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

Authors: John J. Cotter[1], Paul Maguire[2], Felipe Soberon[2], Stephen Daniels[3], James P. O'Gara[4], Eoin Casey[1*]

[1]UCD School of Chemical and Bioprocess Engineering, Centre for Synthesis and Chemical Biology,

[2]Arann Healthcare Ltd, Invent Centre, Glasnevin, Dublin 9, Ireland

[3]School of Electronic Engineering and National Centre for Plasma Science Technology, Dublin City University, Dublin 9, Ireland

[4]UCD School of Biomolecular and Biomedical Science and UCD Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

*Corresponding Author: Eoin Casey.

Address: UCD School of Chemical and Bioprocess Engineering,

Engineering and Materials Science Centre,

University College Dublin, Belfield, Dublin 4, Ireland.

Email: eoin.casey@ucd.ie

Telephone: +353 1 7161877

Fax: +353 1 7161177

Summary

The effective disinfection of hospital surfaces is recognised as an important factor in preventing hospital-acquired infections. The purpose of this study was quantify the disinfection rate of a novel gas plasma system on clinically relevant biofilms. Clinical isolates of *Staphylococcus epidermidis* and methicillin resistant *Staphylococcus aureus* (MRSA) were grown as biofilms on glass surfaces and tested in the a disinfection container remote from the plasma source. The strains used in this study were known to produce substantial quantities of biofilm and average log CFU counts were 9.0 and 9.1 CFU/cm^2 for *S. epidermidis* and MRSA respectively. CFU counts were reduced by between 4 and 4.5 logs after one hour of exposure for MRSA and *S. epidermidis* respectively. More prolonged treatment in case of MRSA biofilms resulted in a 5.5 Log reduction after 90 minutes. Biofilms samples were also placed in medical device packaging bags and similar rates of disinfection were observed.

Keywords

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

Introduction

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

Microorganisms demonstrate a proclivity to adhere to submerged surfaces and produce extracellular polymers that facilitate adhesion and provide a structural matrix. These multi-cellular communities are known as biofilms. Microbial biofilms exhibit a broad spectrum of resistance to antimicrobial treatments. *In vitro*, the minimal bactericidal concentration (MBC) of a drug required for adherent organisms can be three to four orders of magnitude (logs) higher than for planktonic bacteria [1]. Although biofilm formation on medical devices has been recognised as a problem for some time[1], more recent studies have shown a strong link between biofilm formation on hospital surfaces and infection. An outbreak of infection of intensive care patients with a multidrug-resistant strain of *Pseudomonas aeruginosa* was traced to hand hygiene sink drains, where biofilms containing viable organisms were found[2]. When the sink was used for hand-washing, the drain contents splashed at least 1 meter from the sink. In another report[3], a series of laparoscopy port site infections by *Mycobacterium chelonae* were traced to contaminated rinsing water used for washing chemically disinfected instruments. The organism survived and grew within the biofilm at the bottom of disinfectant trays and within the outer sleeves of re-usable laparoscopic instruments. The organisms thriving within biofilm in the bottom of the disinfectant trays were believed to have recontaminated the freshly prepared disinfectant solutions after surviving the commonly employed modes of sterilization and disinfection. An earlier study of *Staphylococcus aureus* contamination of surfaces in a

dermatological ward used scanning electron microscopy used to show *S. aureus* biofilms in the porous surfaces of polyethylene foam within the structure of shower chairs even after disinfection had been undertaken[4]. These studies highlight the need for the development of novel methods for the inactivation of biofilm bacteria on hospital surfaces as a means to prevent the transmission of nosocomial infections.

Low temperature plasma treatment is receiving increased attention as a disinfection system method because conventional methods typically involve exposing contaminated objects to intense heat or to chemicals for prolonged periods. Plasma contains a mixture of charged and chemically reactive species and UV emission which have antimicrobial effects. The chemically active species, also known as plasma radicals, are by-products of the feeding gases to the plasma discharge. Some of the most effective antimicrobial species include highly oxidizing agents such as atomic oxygen, ozone, the hydroxyl radical and nitrogen oxides [5]. These species are generated in air plasma discharges at atmospheric pressure. Multiple gas mixture combinations between oxygen and noble gases or nitrogen at atmospheric pressure have been reported in research work showing the radical antimicrobial effects [6]. Results also suggest that, at atmospheric pressure, it is the oxidizing agents that carry out most of the disinfection action, above any contribution by UV emission in the case of direct plasma exposure [7]. Considering the significant predominance of the oxidizing radical species antimicrobial effect over those of UV and other species in the plasma, a downstream radical exposure system is the most practical approach to a disinfection solution [8]. The downstream radical exposure solution is feasible given the lifetime of some of the strongest oxidizing radicals is in the order of several thousand seconds [9]. This allows the design of a disinfection system with a remote atmospheric plasma source through which an oxygen rich

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

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

Methods

Bacterial strains and media

Staphylococcus aureus and *S. epidermidis* are among the most common hospital pathogens associated with a wide variety of infections including those involving indwelling medical devices. *S. aureus* is a common cause of metal-biomaterial, bone-joint, and soft-tissue infections, while *S. epidermidis* is more common in polymer-associated implant infections. *Staphylococcus epidermidis* 1457 [10] a known strongly adherent biofilm producing strain, was stored in Protect beads at -80°C, and revived in 50ml brain heart infusion (BHI, Oxoid, U.K.) broth overnight in an incubated orbital shaker at 37°C and 150 rpm without aeration. The MRSA strain BH1CC was isolated in Beaumont Hospital, Dublin and was clinically implicated in a central venous catheter-related infection[11]. BH1CC was also stored in Protect beads at -80°C. BH1CC was grown BHI supplemented with 1% glucose, to promote biofilm formation by this strain[12].

Cultivation of biofilm

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

Disinfection Method

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

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

Enumeration/Quantification of biofilm

To quantify biofilm adherence, coverslips were washed and placed in 9 ml sterile Ringers. For the sonication method, the coupons in Ringers were vortexed for 5 min, sonicated for 2 min and then vortexed again for 2 min. The sonication was designed to lift the biofilm from the surface of the coupon, and the vortexing was optimised to provide maximum disruption of the aggregates to create a uniform solution which could be serially diluted and plated on nutrient agar to assess the colony forming units. Plates were incubated at 37°C for 24 h. CFU calculations were performed according to Zelver et al[13].

Results

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

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

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

Discussion

The strains used in this study were known to produce substantial quantities of biofilm and average log CFU counts were 9.0 and 9.1 CFU/cm^2 for *S. epidermidis* and MRSA respectively. 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 formation[14]. 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 al[15] studied plasma inactivation of *P. agglomerans biofil*ms 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 antimicroials 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

Figure Legends

Figure 1 Disinfection System Block Diagram

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

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

References

1. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P., Bacterial biofilms: A common cause of persistent infections. *Science* **1999,** 284, (5418), 1318-1322.

2. Hota, S.; Hirji, Z.; Stockton, K.*et al*., Outbreak of Multidrug-Resistant Pseudomonas aeruginosa Colonization and Infection Secondary to Imperfect Intensive Care Unit Room Design. *Infection Control and Hospital Epidemiology* **2009,** 30, (1), 25-33.

3. Vijayaraghavan, R.; Chandrashekhar, R.; Sujatha, Y.; Belagavi, C. S., Hospital outbreak of atypical mycobacterial infection of port sites after laparoscopic surgery. *Journal of Hospital Infection* **2006,** 64, (4), 344-347.

4. Oie, S.; Yanagi, A.; Matsui, H.; Nishida, T.; Tomita, M.; Kamiya, A., Contamination of environmental surfaces by Staphylococcus aureus in a dermatological ward and its preventive measures. *Biological & Pharmaceutical Bulletin* **2005,** 28, (1), 120-123.

5. Laroussi, M., Low-Temperature Plasmas for Medicine? *Ieee Transactions on Plasma Science* **2009,** 37, (6), 714-725.

6. Morfill, G. E.; Kong, M. G.; Zimmermann, J. L., Focus on Plasma Medicine. *New Journal of Physics* **2009,** 11, 8.

7. Ben Gadri, R.; Roth, J. R.; Montie, T. C.*et al.,* Sterilization and plasma processing of room temperature surfaces with a one atmosphere uniform glow discharge plasma (OAUGDP). *Surface & Coatings Technology* **2000,** 131, (1-3), 528-542.

8. Montie, T. C.; Kelly-Wintenberg, K.; Roth, J. R., An overview of research using the one atmosphere uniform glow discharge plasma (OAUGDP) for sterilization of surfaces and materials. *Ieee Transactions on Plasma Science* **2000,** 28, (1), 41-50.

9. Kossyi, I. A.; Kostinsky, A. Y.; Matveyev, A. A.; Silakov, V. P., Kinetic scheme of the non-equilibrium discharge in nitrogen-oxygen mixtures. *Plasma Sources Science & Technology* **1992,** 1, (3), 207-220.

10. Mack, D.; Siemssen, N.; Laufs, R., Parallel Induction by Glucose of Adherence and a Polysaccharide Antigen Specific for Plastic-Adherent Staphylococcus-Epidermidis - Evidence for Functional Relation to Intercellular-Adhesion. *Infection and Immunity* **1992,** 60, (5), 2048-2057.

11. Fitzpatrick, F.; Humphreys, H.; O'Gara, J. P., Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates. *Journal of Clinical Microbiology* **2005,** 43, (4), 1973-1976.

12. O'Neill, E.; Pozzi, C.; Houston, P.; *et al.*, Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections. *Journal of Clinical Microbiology* **2007,** 45, (5), 1379-1388.

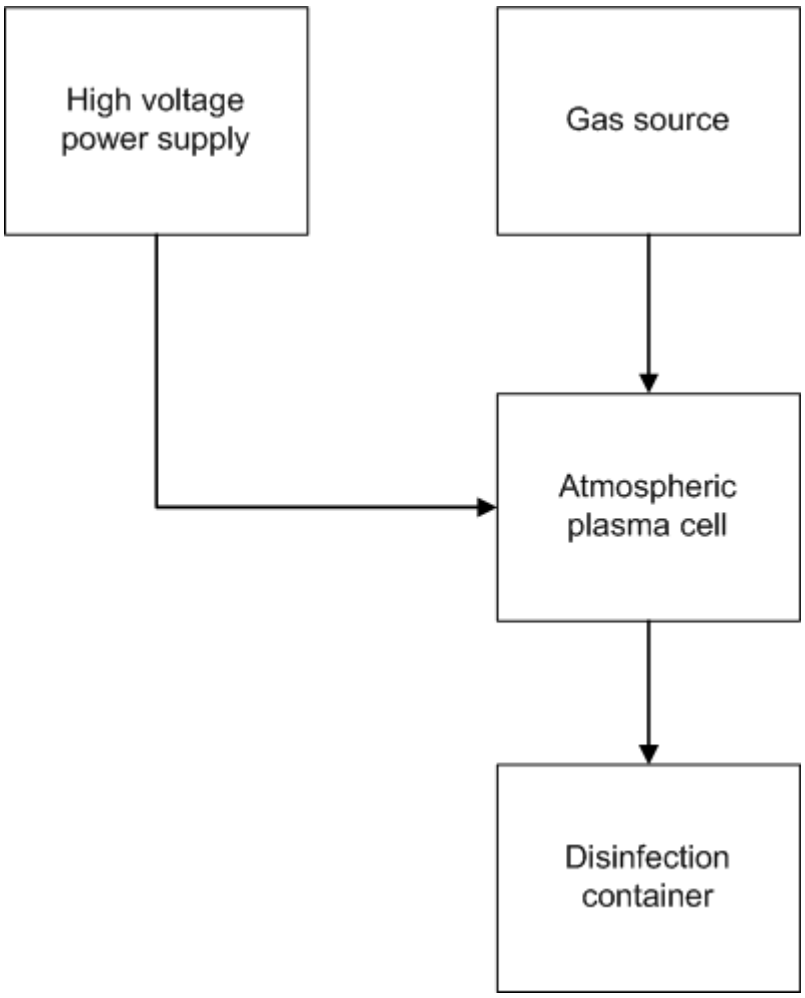
13. Zelver, N.; Hamilton, M.; Pitts, B.; *et al*., Measuring antimicrobial effects on biofilm bacteria: From laboratory to field. In *Biofilms*, Academic Press Inc: San Diego, 1999; Vol. 310, pp 608-628.

14. Cotter, J. J.; O'Gara, J. P.; Mack, D.; Casey, E., Oxygen-Mediated Regulation of Biofilm Development Is Controlled by the Alternative Sigma Factor sigma(B) in Staphylococcus epidermidis. *Applied and Environmental Microbiology* **2009,** 75, (1), 261-264.

15. Vleugels, M.; Shama, G.; Deng, X. T.; Greenacre, E.; Brocklehurst, T.; Kong, M. G., Atmospheric plasma inactivation of biofilm-forming bacteria for food safety control. *Ieee Transactions on Plasma Science* **2005,** 33, (2), 824-828.

16. Rani, S. A.; Pitts, B.; Stewart, P. S., Rapid diffusion of fluorescent tracers into Staphylococcus epidermidis biofilms visualized by time lapse microscopy. *Antimicrobial Agents and Chemotherapy* **2005,** 49, (2), 728-732.

17. Stewart, P. S.; Davison, W. M.; Steenbergen, J. N., Daptomycin Rapidly Penetrates a Staphylococcus epidermidis Biofilm. *Antimicrobial Agents and Chemotherapy* **2009,** 53, (8), 3505-3507.


High voltage power supply
Gas source
Atmospheric plasma cell
Disinfection container

271

272 Fig 1

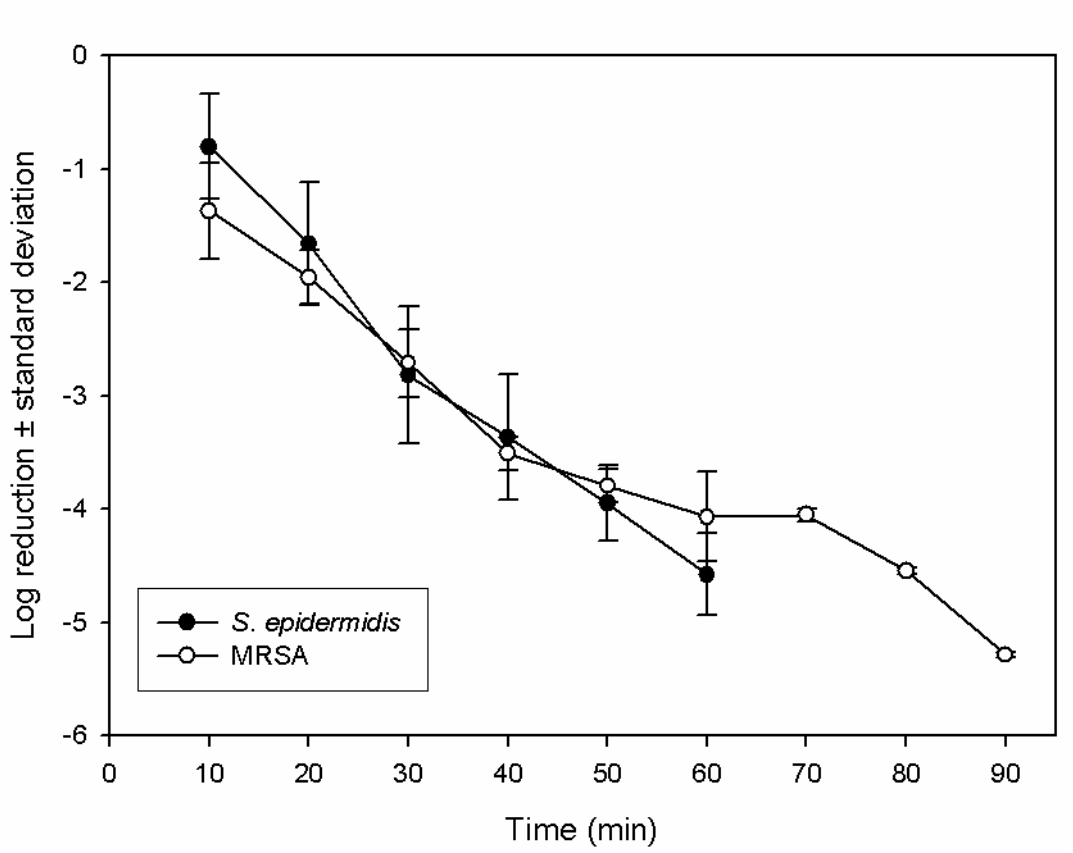
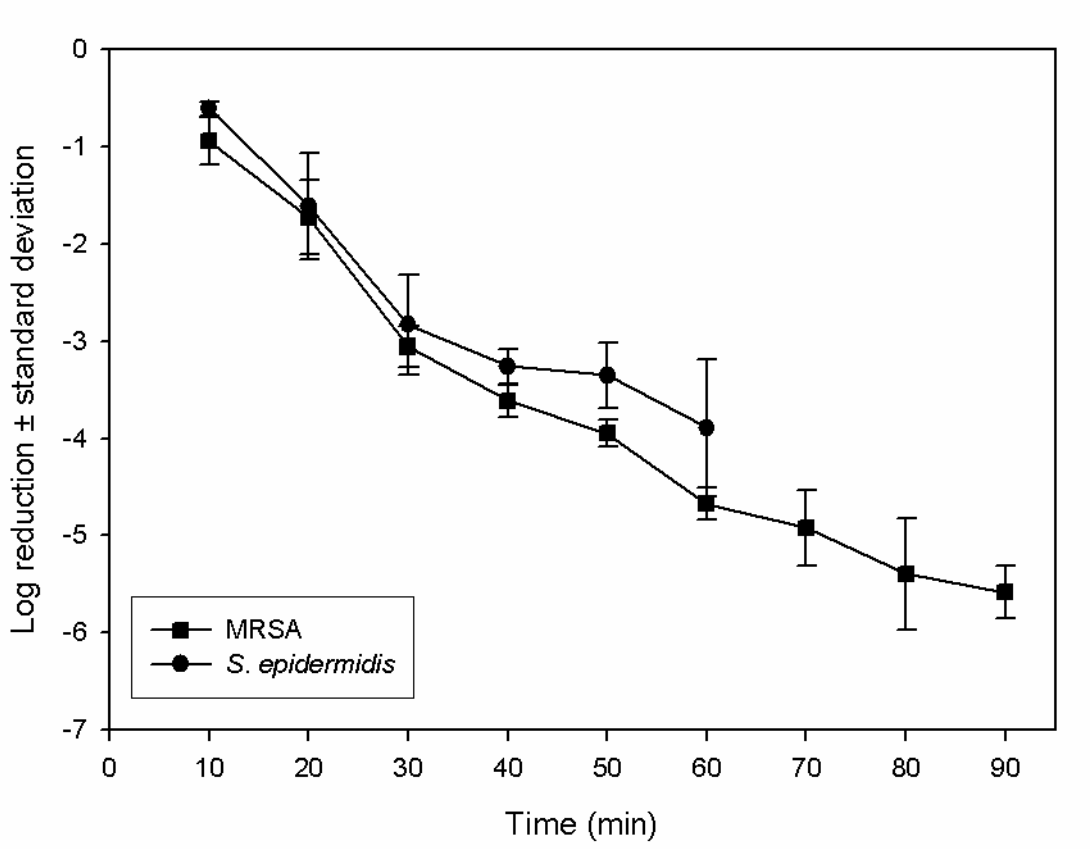

Log reduction ± standard deviation
0
-1
-2
-3
-4
-5
-6
0
10
20
30
40
50
60
70
80
90
Time (min)
S. epidermidis
MRSA

Fig 2



275

276 Fig 3

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