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1 Disinfection of methicillin resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus*
2 *epidermidis* biofilms using a remote non-thermal plasma gas

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22 **Summary**

23 The effective disinfection of hospital surfaces is recognised as an important factor in preventing
24 hospital-acquired infections. The purpose of this study was quantify the disinfection rate of a
25 novel gas plasma system on clinically relevant biofilms. Clinical isolates of *Staphylococcus*
26 *epidermidis* and methicillin resistant *Staphylococcus aureus* (MRSA) were grown as biofilms on
27 glass surfaces and tested in the a disinfection container remote from the plasma source. The
28 strains used in this study were known to produce substantial quantities of biofilm and average
29 log CFU counts were 9.0 and 9.1 CFU/cm² for *S. epidermidis* and MRSA respectively. CFU
30 counts were reduced by between 4 and 4.5 logs after one hour of exposure for MRSA and *S.*
31 *epidermidis* respectively. More prolonged treatment in case of MRSA biofilms resulted in a 5.5
32 Log reduction after 90 minutes. Biofilms samples were also placed in medical device packaging
33 bags and similar rates of disinfection were observed.

34 **Keywords**

35 Biofilm, gas-plasma, disinfection, *Staphylococcus epidermidis*, methicillin resistant
36 *Staphylococcus aureus* (MRSA)

37

38 **Introduction**

39 Healthcare-associated infections (HCAI) are those infections that are acquired by patients when
40 in contact with the healthcare system. HCAs increase the rate of morbidity and mortality
41 amongst patients, while reducing bed capacity and increasing treatment costs per patient.

42 Microorganisms demonstrate a proclivity to adhere to submerged surfaces and produce
43 extracellular polymers that facilitate adhesion and provide a structural matrix. These multi-
44 cellular communities are known as biofilms. Microbial biofilms exhibit a broad spectrum of
45 resistance to antimicrobial treatments. *In vitro*, the minimal bactericidal concentration (MBC) of
46 a drug required for adherent organisms can be three to four orders of magnitude (logs) higher
47 than for planktonic bacteria ¹. Although biofilm formation on medical devices has been
48 recognised as a problem for some time¹, more recent studies have shown a strong link between
49 biofilm formation on hospital surfaces and infection. An outbreak of infection of intensive care
50 patients with a multidrug-resistant strain of *Pseudomonas aeruginosa* was traced to hand hygiene
51 sink drains, where biofilms containing viable organisms were found². When the sink was used
52 for hand-washing, the drain contents splashed at least 1 meter from the sink. In another report³, a
53 series of laparoscopy port site infections by *Mycobacterium chelonae* were traced to
54 contaminated rinsing water used for washing chemically disinfected instruments. The organism
55 survived and grew within the biofilm at the bottom of disinfectant trays and within the outer
56 sleeves of re-usable laparoscopic instruments. The organisms thriving within biofilm in the
57 bottom of the disinfectant trays were believed to have recontaminated the freshly prepared
58 disinfectant solutions after surviving the commonly employed modes of sterilization and
59 disinfection. An earlier study of *Staphylococcus aureus* contamination of surfaces in a

60 dermatological ward used scanning electron microscopy used to show *S. aureus* biofilms in the
61 porous surfaces of polyethylene foam within the structure of shower chairs even after
62 disinfection had been undertaken⁴. These studies highlight the need for the development of novel
63 methods for the inactivation of biofilm bacteria on hospital surfaces as a means to prevent the
64 transmission of nosocomial infections.

65
66 Low temperature plasma treatment is receiving increased attention as a disinfection system
67 method because conventional methods typically involve exposing contaminated objects to
68 intense heat or to chemicals for prolonged periods. Plasma contains a mixture of charged and
69 chemically reactive species and UV emission which have antimicrobial effects. The chemically
70 active species, also known as plasma radicals, are by-products of the feeding gases to the plasma
71 discharge. Some of the most effective antimicrobial species include highly oxidizing agents such
72 as atomic oxygen, ozone, the hydroxyl radical and nitrogen oxides⁵. These species are generated
73 in air plasma discharges at atmospheric pressure. Multiple gas mixture combinations between
74 oxygen and noble gases or nitrogen at atmospheric pressure have been reported in research work
75 showing the radical antimicrobial effects⁶. Results also suggest that, at atmospheric pressure, it
76 is the oxidizing agents that carry out most of the disinfection action, above any contribution by
77 UV emission in the case of direct plasma exposure⁷. Considering the significant predominance
78 of the oxidizing radical species antimicrobial effect over those of UV and other species in the
79 plasma, a downstream radical exposure system is the most practical approach to a disinfection
80 solution⁸. The downstream radical exposure solution is feasible given the lifetime of some of the
81 strongest oxidizing radicals is in the order of several thousand seconds⁹. This allows the design
82 of a disinfection system with a remote atmospheric plasma source through which an oxygen rich

83 gas is continuously fed; the plasma downstream gas mixture containing a significant fraction of
84 oxidizing radicals.

85 This article describes an *in vitro* study where clinical isolates of *Staphylococcus epidermidis* and
86 methicillin resistant *Staphylococcus aureus* (MRSA) were grown as biofilms and used in a novel
87 gas plasma system to determine if this approach can be used for the effective disinfection of
88 biofilm contaminated surfaces. The radical rich plasma gas was supplied to a disinfection
89 chamber, where biofilm samples were exposed to the oxidizing radicals.

90

91 **Methods**

92 ***Bacterial strains and media***

93 *Staphylococcus aureus* and *S. epidermidis* are among the most common hospital pathogens
94 associated with a wide variety of infections including those involving indwelling medical
95 devices. *S. aureus* is a common cause of metal-biomaterial, bone-joint, and soft-tissue infections,
96 while *S. epidermidis* is more common in polymer-associated implant infections. *Staphylococcus*
97 *epidermidis* 1457¹⁰ a known strongly adherent biofilm producing strain, was stored in Protect
98 beads at -80°C, and revived in 50ml brain heart infusion (BHI, Oxoid, U.K.) broth overnight in
99 an incubated orbital shaker at 37°C and 150 rpm without aeration. The MRSA strain BH1CC
100 was isolated in Beaumont Hospital, Dublin and was clinically implicated in a central venous
101 catheter-related infection¹¹. BH1CC was also stored in Protect beads at -80°C. BH1CC was
102 grown BHI supplemented with 1% glucose, to promote biofilm formation by this strain¹².

103

104 ***Cultivation of biofilm***

105 Glass cover slips were immersed in 8 ml of BHI medium for *S. epidermidis*, or BHI with 1%
106 glucose for BH1CC, in each well of 6 well plates. The medium was inoculated with a 80 μ l of a
107 24 h-old culture (adjusted to $A_{660} = 1.0$), and plates were sealed with parafilm (Pechinery PC,
108 U.S.A) prior to incubation at 37°C. After 24 h the cover slips were removed and washed with
109 sterile ringers solution immediately prior to disinfection testing.

110

111 ***Disinfection Method***

112 The disinfection system, known as Radica™, was developed by Arann Healthcare (Dublin,
113 Ireland). The system is based on the generation of oxidizing plasma radicals by a remote plasma
114 source at atmospheric pressure which are subsequently fed into a disinfection container. The
115 novelty of the system lies in the separation of the atmospheric plasma discharge and the
116 downstream radical exposure container: the disinfection container. And the flexibility of the
117 disinfection container which can be tailored to fit any object. Figure 1 shows a block diagram of
118 the system. A dielectric barrier discharge plasma is generated inside an atmospheric plasma cell.
119 The cell has two electrodes: the ground electrode and the high voltage electrode. The high
120 voltage electrode is covered with ceramic layer (alumina) 0.5 mm thick. The air gap between the
121 ceramic and the ground electrode is 1 mm. The atmospheric discharge is sustained by applying
122 high voltage to the high voltage electrode inside the atmospheric cell. The high voltage supply
123 output is 10 kV pulsed at 25 kHz. The inlet gas is air supplied from an air compressor which is
124 fitted with an air dryer and particle filter. The air is feed through the atmospheric plasma cell,
125 through the air gap between the ceramic and the ground electrode, at approximately 30 l/min.
126 The plasma discharge generates oxidizing radicals which are feed into a 7 litre aluminium
127 disinfection container. The radical containing gas output by the atmospheric cell is feed through

128 the bottom of the container while the gas is exhaust to a fume hood extractor through the top of
129 the container. Cover slips were placed on a perforated metal tray at a distance of 2.1cm from the
130 bottom of the container. The disinfection tests were also carried out using general purpose
131 medical device packaging manufactured by Perfecseal Ltd. The packaging consists of breathable
132 coated Tyvek® pouches sealed to 12/50um PET/PE Film. The pouches feature uniform pore size
133 which act as a high bacteriological barrier. Medical device packaging material Tyvek®, a brand
134 by DuPont™, consists of spun-bonded high-density polyethylene. The air permeance of Tyvek®
135 allows efficient Ethylene Oxide, Electron Beam or Gamma Radiation sterilization. The purpose
136 of these experiments were to investigate the effectiveness of the system under conditions in
137 which the objects to be disinfected were in a confined space. Biofilm samples on coverslips were
138 placed inside the pouches; these were heat sealed with a plastic hand sealer prior to initiating
139 disinfection tests.

140

141 *Enumeration/Quantification of biofilm*

142 To quantify biofilm adherence, coverslips were washed and placed in 9 ml sterile Ringers. For
143 the sonication method, the coupons in Ringers were vortexed for 5 min, sonicated for 2 min and
144 then vortexed again for 2 min. The sonication was designed to lift the biofilm from the surface
145 of the coupon, and the vortexing was optimised to provide maximum disruption of the
146 aggregates to create a uniform solution which could be serially diluted and plated on nutrient
147 agar to assess the colony forming units. Plates were incubated at 37°C for 24 h. CFU
148 calculations were performed according to Zilver et al¹³.

149

150 **Results**

151 To rule out the effect of inert gas flow on biofilm, control experiments were undertaken in the
152 disinfection chamber under conditions where air at a flowrate of 30 l/min was used, this airflow
153 was not exposed to plasma. These control experiments resulted in an average 0.5 log reduction in
154 CFU counts for an exposure of one hour.

155
156 Figure 2 shows the effect of exposure time of plasma gas to biofilms of *S. epidermidis* and
157 MRSA on glass cover slips. CFU counts were reduced by 4 and 4.5 logs after one hour of
158 exposure for MRSA and *S. epidermidis* respectively. More prolonged treatment in case of
159 MRSA biofilms resulted in a 5.5 Log reduction after 90 minutes. The results for *S. epidermidis*
160 suggest conventional disinfection kinetics as demonstrated by the linear trend shown in Figure 2.
161 For MRSA the kinetics appear more complex and a plateau effect is apparent from the data
162 between 50 and 60 minutes in Figure 2. This is followed by further substantial decay in CFU
163 counts between 70 and 90 minutes.

164
165 Figure 3 shows results for experiments where the biofilm samples were placed in medical
166 packaging bags within the disinfection chamber. For *S. epidermidis* and MRSA the CFU count
167 reduction after 60 minutes exposure was approximately 4.5 and 4.0 logs respectively. More
168 prolonged treatment in case of MRSA biofilms resulted in a 5.5 Log reduction after 90 minutes.

169

170 **Discussion**

171 The strains used in this study were known to produce substantial quantities of biofilm and
172 average log CFU counts were 9.0 and 9.1 CFU/cm² for *S. epidermidis* and MRSA respectively.
173 The biofilm cultivation method involving 6 well plates was designed to reduce the amount of

174 oxygen available to the organism. The 8ml of inoculated media per well in the sealed plates
175 during incubation reduced the size of the headspace and resulted in a low-oxygen environment
176 for biofilm cultivation. This is relevant because previous work with *S. epidermidis* 1457 showed
177 that reduced oxygen levels increased biofilm formation¹⁴. The results show that gas plasma is
178 an efficient method for the disinfection of biofilms even when the disinfection chamber is remote
179 from the plasma source. The 5.5 log reductions in CFU counts of both *S. epidermidis* and MRSA
180 biofilms is favourable compared to previous reports of biofilm disinfection by gas plasma where
181 up to 3.5 log reduction in viable cell counts were achieved ¹⁵.

182

183 The results from experiments where biofilm samples were placed in the medical packaging bags
184 are comparable to those where the biofilm samples were exposed directly to the gas within the
185 disinfection chamber. This similarity between the two experiments highlights the advantage of
186 gas plasma disinfection and demonstrates the potential for of the system for the disinfection of
187 objects of complex geometric form.

188

189 The survival curve was found to be non-linear in some cases and is particularly noticeable in
190 Figure 2 for the MRSA samples. The shape of the survival curve depends on type of
191 microorganism, type of growth medium, and the method of exposure (direct or remote) ⁵.

192 Vleugels et al¹⁵ studied plasma inactivation of *P. agglomerans* biofilms and noticed that the
193 inactivation kinetics had three different phases. It was speculated that this effect was as a result
194 of a stratification effect associated with the biofilm morphology. It is likely that cells embedded
195 deeper into the biofilm are partially protected from the plasma constituents and thus a partial
196 diffusion barrier is created. However, this effect is only temporary; there is no evidence to suggest that

197 antimicrobials of any type fail to penetrate into the innermost regions of biofilm given sufficient time.
198 Experiments using fluorescently labelled tracers have confirmed this view^{16, 17}.
199 In conclusion, plasma disinfection has many advantages over more conventional methods, the
200 primary advantage is the relatively low temperatures of operation (≤ 50 °C), thus potentially
201 preserving the integrity of polymer-based materials which cannot be placed in autoclaves.
202 Secondly, gas plasma is safer than ethylene oxide. The device used in this study was a prototype
203 and it is likely that improvements will result in enhanced efficiency. Previous studies on gas
204 plasma disinfection systems have generally been tested on planktonic cells. However the present
205 study used biofilm samples due to the increased recognition that biofilms are significantly less
206 susceptible to antimicrobial treatment compared to planktonic cells.

207

208

209

210 **Figure Legends**

211 Figure 1 Disinfection System Block Diagram

212 Figure 2 Survival curves for biofilms of *S epidermidis* and MRSA on cultivated for 24 hours on
213 glass cover slips and directly exposed to the plasma gas in the disinfection container.

214 Figure 3 Survival curves for biofilms of *S epidermidis* and MRSA on cultivated for 24 hours on
215 glass cover slips and placed in medical packaging bags within the disinfection container.

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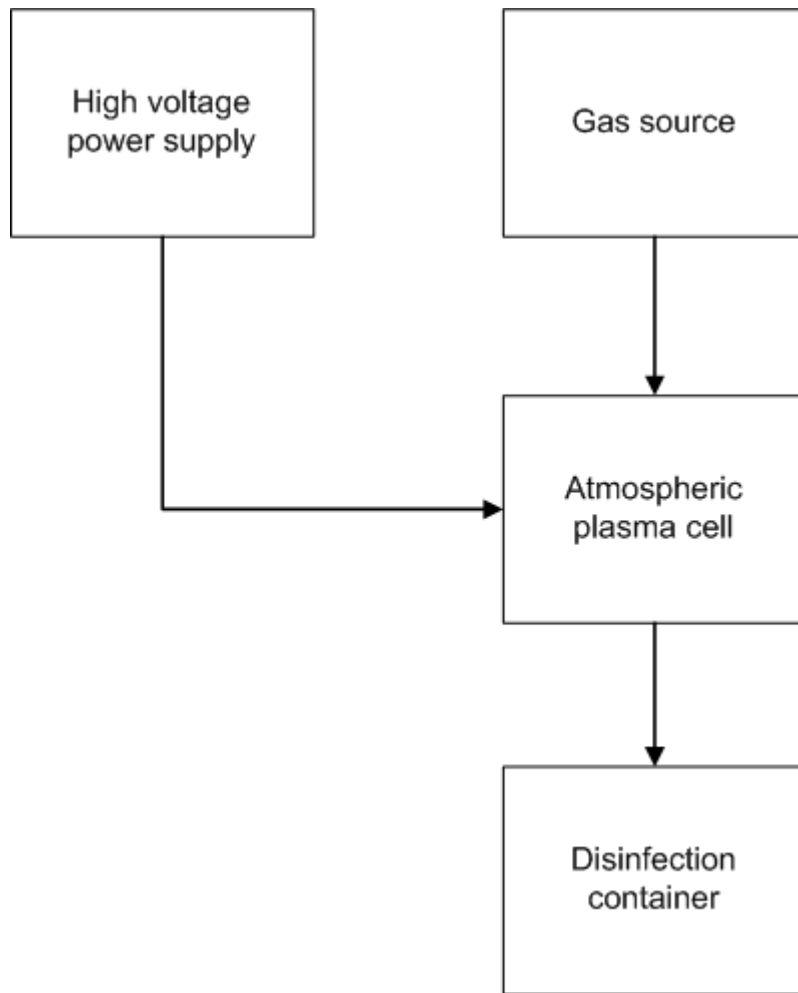
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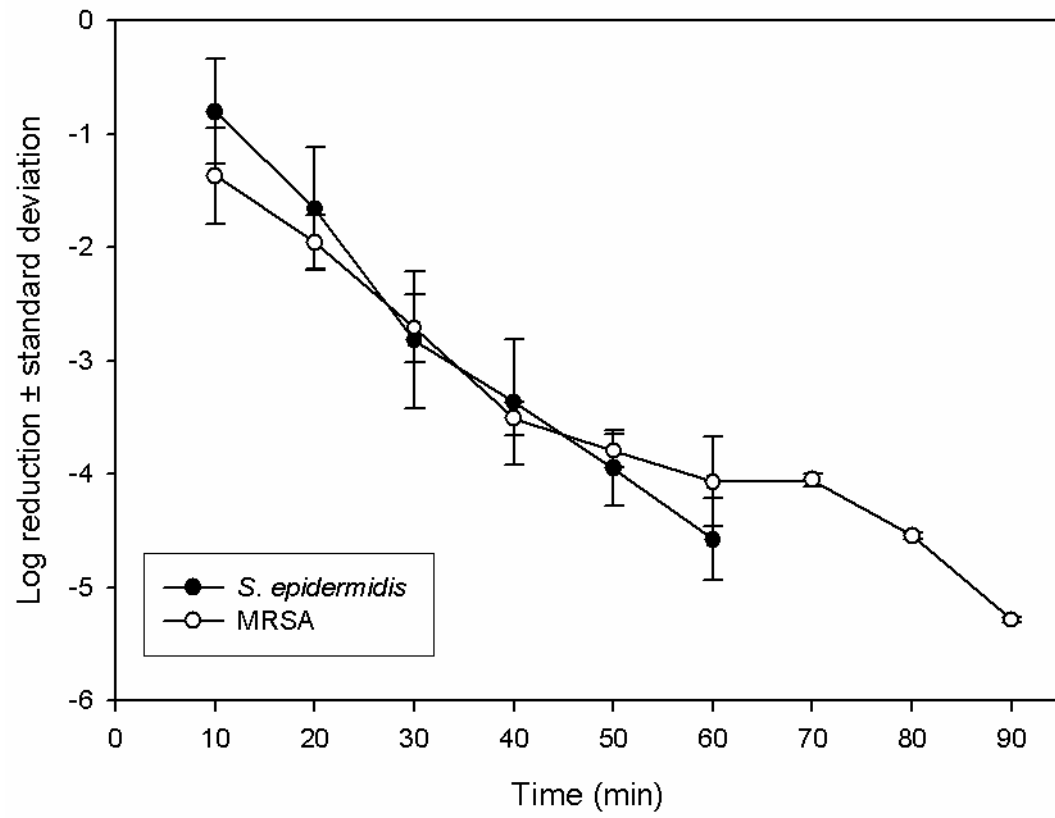
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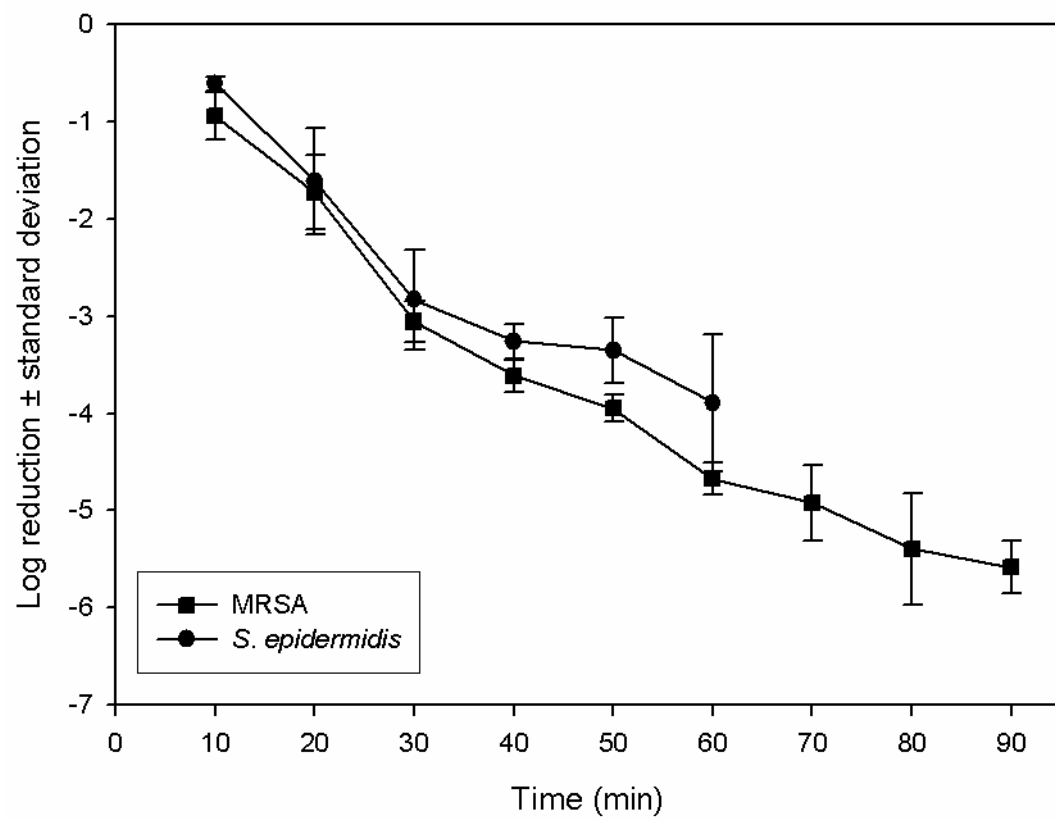
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272 Fig 1



273

274 Fig 2



275

276 Fig 3

277