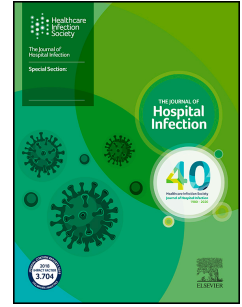


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An automated room disinfection system using ozone is highly active against surrogates for SARS-CoV-2

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

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18

19 Summary

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

27
28 **Aim:** In the following study, the effectiveness of a fully automatic room decontamination sys-
29 tem based on ozone was tested against bacteriophage $\Phi 6$ (phi 6) and bovine coronavirus
30 L9, as surrogate viruses for the pandemic coronavirus SARS-CoV-2.

31
32 **Methods:** For this purpose, various surfaces (ceramic tile, stainless steel surface and furni-
33 ture board) were soiled with the surrogate viruses and placed at two different levels in a gas-
34 tight test room. After using the automatic decontamination device according to the manufac-
35 turer's instructions, the surrogate viruses were recovered from the surfaces and examined by
36 quantitative cultures. Then, reduction factors were calculated.

37
38 **Findings:** The ozone-based room decontamination device achieved virucidal efficacy (re-
39 duction factor $>4 \log_{10}$) against both surrogate organisms regardless of the different surfac-
40 es and positions confirming a high activity under the used conditions.

41
42 **Conclusion:** Ozone is highly active against SARS-CoV-2 surrogate organisms. Further in-
43 vestigations are necessary for a safe application and efficacy in practice as well as integra-
44 tion into routine processes.

45

46

47 **Keywords:** SARS-CoV-2, bovine Coronavirus, bacteriophage Phi 6, surrogate virus, auto-
48 mated room disinfection, ozone,

49 Introduction

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

64

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

70

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

80

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

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

87

88 Coronaviruses form a large and pleomorphic family that is further divided into groups based
89 on serological findings and phylogenetic analysis [20–22]. The bovine coronavirus (BCoV)
90 from the genus *Betacoronavirus* is genetically closely related to SARS-CoV, MERS-CoV and
91 the pandemic SARS-CoV-2 viruses and can be handled outside a BSL-3 safety laboratory.
92 Therefore, we used the BCoV and $\Phi 6$ as surrogate organisms for the present experiments.

93

94

95 **Methods**

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

100

101 Surrogate virus bacteriophage $\Phi 6$ (DSM 21518) and the bacterial host strain *Pseudomonas*
102 *syringae* pv. *Syringae* (DSM 21482) were purchased from Leibniz-Institute DSMZ - Deutsche
103 Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Initial
104 lysate of bacteriophage $\Phi 6$ with a titer of 4×10^{11} plaque forming units (pfu)/mL was pro-
105 duced using a top agar overlay technique as described by the manufacturer. Then, 20 μ L of a
106 1:10 dilution was struck out and dried on ceramic tiles (5x5 cm, #3709PN00, Villeroy&Boch,
107 Mettlach, Germany), stainless steel carriers (#0344818, Modulor GmbH, Berlin) and furniture
108 boards (melamine-coated solid core panels). After each experiment $\Phi 6$ from both, treated
109 and untreated carriers, were recovered by rinsing the surface with 1mL Tryptic Soy Broth
110 (TSB)+ 5mM CaCl₂ medium for 15 times. A quantitative plaque assay was performed using
111 top agar overlay with Tryptic Soy Agar (TSA) + 5 mM CaCl₂ culture media after tenfold serial
112 dilution (detection limit: <10 pfu/mL). Plates were incubated at 23°C for 24 h.

113

114 In the same way further carriers were contaminated with 50 μ L virus inoculum of bovine coro-
115 navirus strain L9 (BCoV). BCoV strain L9 and the host *U373 cells* (passage 8) were obtained
116 by G. Zimmer, Institute of Virology, School of Veterinary Medicine, Hannover, Germany. For
117 preparation of test virus solution, a monolayer of *U373 cells* were infected with BCoV L9.
118 After an incubation period of 24 to 48 hours' cells were lysed by a rapid freeze/thaw cycle.
119 Cellular debris was removed and the supernatant was mixed with bovine serum albumin
120 (BSA) (final concentration: 0.3 g/L BSA). After each experiment an endpoint dilution assay
121 was performed. Therefore, the treated and untreated carriers were rinsed with 1 mL medium
122 without fetal calf serum (FCS). Remaining infectivity was determined by transferring 0.1 mL
123 of appropriate tenfold serial dilutions into eight wells of a microtitre plate with a preformed
124 monolayer of *U373 cells* ($10\text{-}15 \times 10^3$ cells per well), beginning with the highest dilution. Be-
125 fore addition of virus, cells were washed twice with Eagle's minimum essential medium
126 (EMEM) and incubated for 3 h with 100 μ L EMEM with trypsin. Microtitre plates were incubat-
127 ed at 37 °C in a 5 % CO₂-atmosphere. The cytopathic effect was read by using an inverted
128 microscope after five days and the infective dose TCID₅₀/mL was calculated.

129 For the decontamination experiments contaminated carriers were placed horizontally at two
130 different heights on the shelf to represent the efficacy at high and low room levels. Three
131 prepared carriers of each material and surrogate virus were positioned at the high (1.69 m)
132 and two at the low (0.07 m) position. For both surrogate organisms in each experiment two
133 contaminated control carriers were placed in a room without treatment. For bacteriophage
134 $\Phi 6$ additional control experiments at 90% relative humidity (RH) and 22 °C were performed
135 in a climate chamber.

136

137 The disinfection process using the STERISAFE™ Pro system was investigated in two inde-
138 pendent experiments for each organism. According to manufacturer's instructions, the de-
139 contamination time was 60 minutes with a target ozone concentration of 80 ppm and a target
140 RH of 90% generated with the integrated humidifier and ozone generator [23,24]. Ozone
141 concentration and relative humidity were continuously measured by integrated instruments
142 and displayed on a mobile tablet computer outside of the room, as well as recorded in the
143 instrument (supplementary figure S1) [24]. After completion of the disinfection process, the
144 ozone is converted back into pure oxygen (fig. S1) and by-products are removed in an air
145 purification phase. When the process is displayed as finished on the tablet computer, the
146 room can be entered again immediately [24]. The ozone concentration in the treated room
147 then complies to usual limit values of 0.1 ppm (exposure limit for 8 hours per day doing light
148 work) set by Occupational Safety and Health Administration (OSHA) or The National Institute
149 for Occupational Safety and Health (NIOSH) [25]. Both surrogate viruses were investigated
150 together in two independent experiments and reduction factors were calculated by subtract-
151 ing log₁₀ of untreated and treated samples. As defined elsewhere, virucidal efficacy was
152 suggested if the mean reduction factor is $>4\log_{10}$ [26].

153

154 Results

155 The aim of the present study was to evaluate the virus-inactivating properties of ozone in the
156 presence of high relative humidity against surrogate bovine coronavirus (BCoV) and bacteri-
157 ophage $\phi 6$ in a setting of room disinfection. Initial desiccation of bacteriophage $\phi 6$ resulted
158 in mean concentrations of 1.4×10^7 , 3.2×10^7 and 4.5×10^5 plaque forming units (pfu)/mL on
159 ceramic tiles, stainless steel and furniture board, respectively. Initial desiccation of BCoV
160 resulted in mean concentrations of 2.5×10^5 , 4.0×10^5 , and 6.4×10^5 TCID₅₀/mL on ceramic
161 tiles, stainless steel and furniture board, respectively. The stability of both surrogate organ-
162 isms in the desiccation phase allowed further investigations to determine virucidal activity.

163

164 After the decontamination process with STERISAFE™ Pro, independent of the carrier mate-
165 rial used or the room height, no plaque forming units of bacteriophage $\phi 6$ could be recov-
166 ered from the surfaces (fig.1A). The STERISAFE™ Pro achieved mean log₁₀ reduction fac-
167 tors of 6.15 on ceramic tiles, 4.29 on furniture board and 5.31 on stainless steel surfaces for
168 the surrogate virus bacteriophage $\phi 6$ (fig. 1C). Control experiments with high humidity with-
169 out additional ozone as disinfectant revealed a minor decrease of viral activity (supplemen-
170 tary fig S2), indicating that the observed virucidal activity can only be reached by a combina-
171 tion of ozone and humidity.

172

173 For BCoV, post ozone application no residual virus could be detected independent of the
174 carrier material used or the position in the room (corresponding to 3.16 TCID₅₀/mL) (fig. 1B).
175 For the bovine coronavirus, mean log₁₀ reduction factors of 4.88 on ceramic tiles, 5.03 on
176 furniture board and 5.31 on stainless steel surfaces could be determined (fig. 1C). STERIS-
177 AFE™ Pro showed virucidal efficacy (reduction factor $>4\log_{10}$) for both surrogate organisms
178 on all investigated surfaces.

179 Discussion

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

188

189 The present results indicate a virucidal effectiveness (reduction factor $> 4 \log_{10}$) of ozone in
190 combination with high relative humidity for both tested surrogate viruses (bacteriophage $\Phi 6$
191 and BCoV), independent from the surface material. The virucidal effect could be detected at
192 different levels in the test room. Therefore, a distribution of ozone and humidity can be as-
193 sumed as sufficient for successful decontamination. Interestingly, on the furniture board, for
194 bacteriophage $\Phi 6$, the calculated extent of the reduction was lower than on the other materi-
195 als tested. Differences in the reduction of bacteriophage $\Phi 6$ mainly are due to reduced re-
196 covery of phages after initial contamination of control surfaces, which probably results from
197 random fluctuation or specific surface conditions.

198

199 Recent studies have already shown that surface stability and survival time of SARS-CoV-2
200 was influenced by environmental conditions in particular temperature and relative humidity
201 [28–30]. Higher humidity and temperature decrease virus survival time on surfaces [28].
202 However, for bacteriophage $\Phi 6$ we observed only a low decrease of viral activity under hu-
203 mid conditions without the application of ozone. Therefore, it can be assumed that only the
204 combination of ozone with high relative humidity achieves full virucidal efficacy.

205

206 Since bacteriophage $\Phi 6$ is a small enveloped virus it shares similarities with coronavirus.
207 However, it is considered to be more stable than coronavirus because it has a double
208 stranded RNA genome [31]. In contrast, the BCoV belongs to the same family (*Coronaviri-*
209 *dae*) and the same genus *Betacoronavirus* and subgenus *Sarbecovirus* as SARS-CoV-2.
210 Both viruses are likely to have similar properties and can be considered as surrogate viruses
211 for SARS-CoV-2. Therefore, it is assumed, that ozone is also effective against SARS-CoV-2.

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

214

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

223

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

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

249 Conclusion

250 In summary, we found that ozone in combination with high humidity as generated by an au-
251 tomated room decontamination system has a high activity against SARS-CoV-2 surrogate
252 viruses bacteriophage $\phi 6$ and BCoV on different solid surfaces in the hospital environment,
253 confirming the process as a virucidal disinfection. Future work is needed to study compatibil-
254 ity with different surface materials to ensure safe operation of automated room decontamina-
255 tion in the hospital setting.

256

257

258

259

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262

263 Conflict of Interest

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

265 All other authors have no conflict of interest to declare.

266

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269 mercial, or not-for-profit sectors.

270 **Figure Legend**

271 **Fig. 1: Microbial load of bacteriophage Φ 6 (A) and bovine CoV (B) on different surfaces before and post**
272 **ozone decontamination and comparison of the reduction factors achieved (C).** The boxplots represent the
273 variation of contamination with bacteriophage Φ 6 (plaque forming units/mL) on ceramic tile, stainless steel and
274 furniture board examined before and after automated room decontamination (A). The control boxplots result from
275 four samples of each material, whereas post ozone boxplots include 10 values per material. Likewise, variation of
276 viral load on surfaces contaminated with bovine CoV (TCID₅₀/mL) were determined (B). The boxplots result from
277 six (control) and 10 (post ozone) samples for each surface material. All results were calculated from two inde-
278 pendent experiments. The dashed lines (A, B) display the detection limits resulting from the method used. Moreo-
279 ver, reduction factor (R) of bacteriophage Φ 6 and bovine CoV determined for different surfaces is displayed (C).
280 The dashed line (C) represents the log₁₀ reduction factor of four, which means virucidal effectiveness.

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