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Comparison of different disinfection protocols against contamination of ceramic surfaces with *Klebsiella pneumoniae* biofilm

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Environmental contamination with *Klebsiella pneumoniae* biofilm can be a source of healthcare-associated infections. Disinfection with various biocidal active substances is usually the method of choice to remove contamination with biofilm. In this study we tested 13 different disinfection protocols using gaseous ozone, citric acid, and three working concentrations of benzalkonium chloride-based professional disinfecting products on 24-hour-old biofilms formed by two *K. pneumoniae* strains on ceramic tiles. All tested protocols significantly reduced total bacterial counts compared to control, varying from a $\log_{10}$ CFU reduction factor of 1.4 to 5.6. Disinfection combining two or more biocidal active substances resulted in significantly better anti-biofilm efficacy than disinfection with single substances, and the most effective combination for both strains was that of citric acid, gaseous ozone, and benzalkonium chloride. This follow up study is limited to *K. pneumoniae* alone, and to overcome this limitation, future studies should include more bacterial species, both Gram-positive and Gram-negative, and more samples for us to find optimal disinfection protocols, applicable in real hospital settings.

KEY WORDS: benzalkonium chloride; citric acid; *K. pneumoniae*; ozone

Klebsiella pneumoniae often forms biofilm on inanimate surfaces in healthcare facilities, which is a potential source of healthcare-associated infections in immunocompromised patients, including urinary tract infection, pneumonia, and bacteraemia (1–6). Due to the emergence of *K. pneumoniae* multi-drug resistant strains, which limits the availability of effective treatment, these infections can have serious consequences (7, 8).

Once attached to a surface, *K. pneumoniae* easily forms a biofilm, a complex structure surrounded and shielded with self-produced extracellular polymeric substance (EPS) (1, 9). Compared to the planktonic form, biofilms are more resistant to antibiotics, desiccation, and disinfecting products (10–15). In addition, frequent use of the same biocidal active substance or over-dilution may lead to the development of persisters and reduced susceptibility to these substances. Some authors also report cross-resistance to some biocidal substances (18–20). Current control measures in healthcare facilities to battle both planktonic bacteria and biofilm contamination combine mechanical cleaning followed by disinfection, usually with benzalkonium chloride (BAC), member of quaternary ammonium compounds (21–26). Some propose new disinfectants, such as the environmentally-friendly ozone gas, thanks to its strong oxidising properties on cell membrane glycolipids, peptides, proteins, and on nucleic acids (8, 27–31) or combinations (32–34), especially with biocides of natural origin (13), given the reduced bacterial susceptibility to quaternary ammonium compounds if used alone (6, 12, 13, 35–37).

The aim of this study was to further investigate and compare the effects of different combinations of disinfection methods with gaseous ozone, citric acid, and quaternary ammonium compounds, alone and combined, on early *K. pneumoniae* biofilm on ceramic tiles, as a follow up on our previous study of gaseous ozone efficacy against *K. pneumoniae* biofilm formed on ceramics (8).

MATERIALS AND METHODS

Biocidal active substances

For the purposes of this study we compared ceramic tile disinfection with gaseous ozone (O_3), citric acid (CA), and two marketed professional disinfecting products (DP). Gaseous ozone was produced in the laboratory with a mobile ozone generator (Mozon GPF 8008, Mozon d.o.o., Sisak, Croatia) and used in the concentration of 49.914 mg/m^3. Citric acid was purchased from

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manufacturer (Kemig d.o.o., Zagreb, Croatia) and diluted in the laboratory to the working concentration of 15 %. The first disinfecting product (DP1) contains 1 % BAC as the only active substance, while the second (DP2) contains 4.8 % BAC with 0.1 % 2-phenoxyethanol, 0.098 % ethanol, 0.05 % glycolic acid, and 0.02 % *N*-(3-aminopropyl)-*N*-dodecylpropane-1,3-diamine. Both products were obtained from retail and were used in the following working concentrations: 5 % and 20 % for DP1 and 1 % for DP2.

Bacterial strains and biofilm formation

Anti-biofilm efficacy of biocidal active substances alone or in combination was tested against the standard *K. pneumoniae* ATCC 700603 strain and the clinical *K. pneumoniae* 14 strain. The standard strain was obtained from the collection of the University of Rijeka Faculty of Medicine's Department of Microbiology and Parasitology, while the clinical isolate was obtained from a urine sample provided by the Dr. Ivo Pedišić General Hospital in Sisak, Croatia. Both strains were stored in 10 % glycerol broth at -80 °C.

Biofilm was let to form on small ceramic tiles (2.5 × 2.5 cm), which were previously brushed and washed thoroughly and then sterilised in autoclave. The biofilm formation method has been described in detail earlier (8, 32). Briefly, to 250 mL of distilled water we added 5 g of 2 % agar, which was then melted and poured around three ceramic tiles placed in a Petri dish. The upper tile surface was not covered in agar but was layered with diluted overnight bacterial suspension (around 10^5 CFU/mL) and then incubated in the Petri dish placed on an orbital shaker (Unimax model 1010, Heidolph Scientific Products GmbH, Schwabach, Germany) at 30–50 rpm and 25±2 °C for 24 h.

Disinfection protocols

We employed disinfection protocols divided into four groups as follows (Table 1): disinfection with O_3, CA, DP1, or DP2 alone (group A), combined disinfection with O_3 followed by CA, DP1, or DP2 (group B), combined disinfection with CA, DP1, or DP2, followed by O_3 (group C), and combined treatment with CA, O_3, and DP1 (group D). All protocols involved one-hour exposure to O_3 in the concentration of 49.914 mg/m^3. All experiments were done in triplicate. Controls (untreated tiles) were provided for all disinfection protocols.

Disinfection with O_3

Petri dishes with ceramic tiles with formed *K. pneumoniae* biofilm were placed in a sealed experimental chamber (V=0.125 L) and O_3 inserted into the chamber with a silicon tube until it reached the concentration of 49.914 mg/m^3. Exposure lasted 1 h, during which time we monitored the temperature (23.4 °C), relative humidity (56 %), and O_3 concentration with ozone detector Keernuo GT-901 (Keernuo, Shenzhen, China) and Auriol 4-LD5531 weather station (OWIM GmbH, Neckarsulm, Germany). After one hour, the tiles were removed from the agar with sterile pincers, rinsed with 10 mL saline, placed in a Falcon tube (one tile per tube) containing 10 mL sterile saline, and sonicated in an ultrasound bath (BactoSonic, Bandelin, Berlin, Germany) at 40 kHz for 1 min. Falcon tubes with tiles were then vortexed to enhance biofilm detachment from the tiles.

Disinfection with CA

CA was poured over ceramic tiles with formed *K. pneumoniae* biofilm previously removed from agar, washed with sterile saline, and dried in a laminar flow chamber for 1 min. Exposure time to CA was 10 min. After that, the tiles were washed with sterile saline, transferred into a Falcon tube, and prepared for the determination of culturable bacterial count.

Disinfection with DP1 or DP2

DP1 (in either 5 % or 20 % working concentration) or DP2 was poured over the ceramic tiles with formed biofilm and left for 10 min. After exposure, each tile was transferred into a new Petri dish containing a 10 % sodium thiosulphate solution (Kemika d.o.o., Zagreb, Croatia) for 10 min to neutralise BAC. Culturable bacterial count was determined immediately as described below.

Group B combined disinfection protocols

K. pneumoniae biofilm on ceramic tiles was first exposed to O_3 as previously described and then to either CA, DP1, or DP2 as follows: O_3 + CA; O_3 + 5 % DP1; O_3 + 20 % DP1, and O_3 + 1 % DP2.

Group C combined disinfection protocols

Ceramic tiles with *K. pneumoniae* biofilm were first treated with either CA, DP1, or DP2 as described above, and then with O_3 as follows: CA + O_3; 5 % DP1 + O_3; 20 % DP1 + O_3, and 1 % DP2 + O_3. After the pre-treatment with CA, DP 1 and DP2, the tiles were neutralised, rinsed with sterile saline, dried off in laminar flow chamber, and then exposed to O_3 in a sealed chamber for 1 h.

Group D combined disinfection with CA, O_3, and DP1

K. pneumoniae biofilm on ceramic tiles was first treated with 15 % CA for 10 min as described above, then with O_3 for 1 h, and finally with 20 % DP1 for 10 min.

Determination of culturable bacterial counts

After all disinfection protocols, culturable bacterial count was determined using ten-fold serial dilutions prepared and inoculated on Muller Hinton agar. After incubation at 35±2 °C for 24–48 h, culturable bacteria were counted and are expressed as CFU/cm^2.

Crystal violet staining and digital microscopy

For imaging, the ceramic tiles with representative strain *K. pneumoniae* ATCC 700603 biofilm were rinsed with sterile saline to

Table 1 Disinfection protocols

Protocol group	Protocol No.	Protocol abbreviation	Disinfecting product	Biocidal active substance	Working concentration	Exposure time
A	1	O_3	Ozone generated with a mobile ozone generator	Gaseous ozone	49.914 mg/m^3	1 h
A	2	CA	Citric acid	Citric acid	15 %	10 min
A	3	DP1	Disinfecting product 1	1 % benzalkonium chloride	5 % 20 %	10 min
A	4	DP2	Disinfecting product 2	4.8 % benzalkonium chloride 0.1 % 2-phenoxyethanol 0.098 % ethanol 0.05 % glycolic acid 0.02 % *N*-(3-aminopropyl)-*N*-dodecylpropane-1,3-diamine	1 %	10 min
B	5	O_3 + 15 % CA	Combined disinfection with gaseous ozone and citric acid (pre-treatment)	Gaseous ozone Citric acid	49.914 mg/m^3 O_3 15 % CA	1 h 10 min
B	6	O_3 + 5 % DP1	Combined disinfection with gaseous ozone and disinfecting product 1 (pre-treatment)	Gaseous ozone 1 % benzalkonium chloride	49.914 mg/m^3 5 % DP1	1 h 10 min
B	7	O_3 + 20 % DP1	Combined disinfection with gaseous ozone and disinfecting product 1 (pre-treatment)	Gaseous ozone 1 % benzalkonium chloride	49.914 mg/m^3 20 % DP1	1 h 10 min
B	8	O_3 + DP2	Combined disinfection with gaseous ozone and disinfecting product 2 (pre-treatment)	Gaseous ozone 4.8 % benzalkonium chloride 0.1 % 2-phenoxyethanol 0.098 % ethanol 0.05 % glycolic acid 0.02 % N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine	49.914 mg/m^3 1 % DP2	1 h 10 min
C	9	15 % CA + O_3	Combined disinfection with citric acid and gaseous ozone (post-treatment)	Citric acid Gaseous ozone	15 % 49.914 mg/m^3	10 min 1 h
C	10	5 % DP 1 + O_3	Combined disinfection with disinfecting product 1 and gaseous ozone (post-treatment)	1 % benzalkonium chloride Gaseous ozone	5 % DP1 49.914 mg/m^3	10 min 1 h
C	11	20 % DP 1 + O_3	Combined disinfection with disinfecting product 1 and gaseous ozone (post-treatment)	1 % benzalkonium chloride Gaseous ozone	20 % DP1 49.914 mg/m^3	10 min 1 h
C	12	DP 2 + O_3	Combined disinfection with disinfecting product 1 and gaseous ozone (post-treatment)	4.8 % benzalkonium chloride 0.1 % 2-phenoxyethanol 0.098 % ethanol 0.05 % glycolic acid 0.02 % N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine Gaseous ozone	1 % DP 2 49.914 mg/m^3	10 min 1 h
D	13	15 % CA + O_3 + 20 % DP 1	Combined disinfection with citric acid, gaseous ozone and disinfecting product 1	Citric acid Gaseous ozone 1 % benzalkonium chloride	15 % 49.914 mg/m^3 20 % DP 1	10 min 1 h 10 min

CA – citric acid; DP 1 – disinfection product 1; DP 2 – disinfection product 2; O_3 – ozone

remove excess material, fixated in a dry heat steriliser (ST-01/02, Instrumentaria, Zagreb, Croatia) at 80 °C for 30 min, and stained with 0.1 % crystal violet (CV) dye for 30 min. Images were taken with a DSX 1000 digital microscope (Olympus, Tokyo, Japan) at 20× magnification and the stained tiles are presented as 3D images.

Statistical analysis

For statistical analysis we used the TIBCO Statistica 14.0.1 (TIBCO Software Inc., Palo Alto, CA, USA). The normality of data distribution was tested with the Shapiro-Wilk test. Statistical differences in bacterial counts between control and treated samples were tested using the non-parametric Wilcoxon signed-rank test for paired samples. Differences in bacterial counts between treatments were tested with the non-parametric Mann-Whitney *U* test, and the average of rank was determined with Friedman's ANOVA and Kendall's coefficient of concordance.

RESULTS

Anti-biofilm efficacy of different groups of disinfection protocols on the 24-h biofilm produced by the two *K. pneumoniae* strains on ceramic tiles is shown in Tables 2 and 3. As expected, the combination of all three methods (CA + O_3 + 20 % DP1) achieved the highest $\log_{10}$ CFU reduction factor of 5.2 for *K. pneumoniae* ATCC 700603 (Table 2) and 5.6 for *K. pneumoniae* 14 (Table 3), which was significantly higher than with single biocidal active substance treatments (group A) (P=0.00021) and groups B and C combined disinfection protocols (P=0.0051).

In addition, combination groups B and C achieved significantly better efficacy in reducing bacterial count than single substance treatment (group A) (P=0.00016) for both *K. pneumoniae* strains but did not significantly differ between themselves.

Figure 1 shows visualisations obtained with digital microscopy of stained *K. pneumoniae* ATCC700603 biofilms treated with protocols 1, 5, 7, 9, 11, and 13 compared to control. The absence of crystal violet dye marks areas of destroyed and detached biofilm. Again, the most effective biofilm destruction is observed for protocol 13, that is, the triple combination of CA, O_3, and 20 % DP2 (Figure 1, slide 13).

DISCUSSION

As expected, disinfection protocols that combined O_3 with CA or BAC significantly reduced total culturable bacterial counts compared to treatment with a single biocidal active substance. This finding is in line with previous reports (32, 38–42) showing improved anti-biofilm effect of combined disinfectant treatments.

Even more effective was the combination involving pre-treatment with CA, treatment with O_3, and post-treatment with BAC. This protocol was significantly more effective than the rest. With a $\log_{10}$ CFU reduction factor higher than 5 it meets the requirement of the European Standard EN 13727:2015 (43) for biocidal active substance to be considered effective against bacteria in planktonic form. However, there are no standards for biofilm, even though the biocidal action can be impaired by interaction with EPS (44). This lack of biocidal efficacy standard against biofilm can

Table 2 Average $\log_{10}$ CFU reduction ranks of disinfection protocol groups against *K. pneumoniae* ATCC 700603

Protocol group	Average rank	Median	Disinfectant	Median	SE
A (single biocidal active substance)	2.000	1.743[a]	O_3	2.689[A]	0.065
			15 % CA	1.665[B]	0.142
			20 % DP1	1.865[B]	0.152
			1 % DP2	1.438[B]	0.168
B (combined biocidal active substance)	2.583	3.161[b]	O_3 + 15 % CA	4.161[A]	0.476
			O_3 + 5 % DP1	3.113[B]	0.079
			O_3 + 20 % DP1	5.255[A]	0.019
			O_3 + 1 % DP2	1.945[C]	0.229
C (combined biocidal active substance)	2.750	5.078[b]	15 % CA + O_3	5.190[A]	0.083
			5 % DP1 + O_3	4.929[A]	0.073
			20 % DP1 + O_3	5.190[A]	0.031
			1 % DP2 + O_3	2.088[B]	0.159
D (combined biocidal active substance)	3.667	5.290[c]	CA + O_3 + 20 % DP1	5.290	0.024

CA – citric acid; DP 1 – disinfection product 1; DP 2 – disinfection product 2; O_3 – ozone; SE – standard error. Different lowercase letters in superscript indicate statistically significant difference between groups (P<0.05). Different uppercase letters in superscript indicate statistically significant difference between biocidal substances used in protocol (P<0.05)

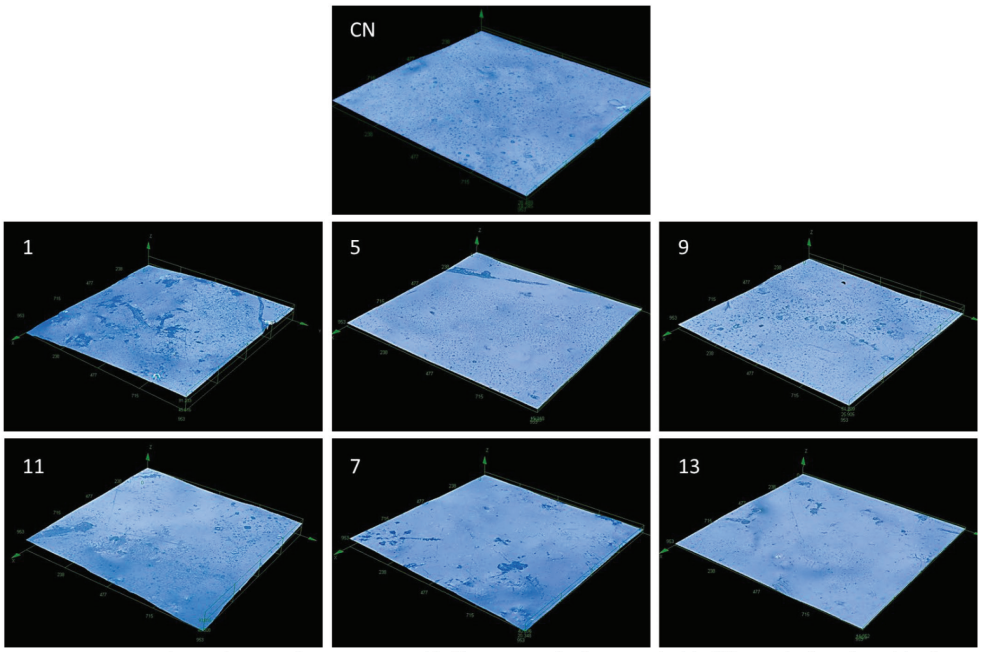

Figure 1 Representative 3D images of *K. pneumoniae* biofilm on ceramic tiles treated with different single and combined disinfection protocols using digital microscopy according to disinfection efficacy (20× magnification); 1 – O_3; 5 – O_3 + 15 % CA; 9 – CA + O_3; 11 – 20 % DP1 + O_3; 7 – O_3 + 20 % DP1; 13 – CA + O_3 + 20 % DP1; CN – control (no treatment); CA – citric acid; DP 1 – disinfection product 1; DP 2 – disinfection product 2; O_3 – ozone

Table 3 Average log log_{10} CFU reduction ranks of disinfection protocol groups against *K. pneumoniae* 14

Protocol group	Average rank	Median	Disinfectant	Median	SE
A (single biocidal active substance)	1.000	1.761[a]	O_3	1.628	0.131
			15 % CA	1.707	0.143
			20 % DP1	1.889	0.140
			1 % DP2	1.673	0.143
B (combined biocidal active substance)	2.500	3.663[b]	O_3 + 15 % CA	5.128[A]	0.122
			O_3 + 5 % DP1	2.415[B]	0.131
			O_3 + 20 % DP1	4.929[A]	0.078
			O_3 + 1 % DP2	2.184[B]	0.129
C (combined biocidal active substance)	2.667	4.923[b]	15 % CA + O_3	5.124[A]	0.053
			5 % DP1 + O_3	4.801[A]	0.135
			20 % DP1 + O_3	5.127[A]	0.124
			1 % DP2 + O_3	1.938[B]	0.084
D (combined biocidal active substance)	3.833	5.699[c]	CA + O_3 + 20 % DP1	5.699	0.098

CA – citric acid; DP 1 – disinfection product 1; DP 2 – disinfection product 2; O_3 – ozone; SE – standard error. Different lowercase letters in superscript indicate statistically significant difference between groups ($P<0.05$). Different uppercase letters in superscript indicate statistically significant difference between biocidal substances used in protocol ($P<0.05$)

be a potential problem, leading to the overuse of certain chemical biocides and subsequently contributing to exposure to hazardous substances, pollution, disinfectant resistance, cross-resistance, and waste management issues (13).

Interestingly, we found no significant difference in efficacy between combined treatments of group B and C protocols, regardless of the used biocidal active substance combined with O_3 or bacterial strain. In other words, it made no difference whether O_3 was applied in pre- or post-treatment. Our findings are similar to those reported on combined disinfection with O_3 and CA on *A. baumannii* biofilm (32), indicating that the order of application of disinfectants does not affect the antimicrobial effect.

Among combination protocols, the least effective was the combination of O_3 and DP2, regardless of the application order, most likely because DP2 contains the lowest BAC concentration.

Single disinfectant protocols also significantly reduced culturable bacterial counts in both *K. pneumoniae* strains compared to control, with the exception of O_3 against the clinical *K. pneumoniae* 14 strain. This is in line with our previous study (8) and earlier reports on other multi-drug resistant Gram-negative pathogens like *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterococcus faecalis*, which highlight the fact that ozone applied alone fails to completely remove biofilm from the surface (31, 32, 45).

By destroying bacterial cells, all disinfection protocols caused morphological changes in the biofilms and partial detachment from the tile surface, which is in line with earlier reports on anti-biofilm effects against several bacteria (8, 32, 45–478).

CONCLUSION

To conclude, our study confirms that combined disinfection using two or more different biocidal active substances is more effective in removing biofilm contamination from surfaces than using only one active substance. It has also singled out the triple combination of CA, O_3, and 20 % DP2 as the most effective. Furthermore, to completely remove biofilm, we recommend that such combined disinfection should always be preceded by mechanical cleaning of the surfaces.

Regarding the practical application of biocidal active substances used in this study, gaseous ozone and citric acid are cheap to produce and considered environmentally friendly replacements of toxic chemicals with equally effective biocidal properties. Considering, however, that gaseous ozone can be toxic to humans, all precaution measures must be implemented during disinfection.

This follow up study is limited to *K. pneumoniae* alone, and to overcome this limitation, future studies should include more bacterial species, both Gram-positive and Gram-negative, and more samples for us to find optimal disinfection protocols, applicable in real hospital settings.

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Conflict of interests

None to declare.

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Usporedba različitih protokola dezinfekcije na biofilmu *K. pneumoniae* na keramici

Kontaminacija bolničkoga okoliša biofilmom *Klebsiella pneumoniae* može utjecati na širenje bolničkih infekcija. Dezinfekcija različitim biocidnim aktivnim tvarima obično je metoda izbora za uklanjanje biofilma s površina. U ovoj smo studiji testirali 13 različitih protokola dezinfekcije koristeći plinoviti ozon, limunsku kiselinu i tri radne koncentracije profesionalnih dezinfekcijskih proizvoda na bazi benzalkonijeva klorida na 24-satnom biofilmu dvaju sojeva *K. pneumoniae* na keramičkim pločicama. Svi testirani protokoli značajno su smanjili ukupni broj bakterija u usporedbi s kontrolom, varirajući od čimbenika smanjenja $\log_{10}$ CFU od 1,4 do 5,6. Dezinfekcija kombinacijom dviju ili više biocidnih aktivnih tvari rezultirala je značajno boljim antibiofilm učinkom od dezinfekcije jednom tvari, a najučinkovitija kombinacija za oba soja bila je kombinacija limunske kiseline, plinovitog ozona i benzalkonijeva klorida.

KLJUČNE RIJEČI: benzaklonijev klorid, biofilm, *K. pneumoniae*, limunska kiselina, ozon