportional to genome length, with a single lesion within an amplified region sufficient to prevent its recovery [46]. Three observations follow this expectation. The long-range product of the PA gene did not amplify after irradiation (Fig. 5); in the irradiated IAV sample every segment longer than 1.5 kb fell to a median read depth of at most one read, while the two shortest segments, M and NS, retained a breadth of coverage close to the control (Fig. 6); and the reference mapping rate fell without reaching zero, from 99.5% to 57.8% for IAV and from 99.9% to 39.4% for IBV, as expected when short fragments remain amplifiable. Two features are accounted for by the amplicon architecture alongside genome length. The incomplete recovery of the IAV NA segment reflects the recognised heterogeneity of per-segment coverage in multi-segment

influenza sequencing, in which the longer amplicons are amplified less efficiently and the baseline depth of individual segments varies appreciably [47], so a segment with lower starting coverage falls below the reporting threshold at a lower level of damage. The more extensive loss in IBV, which affected all segments, is concordant with its lower starting median depth relative to IAV and with the dependence of photoproduct yield on pyrimidine-dinucleotide composition, which differs between the two genomes [46]; the latter is quantifiable *in silico* on the reference sequences. Amplicon sequencing reports the amplifiability of each region and not genome integrity as such, so coverage loss is an indirect measure of damage, and the agreement between the long-range product and the segment-resolved coverage provides the stronger evidence [48].

The molecular readouts obtained on the same samples differ in the length of the region they interrogate, and together they show why the choice of target governs the apparent efficacy of a nucleic-acid assay. On the short diagnostic amplicon, real-time RT-PCR recorded a reduction of less than $1 \log_{10}$ (ΔCq 2.70 for IAV, 3.04 for IBV), because a target of 70 to 150 bp has a low probability of carrying a lesion at this fluence. The long-range product on the PA gene was abolished, amplicon sequencing lost coverage first on the longer segments, and the infectious titre, which requires an intact and translatable genome, showed the largest reduction. This ordering is the expected consequence of lesions accumulating in proportion to target length [46], and it quantifies, within a single set of samples, the extent to which a short-amplicon assay underestimates inactivation. Diagnostic and environmental assays typically amplify targets of this short length, so they can continue to detect nucleic acid that no longer corresponds to infectious virus and overestimate the residual load [49]; a long-range assay [36] offers an intermediate between cell-culture titration and short-target PCR.

The operational rationale for an operator-independent method follows from the gap between recommended and observed practice: disinfection consistent with guidance is performed in fewer than 4% of encounters, and educational or reminder-based interventions produce limited and inconsistent improvement [7, 10, 11]. A 150 s cycle is compatible with the interval during which the instrument is idle between patients, and the absence of a consumable removes the dependence on the point-of-care availability of wipes, a recognised contributor to omitted disinfection [8, 9, 19]. The result belongs to the emission and to the delivered dose, not to the unit that houses them. These are *in-vitro* findings, and their operational value requires confirmation in a clinical field study with sampling of the diaphragm before and after use.

Several constraints frame the interpretation. The reductions for the culturable organisms are bounded by the inoculum and the detection limit, so the values reported are conservative and the true efficacy is likely to exceed the demonstrable range. Exposures were performed in PBS on an optically favourable quartz carrier under clean conditions; testing under the interfering protein load defined by the surface-disinfectant standards, and on a matrix reproducing the working membrane and the geometry of the chest-piece rim, is the appropriate next tier and is not expected to alter the mechanistic conclusions. A single strain was examined per species, and a single clade for *C. auris*, in keeping with a proof-of-concept evaluation. Genomic damage was characterised for the influenza viruses through the

amplifiability of the genome, an established indirect measure that could be complemented by direct integrity assays. Three independent runs per condition are adequate to estimate the magnitude of the reductions, with Poisson-based intervals, though not to support formal hypothesis testing.

In conclusion, a single automatic UVC-LED cycle inactivated a microbiologically diverse panel and reduced the infectious titre of four enveloped respiratory viruses. Its practical interest lies less in the reduction itself, which chemical disinfectants also achieve, than in the fact that the cycle runs to completion without depending on the operator, the point at which stethoscope disinfection most often fails. The breadth of the panel, which included a rapidly growing mycobacterium, a multidrug-resistant yeast and the UV-resistant conidia of a mould, indicates that the delivered fluence covered organisms well beyond the vegetative bacteria that dominate routine contamination. For the influenza viruses the loss of infectivity corresponded to length-dependent damage of the genome and not to residual amplifiable nucleic acid, so the inactivation is genuine rather than a matter of lost culturability. The same length dependence carries a methodological consequence: a short diagnostic amplicon survives the damage that abolishes infectivity, and assays of that kind will therefore overestimate the residual contamination of a treated surface, a point worth keeping in mind whenever molecular methods are used to monitor UVC disinfection. Evaluation under soiled conditions and in clinical use remains the necessary step toward routine application.

Declarations

Author Contributions: D.A.: conceptualization, methodology, investigation, formal analysis, software, visualization, writing – original draft. A.D.C., G.N., D.M.C., C.F.: investigation, data curation, writing – review and editing. A.M.: methodology, investigation, writing – review and editing. E.M.: resources, supervision, funding acquisition, writing – review and editing. I.M.: conceptualization, methodology, formal analysis, writing – review and editing. S.M.: conceptualization, methodology, investigation, formal analysis, writing – review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive repository, under BioProject accession PRJNA1507286 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1507286>), which comprises four sequencing runs:

SRR39998434, SRR39998435, SRR39998436 and SRR39998437. The reads deposited are those retained by the DRAGEN Microbial Amplicon pipeline after quality filtering, primer trimming and duplicate removal. All other datasets generated or analysed during this study are included in this published article and its Supplementary Information file.

Conflicts of Interest: A.M. is employed by VisMederi Srl. E.M. has shares of VisMederi Srl. The other authors have no relevant financial or non-financial interests to disclose.

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Figures

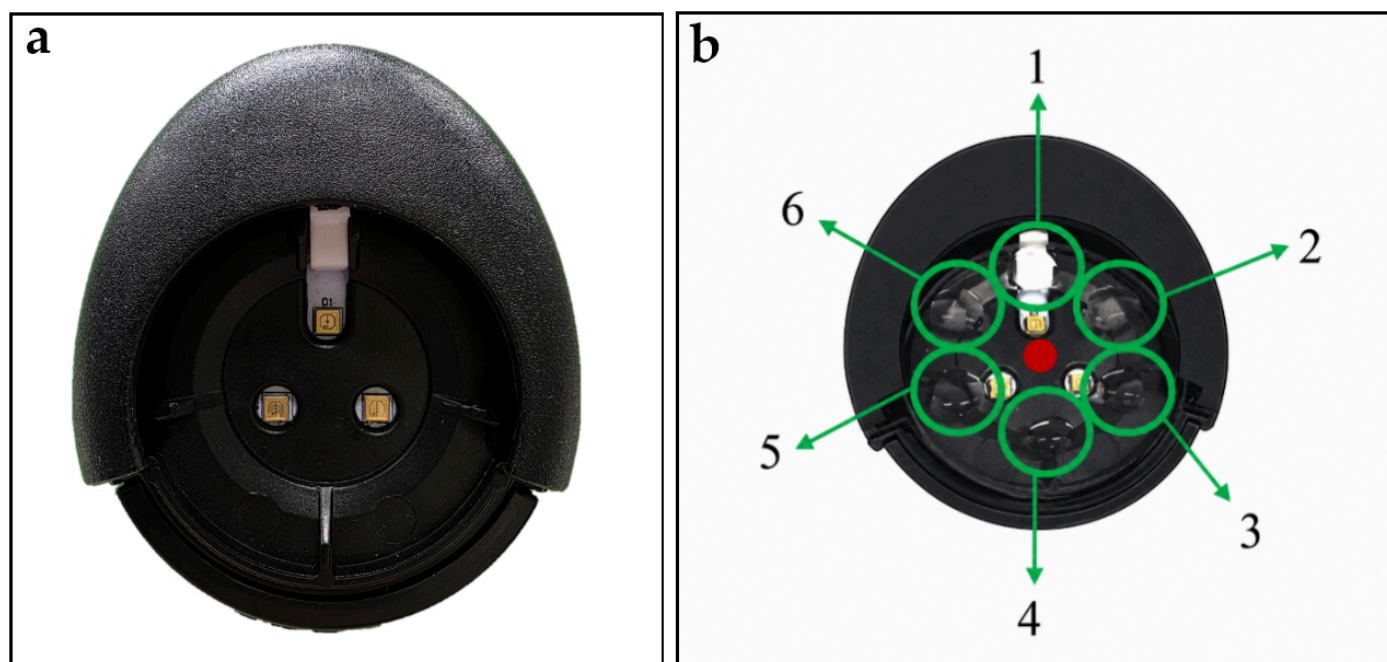


Figure 1

Experimental configuration of the UVC-LED disinfection device and inoculum droplet positioning.

(a) This top view shows the test device equipped with three fixed-emission UVC LEDs (peak wavelength 275 nm), which are arranged to irradiate the internal treatment chamber. (b) Six 50 μ L droplets of bacterial or viral suspension were deposited on a fused-silica quartz disc inside the device, corresponding to the area normally occupied by a stethoscope diaphragm. The radial arrangement of the droplets enables the antimicrobial efficacy to be evaluated under different irradiation positions, including lateral points located approximately 2 cm from the central emission zone. The red dot represents the measurement point in the central emission zone of the device.

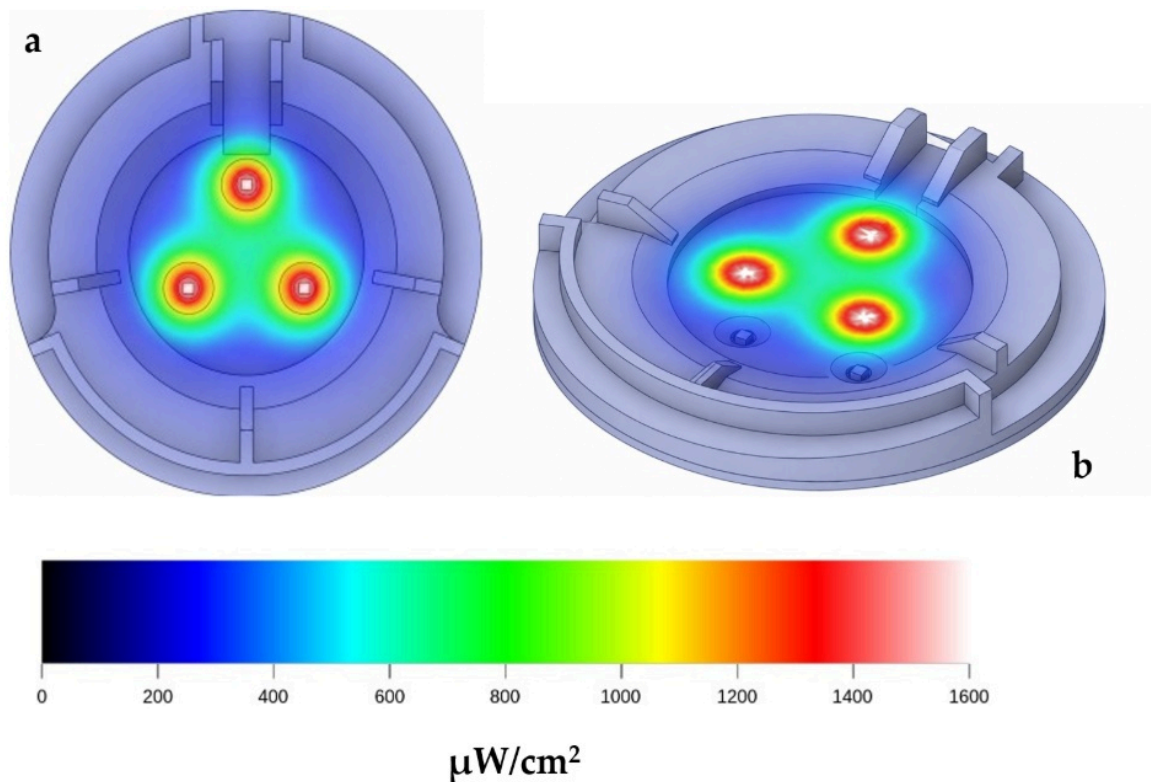


Figure 2

Optical simulation of the irradiance distribution generated by three UVC LEDs inside the device chamber. Photometric simulation was performed using Ansys Speos 2024 to model the spatial irradiance distribution produced by the three fixed-emission, 275 nm UVC LEDs. Panel (a) shows the two-dimensional irradiance map projected onto the quartz exposure plane. Panel (b) illustrates the three-dimensional propagation of optical energy within the internal chamber geometry. The colour scale represents relative irradiance intensity, demonstrating the overlap of the three emission fields and the homogeneous exposure region that corresponds to the positions selected for inoculum deposition during experimental testing.

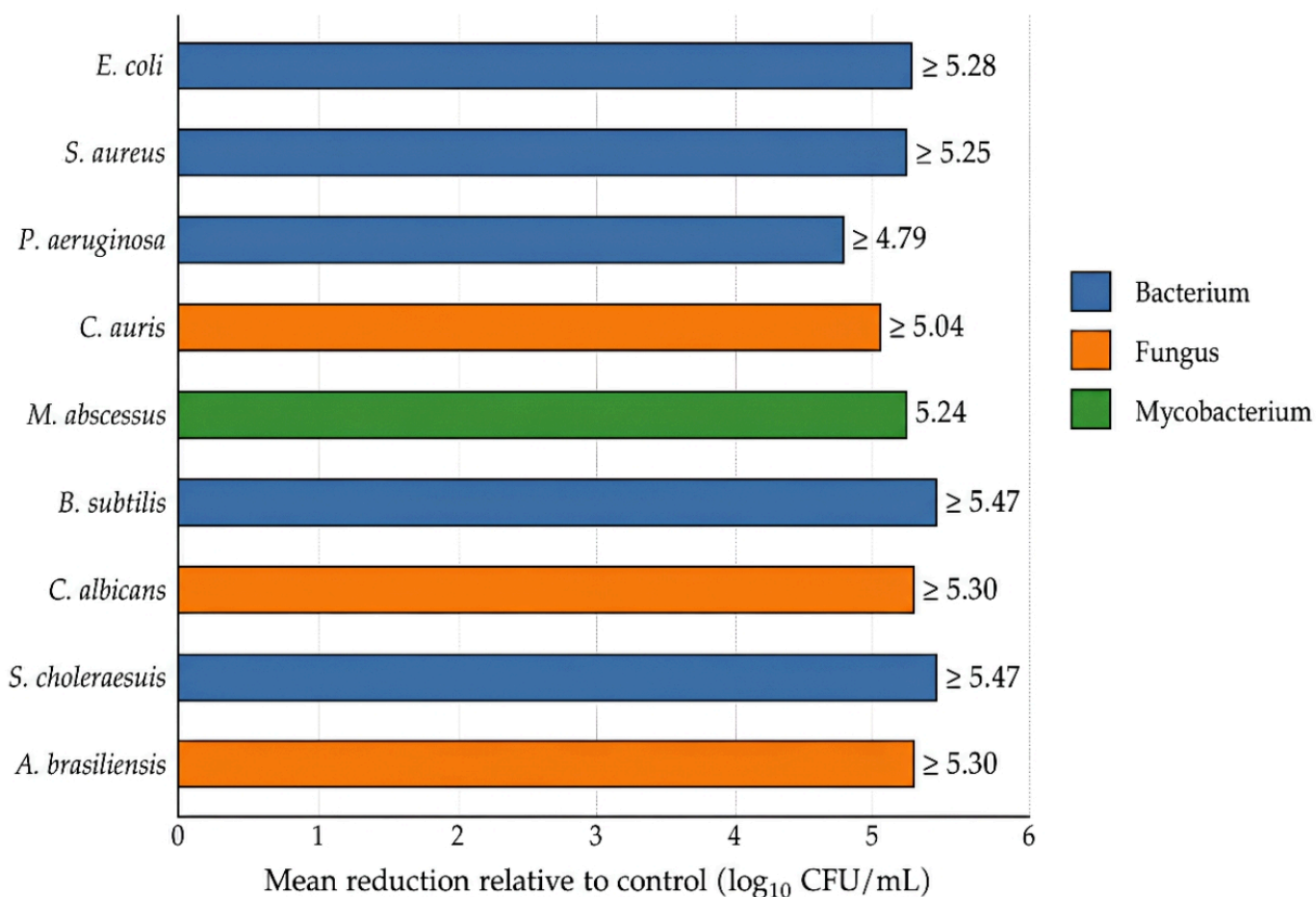


Figure 3

Antimicrobial efficacy of the test device against bacterial and fungal strains. Mean $\log_{10}$ reduction in viable count (CFU/mL) for each strain after a single 150 s UVC-LED cycle, relative to the paired non-irradiated control. Values marked $\geq$ are lower bounds: no colonies were recovered after treatment, so the reduction is set by the control count and the limit of detection. *M. abscessus* is a point estimate, colonies having been recovered. Colours denote the microbial group.

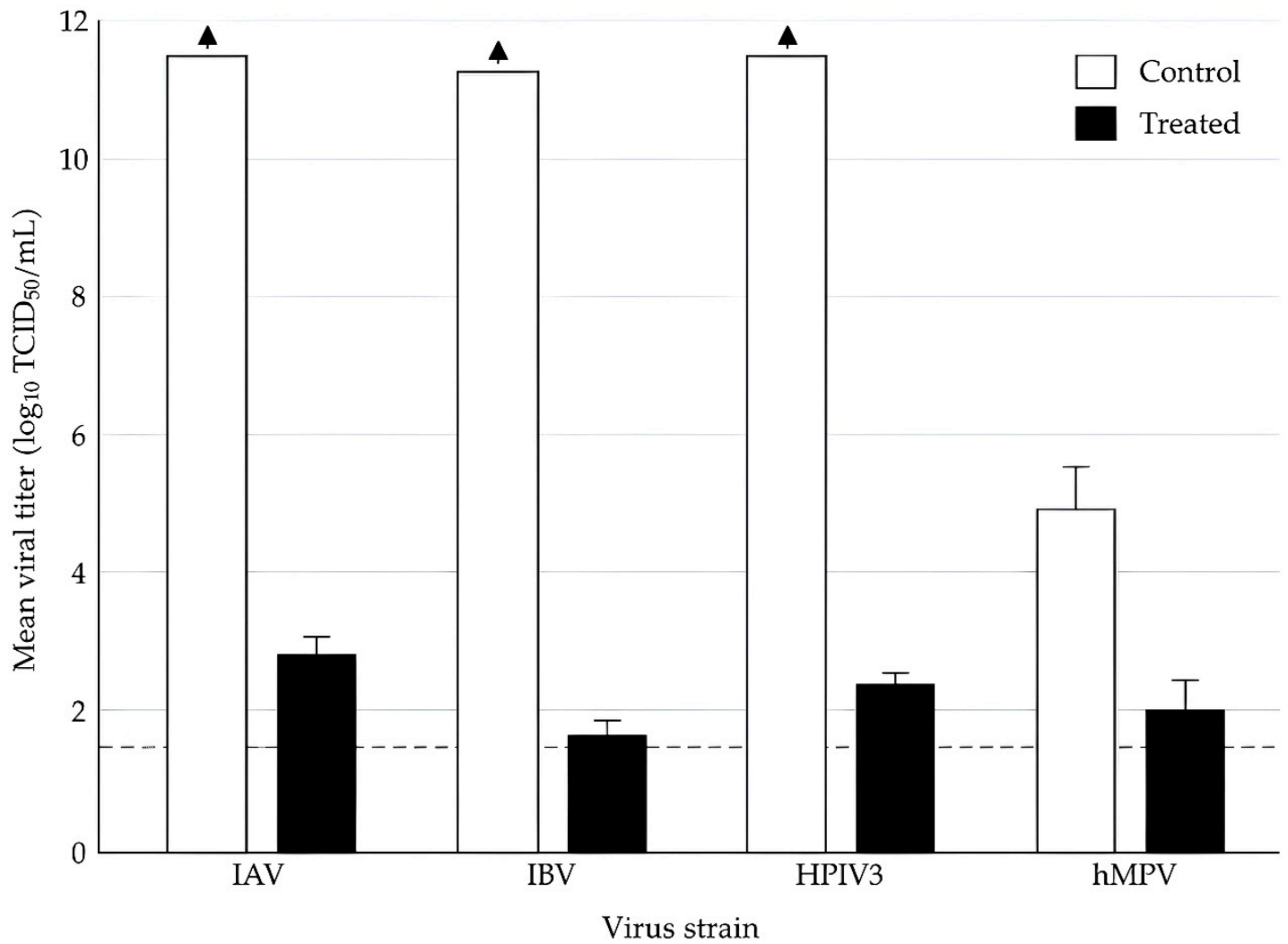


Figure 4

Virucidal efficacy of the UVC device against IAV, IBV, HPIV3 and hMPV. Mean infectious titre (log₁₀ TCID₅₀/mL) of the four respiratory viruses before (Control) and after (Treated) a single 150-second UVC-LED cycle. Bars are the mean of three independent runs; error bars show the standard deviation of the treated samples and hMPV virus control. The control titres of IAV, IBV and HPIV3 exceeded the upper quantifiable range of the endpoint titration and are marked as censored (↑). The dashed line marks the assay detection floor (1.50 log₁₀ TCID₅₀/mL).

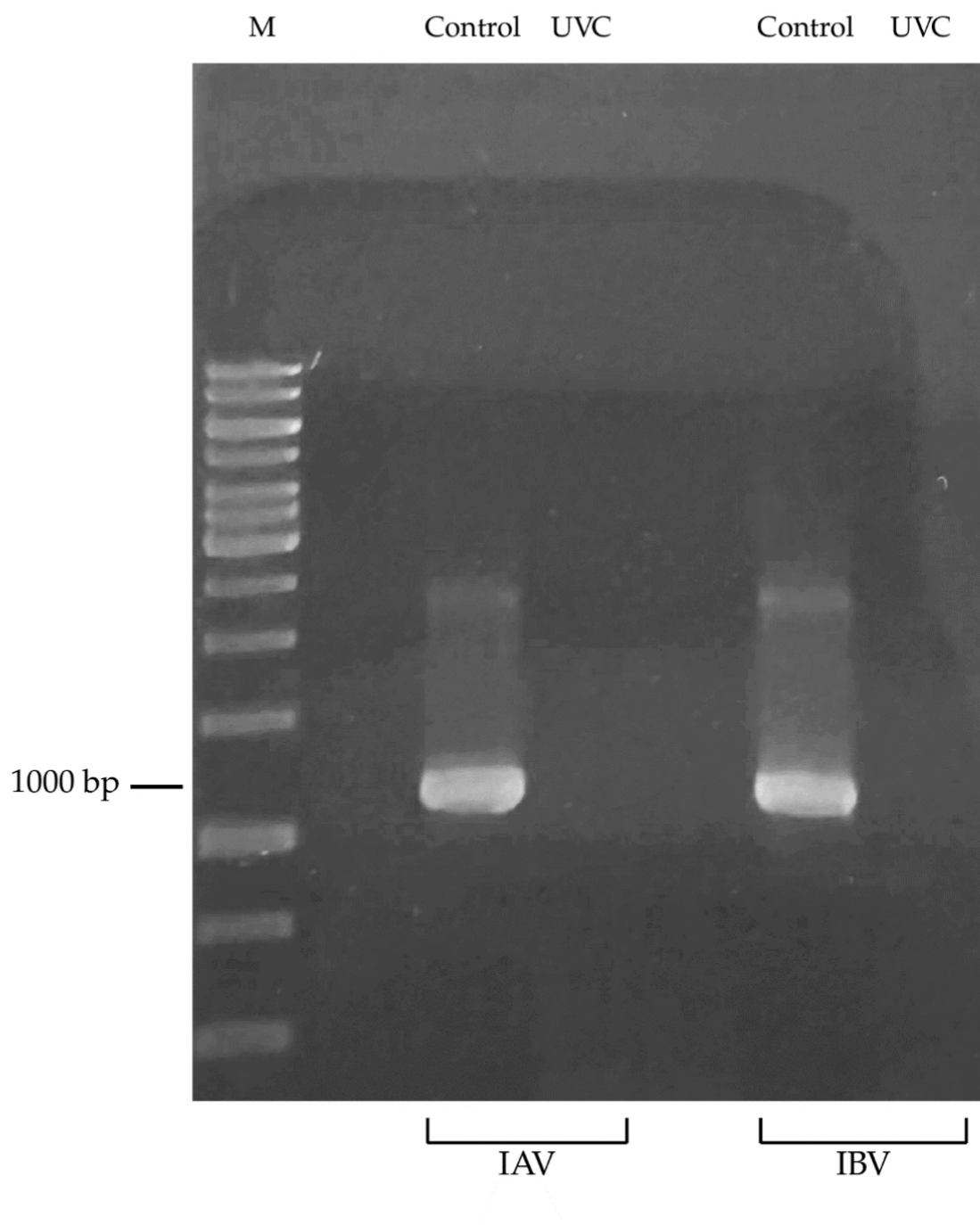


Figure 5

Agarose gel electrophoresis of the long-range RT-PCR products spanning the PA gene of IAV and IBV. Non-irradiated (Control) and UVC-exposed (UVC) samples; M, molecular size marker; the 1000 bp position is indicated. Expected product size 1000–1500 bp.

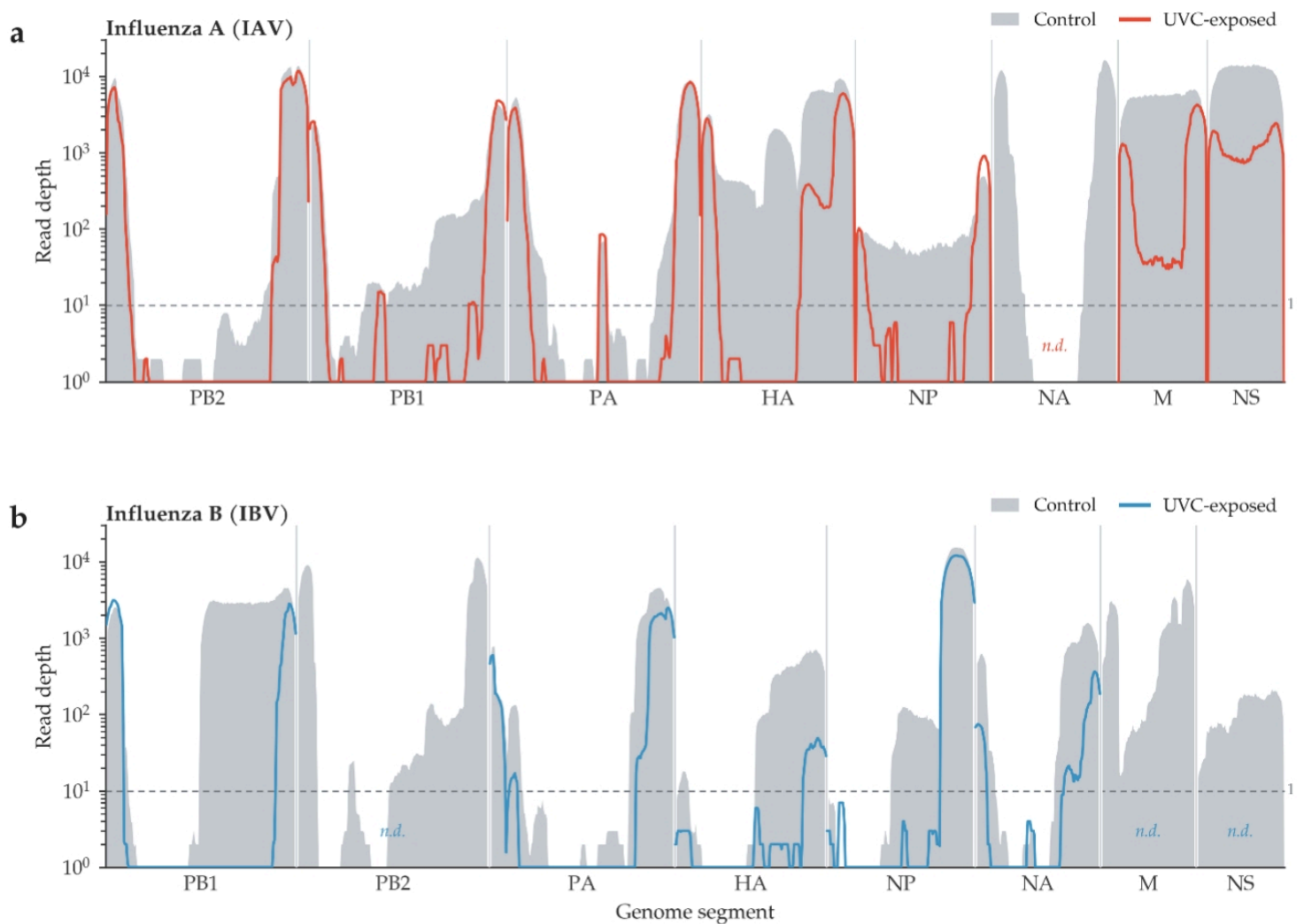


Figure 6

Read depth along the influenza genome before and after UVC. (a, b) Depth on a log scale across the eight genome segments of IAV (a) and IBV (b); the grey area is the non-irradiated control, and the coloured line is the UVC-exposed sample. Segments are labelled by gene and separated by vertical lines, and the dashed line marks the 10x depth used to define breadth of coverage. Segments not recovered after irradiation are marked n.d. Reads were mapped to the genome selected by DRAGEN from its reference panel: A/Human/New York City/PV34639/2021 (H1N1)pdm09 for both the IAV control and for

the irradiated IAV sample, B/Florida/29/2016 (Victoria lineage) for the IBV control and for the irradiated IBV sample. Segments are aligned by gene.

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