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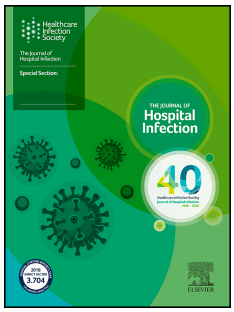

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An automated room disinfection system using ozone is highly active against surrogates for SARS-CoV-2

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Summary

Background: The presence of coronaviruses on surfaces in the patient environment is a potential source of indirect transmission. Manual cleaning and disinfection measures do not always achieve sufficient removal of surface contamination. This increases the importance of automated solutions in the context of final disinfection of rooms in the hospital setting. Ozone is a highly effective disinfectant which, combined with high humidity, is an effective agent against respiratory viruses. Current devices allow continuous nebulization for high room humidity as well as ozone production without any consumables.

Aim: In the following study, the effectiveness of a fully automatic room decontamination system based on ozone was tested against bacteriophage *Φ6* (phi 6) and bovine coronavirus L9, as surrogate viruses for the pandemic coronavirus SARS-CoV-2.

Methods: For this purpose, various surfaces (ceramic tile, stainless steel surface and furniture board) were soiled with the surrogate viruses and placed at two different levels in a gas-tight test room. After using the automatic decontamination device according to the manufacturer's instructions, the surrogate viruses were recovered from the surfaces and examined by quantitative cultures. Then, reduction factors were calculated.

Findings: The ozone-based room decontamination device achieved virucidal efficacy (reduction factor >4 log10) against both surrogate organisms regardless of the different surfaces and positions confirming a high activity under the used conditions.

Conclusion: Ozone is highly active against SARS-CoV-2 surrogate organisms. Further investigations are necessary for a safe application and efficacy in practice as well as integration into routine processes.

Keywords: SARS-CoV-2, bovine Coronavirus, bacteriophage Phi 6, surrogate virus, automated room disinfection, ozone,

Introduction

The spread of viruses with pandemic potential due to indirect contact transmission is controversial discussed. Even in the current pandemic situation of Covid-19 disease, the persistence of SARS-CoV-2 on inanimate surfaces and the role of contaminated surfaces as transmission pathway is not clear. A current study showed a stability of SARS-CoV-2 on different surface material (copper, cardboard, stainless steel and plastic) for 8 to 72 hours under experimental conditions [1]. Therefore, touching contaminated surfaces might be a potential source of viral transmission [2]. Recent studies conducted in China and Hong Kong during the SARS-CoV-2- pandemic showed viral RNA in the patient environment [3,4]. It therefore seems rational to reduce the microbial load by disinfection. This assumption was supported by investigations, which revealed contamination with viral RNA on surfaces even after final cleaning and disinfection of a patient room [5,6]. In addition, several studies demonstrated that environmental cleaning in hospitals is frequently lacking. It was shown, that less than 50% [7] respectively averagely 57% [8] of surfaces were cleaned adequately following patients discharge.

To improve this problem and prevent environmental-borne transmission, the usage of automated room disinfection systems could be an additional method of disinfection in hospital settings [5]. Currently aerosolized and vapored hydrogen peroxide, ozone, chlorine dioxide and ultraviolet radiation are mechanisms, which were used for room decontamination after the discharge of patients [9,10].

Ozone is not a common reagent, because of the need of permanent moisture to achieve effectiveness [11]. Consequently, only a few studies reported using ozone for room decontamination in general but not yet in the hospital setting [10,12,13]. In a current study, Dubuis *et al* showed that ozone combined with high relative humidity is an effective disinfectant for respiratory viruses [14]. Because of recent technologies, which enable generating ozone from atmospheric oxygen in combination with an integrated nebulizer for controlled increase of room humidity, the aim of this study is to evaluate the effectiveness of an automatic room disinfection unit based on ozone combined with high relative humidity against SARS-CoV-2 surrogates.

As a consequence of biosafety concerns and high demands for working with SARS-CoV-2, surrogate viruses were used in this study. Bacteriophages are known as suitable surrogates for human respiratory viruses owing to great similarities in size, shape, surface properties

and environmental persistence, however they are non-pathogenic to humans [15]. Due to his lipid envelope, bacteriophage *Φ6* (phi 6) from the family of the *Cystoviridae* has been suggested as a surrogate for coronaviruses [16–19].

Coronaviruses form a large and pleomorphic family that is further divided into groups based on serological findings and phylogenetic analysis [20–22]. The bovine coronavirus (BCoV) from the genus *Betacoronavirus* is genetically closely related to SARS-CoV, MERS-CoV and the pandemic SARS-CoV-2 viruses and can be handled outside a BSL-3 safety laboratory. Therefore, we used the BCoV and *Φ6* as surrogate organisms for the present experiments.

Methods

To evaluate the efficacy of an ozone based device for automated room disinfection (STERISAFE™ Pro version 1.0, STERISAFE ApS, Ole Maaløe's vej 5, DK – 2200 Copenhagen), carriers contaminated with two different surrogate viruses of SARS-CoV-2 were decontaminated in a 6 m³ gas-tight test room furnished with a shelf.

Surrogate virus bacteriophage *Φ6* (DSM 21518) and the bacterial host strain *Pseudomonas syringae* pv. *Syringae* (DSM 21482) were purchased from Leibniz-Institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Initial lysate of bacteriophage *Φ6* with a titer of 4×10^{11} plaque forming units (pfu)/mL was produced using a top agar overlay technique as described by the manufacturer. Then, 20µL of a 1:10 dilution was striked out and dried on ceramic tiles (5x5 cm, #3709PN00, Villeroy&Boch, Mettlach, Germany), stainless steel carriers (#0344818, Modulor GmbH, Berlin) and furniture boards (melamine-coated solid core panels). After each experiment *Φ6* from both, treated and untreated carriers, were recovered by rinsing the surface with 1mL Tryptic Soy Broth (TSB)+ 5mM $CaCl_2$ medium for 15 times. A quantitative plaque assay was performed using top agar overlay with Tryptic Soy Agar (TSA) + 5 mM $CaCl_2$ culture media after tenfold serial dilution (detection limit: <10 pfu/mL). Plates were incubated at 23℃ for 24 h.

In the same way further carriers were contaminated with 50µL virus inoculum of bovine coronavirus strain L9 (BCoV). BCoV strain L9 and the host *U373 cells* (passage 8) were obtained by G. Zimmer, Institute of Virology, School of Veterinary Medicine, Hannover, Germany. For preparation of test virus solution, a monolayer of *U373 cells* were infected with BCoV L9. After an incubation period of 24 to 48 hours' cells were lysed by a rapid freeze/thaw cycle. Cellular debris was removed and the supernatant was mixed with bovine serum albumin (BSA) (final concentration: 0.3 g/L BSA). After each experiment an endpoint dilution assay was performed. Therefore, the treated and untreated carriers were rinsed with 1 mL medium without fetal calf serum (FCS). Remaining infectivity was determined by transferring 0.1 mL of appropriate tenfold serial dilutions into eight wells of a microtitre plate with a preformed monolayer of *U373* cells (10-15 x 10^3 cells per well), beginning with the highest dilution. Before addition of virus, cells were washed twice with Eagle`s minimum essential medium (EMEM) and incubated for 3 h with 100µL EMEM with trypsin. Microtitre plates were incubated at 37 ℃ in a 5 % CO_2-atmosphere. The cytopathic effect was read by using an inverted microscope after five days and the infective dose $TCID_{50}$/mL was calculated.

For the decontamination experiments contaminated carriers were placed horizontally at two different heights on the shelf to represent the efficacy at high and low room levels. Three prepared carriers of each material and surrogate virus were positioned at the high (1.69 m) and two at the low (0.07 m) position. For both surrogate organisms in each experiment two contaminated control carriers were placed in a room without treatment. For bacteriophage *Φ6* additional control experiments at 90% relative humidity (RH) and 22 ℃ were performed in a climate chamber.

The disinfection process using the STERISAFE^{TM} Pro system was investigated in two independent experiments for each organism. According to manufacturer's instructions, the decontamination time was 60 minutes with a target ozone concentration of 80 ppm and a target RH of 90% generated with the integrated humidifier and ozone generator [23,24]. Ozone concentration and relative humidity were continuously measured by integrated instruments and displayed on a mobile tablet computer outside of the room, as well as recorded in the instrument (supplementary figure S1) [24]. After completion of the disinfection process, the ozone is converted back into pure oxygen (fig. S1) and by-products are removed in an air purification phase. When the process is displayed as finished on the tablet computer, the room can be entered again immediately [24]. The ozone concentration in the treated room then complies to usual limit values of 0.1 ppm (exposure limit for 8 hours per day doing light work) set by Occupational Safety and Health Administration (OSHA) or The National Institute for Occupational Safety and Health (NIOSH) [25]. Both surrogate viruses were investigated together in two independent experiments and reduction factors were calculated by subtracting log10 of untreated and treated samples. As defined elsewhere, virucidal efficacy was suggested if the mean reduction factor is >4log10 [26].

Results

The aim of the present study was to evaluate the virus-inactivating properties of ozone in the presence of high relative humidity against surrogate bovine coronavirus (BCoV) and bacteriophage *Φ6* in a setting of room disinfection. Initial desiccation of bacteriophage *Φ6* resulted in mean concentrations of 1.4×10^7, 3.2×10^7 and 4.5×10^5 plaque forming units (pfu)/mL on ceramic tiles, stainless steel and furniture board, respectively. Initial desiccation of BCoV resulted in mean concentrations of 2.5×10^5, 4.0×10^5, and 6.4×10^5 $TCID_{50}$/mL on ceramic tiles, stainless steel and furniture board, respectively. The stability of both surrogate organisms in the desiccation phase allowed further investigations to determine virucidal activity.

After the decontamination process with STERISAFE™ Pro, independent of the carrier material used or the room height, no plaque forming units of bacteriophage *Φ6* could be recovered from the surfaces (fig.1A). The STERISAFE™ Pro achieved mean log10 reduction factors of 6.15 on ceramic tiles, 4.29 on furniture board and 5.31 on stainless steel surfaces for the surrogate virus bacteriophage *Φ6* (fig. 1C). Control experiments with high humidity without additional ozone as disinfectant revealed a minor decrease of viral activity (supplementary fig S2), indicating that the observed virucidal activity can only be reached by a combination of ozone and humidity.

For BCoV, post ozone application no residual virus could be detected independent of the carrier material used or the position in the room (corresponding to 3.16 $TCID_{50}$/mL) (fig. 1B). For the bovine coronavirus, mean log10 reduction factors of 4.88 on ceramic tiles, 5.03 on furniture board and 5.31 on stainless steel surfaces could be determined (fig. 1C). STERISAFE™ Pro showed virucidal efficacy (reduction factor >4log10) for both surrogate organisms on all investigated surfaces.

Discussion

Previous studies have shown the distribution and transmission of nosocomial pathogens due to surface contamination [11,27]. A common reason seems to be inadequate manual cleaning and disinfection, which fail to remove surface bioburden [9,11,27]. To improve the effectiveness of surfaces disinfection and to increase patient and occupational safety, automated room disinfection systems could be a useful method. Based on previous studies showing the efficacy of ozone against respiratory viruses, the aim of the present study was to test the efficacy of an ozone-based automatic room decontamination device against surrogate viruses of the pandemic coronavirus SARS-CoV-2 [14].

The present results indicate a virucidal effectiveness (reduction factor > 4 log10) of ozone in combination with high relative humidity for both tested surrogate viruses (bacteriophage *Φ6* and BCoV), independent from the surface material. The virucidal effect could be detected at different levels in the test room. Therefore, a distribution of ozone and humidity can be assumed as sufficient for successful decontamination. Interestingly, on the furniture board, for bacteriophage *Φ6,* the calculated extent of the reduction was lower than on the other materials tested. Differences in the reduction of bacteriophage *Φ6* mainly are due to reduced recovery of phages after initial contamination of control surfaces, which probably results from random fluctuation or specific surface conditions.

Recent studies have already shown that surface stability and survival time of SARS-CoV-2 was influenced by environmental conditions in particular temperature and relative humidity [28–30]. Higher humidity and temperature decrease virus survival time on surfaces [28]. However, for bacteriophage *Φ6* we observed only a low decrease of viral activity under humid conditions without the application of ozone. Therefore, it can be assumed that only the combination of ozone with high relative humidity achieves full virucidal efficacy.

Since bacteriophage *Φ6* is a small enveloped virus it shares similarities with coronavirus. However, it is considered to be more stable than coronavirus because it has a double stranded RNA genome [31]. In contrast, the BCoV belongs to the same family (*Coronaviridae*) and the same genus *Betacoronavirus* and subgenus *Sarbecovirus* as SARS-CoV-2. Both viruses are likely to have similar properties and can be considered as surrogate viruses for SARS-CoV-2. Therefore, it is assumed, that ozone is also effective against SARS-CoV-2.

This assumption is also supported by current literature reviews and initial results from laboratory experiments that were able to show an efficacy of ozone against SARS-CoV-2 [32–34].

The tested ozone room disinfection system represents a safe and useful additional disinfection method that can be implemented after the discharge of patients infected with contagious and environmentally resistant pathogens such as SARS-CoV-2. However, due to toxicity of ozone, doors, ventilation diffusers must be strictly sealed to prevent unintentional dissemination [24], resulting in an additional work load for the operating person. Additionally, due to the generated water aerosol smoke detectors must also be covered to avoid unwanted alarms. During the disinfection cycle a concept is needed, to prevent unauthorized room entrance during disinfection process.

Our study has several limitations, which should be noted. In this study only clean conditions were used for the experiments on solid surfaces. It has been demonstrated that organic loading could have an inhibitory effect on the efficacy of disinfection methods [35,36]. Further experiments using test soiling for dirty conditions (Bovine albumin 3.0 g/L + sheep erythrocytes 3 mL/L [26]) as well as experiments with absorbent items have to be done, to evaluate the virucidal effect for applications were insufficient cleaning prior the disinfection process is expected. Secondly, it must be taken into account that the experiments were conducted in a small room with a simple room structure and only a few furnishings. However, in a recent study, effectiveness against environmental resistant *Enterococcus faecium* was analyzed within complex room conditions. A position-independent bactericidal effectiveness could be shown, confirming a sufficient distribution of ozone and humidity even in a furnished room with anteroom and bathroom [37]. Furthermore, in order to achieve conditions that are as close to reality as possible, we did not use standardized but realistic room conditions for the untreated control panels that prevailed at the time of the test. Spontaneous reductions that could be caused by temperature and humidity fluctuations will therefore not be excluded and assessed. Finally, before the general implementation of such an ozone generating device can be recommended, further studies are needed to ensure the safe operation in the hospital environment. The oxidizing properties of ozone can lead to damage of many materials and thus to a shortening of the life cycle of products [38]. Elastomers and surface coatings in particular can be damaged [34]. The compatibility of ozone in connection with electronic medical devices should be clarified with the manufacturers, as is the case for all airborne disinfection processes. Due to this fact, further experiments are necessary to ensure compatibility with common furnishing and medical device materials in hospitals [11,39] To verify safety opera-

tion and efficacy, logging of process data independent from disinfection device should be recommended for practical application.

Conclusion

In summary, we found that ozone in combination with high humidity as generated by an automated room decontamination system has a high activity against SARS-CoV-2 surrogate viruses bacteriophage *Φ6* and BCoV on different solid surfaces in the hospital environment, confirming the process as a virucidal disinfection. Future work is needed to study compatibility with different surface materials to ensure safe operation of automated room decontamination in the hospital setting.

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Conflict of Interest

BK and JK received a travel grant from Infuser Deutschland GmbH, Mannheim, Germany.

All other authors have no conflict of interest to declare.

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Figure Legend

Fig. 1**: Microbial load of bacteriophage Φ6 (A) and bovine CoV (B) on different surfaces before and post ozone decontamination and comparison of the reduction factors achieved (C).** The boxplots represent the variation of contamination with bacteriophage Φ6 (plaque forming units/mL) on ceramic tile, stainless steel and furniture board examined before and after automated room decontamination (A). The control boxplots result from four samples of each material, whereas post ozone boxplots include 10 values per material. Likewise, variation of viral load on surfaces contaminated with bovine CoV (TCID50/mL) were determined (B). The boxplots result from six (control) and 10 (post ozone) samples for each surface material. All results were calculated from two independent experiments. The dashed lines (A, B) display the detection limits resulting from the method used. Moreover, reduction factor (R) of bacteriophage Φ6 and bovine CoV determined for different surfaces is displayed (C). The dashed line (C) represents the log10 reduction factor of four, which means virucidal effectiveness.

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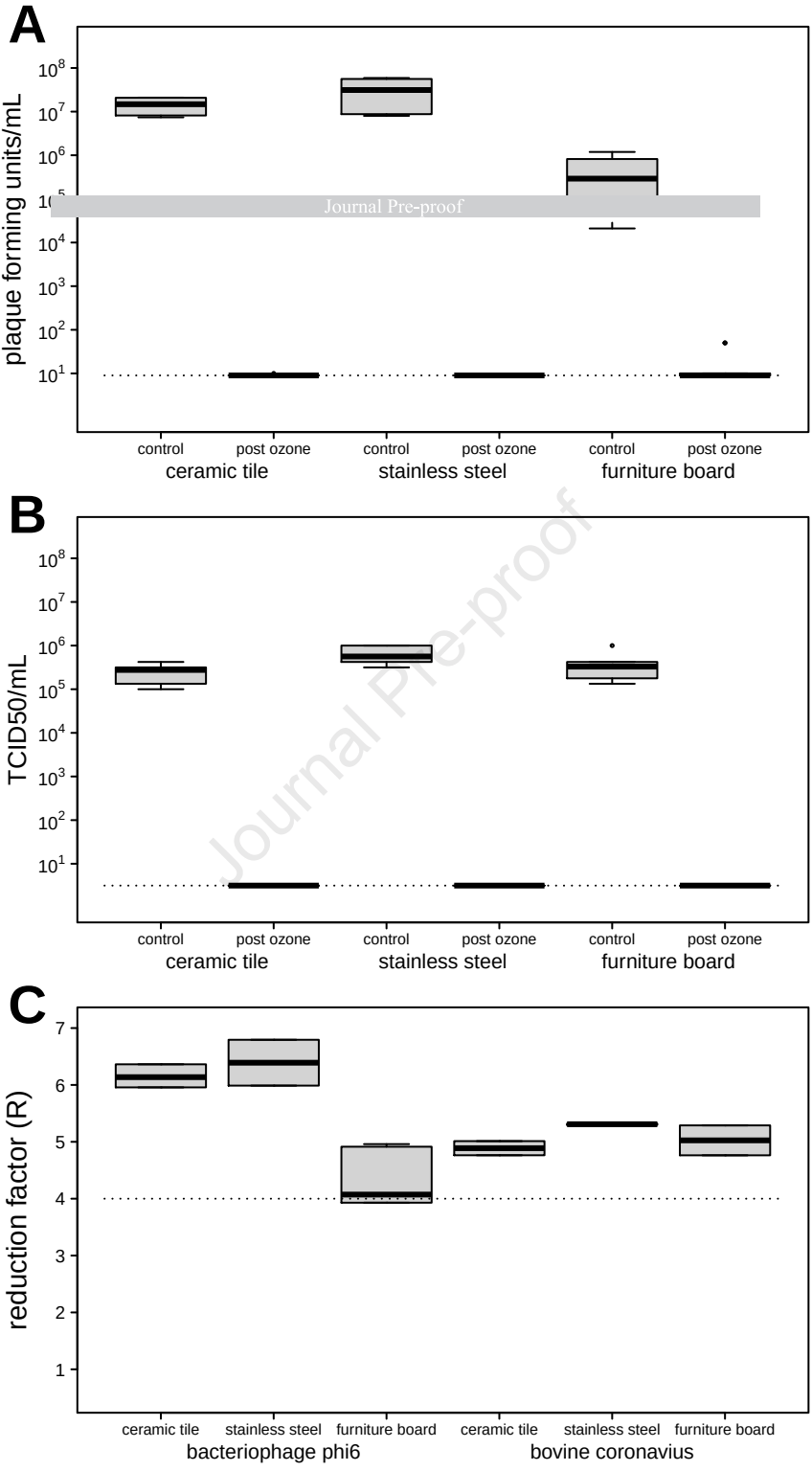
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