Test for bacterial resistance build-up against plasma treatment

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Abstract. It is well known that the evolution of resistance of microorganisms to a range of different antibiotics presents a major problem in the control of infectious diseases. Accordingly, new bactericidal ‘agents’ are in great demand. Using a cold atmospheric pressure (CAP) plasma dispenser operated with ambient air, a more than five orders of magnitude inactivation or reduction of Methicillin-resistant Staphylococcus aureus (MRSA; resistant against a large number of the tested antibiotics) was obtained in less than 10 s. This makes CAP the most promising candidate for combating nosocomial (hospital-induced) infections. To test for the occurrence and development of bacterial resistance against such plasmas, experiments with Gram-negative bacteria (Escherichia coli) and Gram-positive bacteria (Enterococcus mundtii) were performed. The aim was to determine quantitative limits for primary (naturally) or secondary (acquired) resistance against the plasma treatment. Our results show that E. coli and E. mundtii possess no primary resistance against the plasma treatment. By generating four generations of bacteria for every strain, where the survivors of the plasma treatment were used for the production of the next generation, a lower limit to secondary resistance was obtained. Our results indicate that

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CAP technology could contribute to the control of infections in hospitals, in outpatient care and in disaster situations, providing a new, fast and efficient broad-band disinfection technology that is not constrained by bacterial resistance mechanisms.

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1. Introduction

Due to the rapid growth of nosocomial infections and the increase of antibiotically resistant bacteria, disinfection and sterilization are an important and growing problem in hospitals and healthcare service units.

It was a historic milestone when in 1928 Sir Alexander Fleming discovered that colonies of the bacterium Staphylococcus aureus could be destroyed by the mold Penicillium notatum, thereby proving its antibiotic properties. Fleming’s work eventually led to the large-scale production of penicillin in the 1940s, followed by many antibiotic agents with different inhibition processes in bacterial growth. Resistant strains of bacteria first began to appear as early as the late 1940s. Currently, it is known that some bacterial resistance against every antibiotic agent used for curing infections has been evolving during the last few decades [1]. In 2007, the European Antimicrobial Resistance Surveillance System (EARSS) announced in their yearly report [2] that the antimicrobial resistance of different bacterial strains such as Streptococcus pneumoniae, S. aureus, Enterococcus faecalis, Clostridium difficile, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa is increasing drastically and therefore becoming a larger public health problem every year. Focusing on multi-resistant bacteria, global health care institutions especially consider Methicillin-resistant Staphylococcus aureus (MRSA) as a public health threat which results in 100,000 new infections and 19,000 deaths annually in the USA [3]. Another problem is the continuous decrease in production of new antibiotics (see footnote 5).

5 Centers for Disease Control and Prevention (CDC), www.cdc.gov.
Resistance among bacteria can be classified as primary (naturally) or secondary (acquired). Bacteria, which exhibit an inherent impact gap for a certain antibiotic, are called primary (naturally) resistant. Primary non-resistant bacteria, which evolve resistance against the antibiotic by means of mutations in their own DNA or the acquisition of resistance-conferring DNA from another source, are called secondary (acquired) resistant [4]. This resistance formation can result either from spontaneous mutations (vertical gene transfer) or from the horizontal transfer of genetic information. The spontaneous mutation rate in bacteria (E. coli) is of the order of $10^{-6}$–$10^{-9}$. This means that one in every $10^{6}$–$10^{9}$ bacteria could evolve a primary resistance due to the process of mutation. This resistance gene will then be directly passed to the bacteria’s offspring during DNA replication, which will lead to resistant bacteria descendants. The second mechanism responsible for developing antibiotic resistance in bacteria is based on the horizontal exchange of resistance genes via conjugation, transformation or transduction. Conjugation occurs when resistant genes, which are part of a plasmid, are transferred from a donor cell to a receptor cell which are in direct contact. Transformation is a process where genetic alteration results from the bacterial surroundings. Transduction describes the transfer of DNA via the reproduction cycle of bacteriophages (see footnote 6).

As mentioned already, the rapid evolution of bacterial resistance to antibiotics has developed into an increasingly severe health problem in hospitals, a development that has to be contained [3]. In addition to the reduction of selection pressure by reasonable usage of antibiotics, the most effective method of containment is disinfection of instruments, hospital staff and visitors.

The result that cold atmospheric pressure plasmas (CAPs) have antibacterial properties regardless of the kind of bacteria and their resistance level in vivo was first published by Isbary et al [5, 6], who treated chronic infected wounds in patients and a patient with the so-called Hailey–Hailey disease. However, the microwave technology-driven plasma device used for these studies uses argon gas as a carrier gas, is very large and expensive in production and is therefore not suitable for large-area (for example, hand) disinfection purposes.

In Morfill et al [7], a CAP plasma dispenser based on surface microdischarge technology (SMD) was developed and specifically designed for the disinfection of larger areas. This SMD plasma dispenser was used in this paper to investigate the bacterial resistance—primary and secondary build-up—toward Gram-positive and Gram-negative bacteria.

2. The principal mechanisms of bactericidal action of atmospheric pressure plasma

For an overview, see the review by Kong et al [8]. There is no doubt that plasma is a very efficient bactericidal agent. Although the detailed processes are still somewhat speculative, nevertheless it is instructive to summarize the possibilities—in the hope that this may stimulate more research. Three principal processes are cell wall permeabilization, penetration of reactive species and chemical action inside the cell:

1. Cell-wall permeabilization by electromechanical (e.g. electrical stress and electroporation), thermal (e.g. by heat released in surface recombination and de-excitation) and chemical processes (e.g. hydrogen denaturation of the membrane via OH radicals).
Electroporation, which disrupts the cell membrane, becomes important at high electric fields of the order $E \gtrsim 30 \text{kV cm}^{-1}$. Such values can be attained, for instance, with dielectric barrier (DBD) plasmas, where the cell wall forms the second electrode [9–11].

Thermoporation is a well-studied process and has been utilized in sterilization for many years [12–15]. In the case of plasmas, thermoporation is a very localized process. It is caused by electron–ion recombination on the cell surface or by de-excitation of molecules. The heat released locally is of the order of a few eV and the release is essentially instantaneous. If the energy is absorbed, i.e. it is not lost by radiation, then it is transported from the source by conduction. Solving the simple cylindrical heat conduction equation with a δ-function source $Q(\delta(r)\delta(t))$, we obtain the result that a localized temperature increase of about 40°C above ambient is possible, which will most likely induce short-lived microporation of the membrane of size 5–10 nm. Time scales for keeping these micropores open before repairs set in are believed to be of the order of ms, perhaps even longer up to seconds. A simple back of the envelope calculation, which is quite instructive, is the following. Assume an energy deposition of, for example, 5 eV into the membrane homogeneously in a volume of $5 \times 5 \times 5 \text{nm}^3$. For a membrane material density of 0.2 g cm$^{-3}$ this implies a mass of $2.5 \times 10^{-20}$ g. For a typical molecule of mass number 18 (e.g. H$_2$O) this implies $10^3$ molecules. Then, we have an energy per molecule of $5 \times 10^{-3}$ eV, resulting in a temperature increase of $\Delta T \approx 40^\circ \text{C}$.

Hydrogen denaturation of the membrane, in particular due to the chemical reaction OH + H $\rightarrow$ H$_2$O, is another studied process, which also leads to permeabilization of the membrane. Similar to the previous case of thermoporation, the micropores are also in the range of a few nm and the damage is not permanent [16–18].

Electrical stress can be induced due to charging of the bacteria. The equilibrium configuration of a ‘soft body’ is spherical when charge is applied. It has been estimated, given the tensile strength of the membrane [19, 20], that potentials of the order of 600 V could be sufficient for causing fractures in bacteria.

2. **Cell penetration of reactive oxygen and nitrogen species** (produced in some 600 non-equilibrium plasma–chemical reactions in air [21–23]). Since these reactive species (R) are gaseous, their mobility velocity $v_R$ is high, of the order of 100 m s$^{-1}$, and hence the fluxes onto a surface ($F_R = n_R v_R$) can be substantial even for small concentrations $c_R = n_R / n_{\text{Air}}$.

We can estimate the plasma density required to (a) produce membrane permeabilization and (b) allow penetration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) into the bacterium to damage the DNA if we only consider ‘distant’ plasma sources—so that only thermoporation and hydrogen denaturation need to be considered. The rate at which membrane permeabilization occurs is then

$$\dot{N} = \sum_i n_i v_i A, \quad (1)$$

where $i$ stands for $e - i$ recombination ($n_{i=1} = \text{ion density}$) for excited atoms and molecules ($n_{i=2} = \text{atom/molecule density}$) and for hydrogen denaturation ($n_{i=3} = \text{OH density}$), which we call ‘interactive species’.

The corresponding $v_i$ is the thermal velocity and $A$ is the surface area of a bacterium. Assuming that each ‘event’ opens up a micropore of the area $A_H$, which lasts for a typical time $\tau$ before repair, then the surface permeabilization fraction is

$$f_H = A_H \dot{N} \tau / A. \quad (2)$$

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If the efficiency for microporation for the different processes is given by $\varepsilon_i \leq 1$, then from (1) we obtain

$$f_H = A_H \tau \sum_i n_i \varepsilon_i v_i. \quad (3)$$

To destroy the bacterium, a reactive species has to penetrate the membrane. The density of reactive species produced by non-equilibrium plasma reactions (in air) is

$$n_{RS} = \sum_j n_{RS,j}, \quad (4)$$

where we may consider a number of these (e.g. NO, OH, NO$_2$ and H$_2$O$_2$). The flux of these species onto a surface is

$$F_{RS} = \sum_j n_{RS,j} v_{RS,j}. \quad (5)$$

The number of penetrating ROS/RNS molecules per second is then

$$\dot{N}_{RS} = F_{RS} f_H A = \left( \sum_j n_{RS,j} v_{RS,j} \right) A_H \tau \left( \sum_i n_i v_i \varepsilon_i \right). \quad (6)$$

Without introducing too much error, we may put $\sum_j n_{RS,j} v_{RS,j} = n_{RS} v_{RS}$ and $\sum_j n_i v_i \varepsilon_i = n v \varepsilon$, where $n_{RS} v_{RS} \approx n v$.

This assumes that the total density of reactive species $n_{RS,j}$ is roughly the same as the total density of interactive species, $n$, which is a reasonable assumption. Also, for similar mass molecules, $v_i \approx v_{RS} \approx v$.

Then, (6) simplifies to

$$\dot{N}_{RS} = n^2 v^2 A_H \tau \varepsilon \quad (7)$$

and the total over a plasma application period $t_p (\geq \tau)$ is

$$N_{RS} = \dot{N}_{RS} t_p. \quad (8)$$

In order to be effective in killing the bacteria, $N_{RS}$ has to exceed a certain threshold or dose $N_{crit}$.

Hence, the condition for plasma disinfection becomes (using (7) and (8))

$$n^2 v^2 A_H A \cdot \tau \cdot \varepsilon \cdot t_p > N_{crit},$$

$$n > \frac{N_{crit}}{\sqrt{v^2 A_H A \cdot \tau \cdot \varepsilon \cdot t_p}}. \quad (9)$$

Using typical values: $v = 10^5$ cm s$^{-1}$, $A_H = 2.5 \times 10^{-13}$ cm$^2$, $A = 3 \times 10^{-8}$ cm$^2$, $\tau = 10^{-3}$ s, $\varepsilon = 0.1$ and $t_p = 10$ s, we obtain

$$n \geq 3 \times 10^6 N_{crit} \text{ cm}^{-3}. \quad (10)$$

Typical plasma densities obtained with low-power CAPs are $10^9$–$10^{10}$ cm$^{-3}$. Therefore, provided that 1000 ROS/RNS produce enough ‘overkill’ for bacteria, CAPs should be well placed to provide very efficient disinfection in timeframes of seconds, as has been observed repeatedly.
Figure 1. Antibiotic resistance of the used MRSA strain. The MRSA strain shows resistance against penicillin, ampicillin and cefoxitin (all beta-lactam antibiotics) and additionally to gentamicin (aminoglycosides), erythromycin (macrolides), doxycyclin (tetracyclines), ciprofloxacin (chinolones) and clindamycin (lincosamines).

3. Chemical reactions inside the cell (e.g. Fenton’s reaction—see [24]) which in the case of bacteria can lead to direct destruction of the DNA, which is located in the cytoplasm. The DNA of human cells is additionally protected both by chemical enzyme reactions [25–28] and by the cell nucleus membrane. Accordingly, a lethal dose for bacteria need not harm or cause permanent damage to human cells.

3. Experimental setup

3.1. Pretest for estimating the antibiotic resistance of Methicillin-resistant Staphylococcus aureus (MRSA)

To analyze the resistance of MRSA against different antibiotics, we used the Kirby–Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [29]. An overnight culture of a laboratory strain of MRSA was suspended in physiological saline and adjusted to a density of McFarland 0.5 (1.5 × 10^8 colony-forming units (CFU) ml^-1). Müller–Hinton agar plates were inoculated with the suspension using a swab. Paper discs, each impregnated with a defined concentration of a representative antibiotic, were placed on the agar surface. After incubation for 18 h in a 35°C incubator, the zones of inhibition of bacterial growth surrounding the discs were measured and interpreted according to the CLSI standards as resistant or susceptible. Figure 1 shows the experimentally obtained results. The used MRSA strain possesses resistance against penicillin, ampicillin and cefoxitin (all beta-lactam antibiotics) and additionally against gentamicin (aminoglycosides), erythromycin (macrolides), doxycyclin (tetracyclines), ciprofloxacin (chinolones) and clindamycin (lincosamines).
Figure 2. (a) Cross-sectional view of one SMD electrode. (b) Image of the plasma discharge. The wire electrode is clearly visible. The plasma micro-discharges occur inside the mesh very close to the surface.

4. Plasma dispenser

In this study, a CAP plasma device (HandPlaSter) published by Morfill et al [7] was used under ambient conditions (temperature ~20°C and relative humidity ~40%). This plasma device consists of two surface micro-discharge (SMD) plasma electrodes—each electrode containing an insulator plate made of Teflon (thickness 1 mm) sandwiched by a copper plate (thickness 1 mm) and an electrically grounded mesh grid (6 mesh per inch) (figure 2). For the plasma treatment of bacteria, only the lower electrode of 10×10 cm$^2$ was used (figure 2(a)). The plasma was produced between the mesh grid by applying a high ac voltage of 18–19 kV$_{pp}$ at 12.5 kHz between the metal plate and the mesh grid. The power consumption of this electrode is 0.5 W cm$^{-2}$ evaluated by the Lissajous figure method using a 1 µF capacitor. (For the treatment with plasma, bacteria inoculated on agar plates in Petri dishes were placed upside down on the lower electrode, so that the distance between the electrode and the agar surface equaled 6 mm.) As reported in Morfill et al [7], this plasma dispenser has bactericidal and fungicidal properties. The increase in gas temperature (measured with a thermocouple) was less than 2°C for the maximum treatment time, so that thermal bactericidal effects can be ruled out.

The ultraviolet (UV) emission spectrum of the plasma dispenser was measured with an AvaSpec-2048 fiber optic spectrometer (Avantes, USA) at a distance of 6 mm from the electrode as shown in figure 3 (the acquisition time was 500 ms). The small peaks in the UVC region of the spectrum correspond to NO and the peak at 309 nm results from OH. The other peaks in the UVA region correspond to nitrogen molecules. Furthermore, the UV power density in the wavelength range of 170–340 nm was measured with a Hamamatsu UV-power meter C8026 (Hamamatsu Photonics KK, Japan). The measured density at a distance of 6 mm was 2.1 µW cm$^{-2}$ and the effective light power integrated with the International Commission on Non-Ionizing Radiation Protection (ICNIRP) weighting function was 0.50 µW cm$^{-2}$.
5. Results

5.1. MRSA

For the performance tests with MRSA, a bacteria suspension of approximately $10^8$ cells ml$^{-1}$ was prepared in physiological saline using the Vitek Densicheck System (BioMerieux).

For the control, this bacteria suspension was diluted by a factor of $10^5$. Then, 200 $\mu$l of this diluted suspension was smeared out on a Müller–Hinton agar plate (8.6 cm diameter). After incubation at $35^\circ$C for 18 h, the resulting colony-forming units were counted.

The plasma performance tests were carried out with the undiluted original bacteria suspension. After 200 $\mu$l of this suspension (per plate) was applied to Müller–Hinton agar plates, the bacteria on the agar plates were then exposed to plasma (voltage $= 18$ kV, frequency $= 2.5$ kHz and power $= 0.5$ W cm$^{-2}$) for 2, 5, 15 and 30 s using the plasma system described previously [7]. (For treatment with plasma, bacteria inoculated on agar plates in Petri dishes were placed upside down on the lower electrode, so that the distance between the electrode and the agar surface was equal to 6 mm.) The plasma-treated agar plates were then incubated at $35^\circ$C for 18 h and the number of surviving bacteria, or CFUs, was counted afterwards. From this measurement the quantitative reduction of MRSA can be obtained (figure 4). It is evident that MRSA has no primary resistance against plasma treatment. There is an initial rapid decrease in bacterial load (log 5 in 2–5 s) followed by a slower decrease (log 1 in $\sim$10 s). This slower decrease is most likely due to bacteria that are embedded in the agar (the bacteria were spread using a spatula, and sometimes it is possible to mechanically push them into the substrate).
Figure 4. (a) Photographs of colonies formed by single surviving MRSA bacteria on agar plates after plasma treatment times of 2–30 s and incubation, as indicated. After incubation each surviving bacteria multiplied to form a colony that is clearly visible (CFU). For comparison a $10^5$ diluted untreated bacterial population is shown (top panel left). A log 5 reduction in bacterial load is obtained in 2–5 s. (b) The number of bacteria (i.e. the number of MRSA colonies) that survived after different plasma treatment times (red data points). For comparison, the $10^5$ diluted control sample is shown (blue data point).

5.2. Bacterial resistance tests with E. coli and E. mundtii

For the bacterial resistance tests, a suspension of the respective bacteria of approximately $10^8$ cells ml$^{-1}$ in physiological saline was prepared. As described previously, 200 $\mu$l of this suspension was smeared out on each of three agar plates, which were prepared in parallel. These agar plates were then exposed to plasma (voltage = 18 kV, frequency = 12.5 kHz, power = 0.5 W cm$^{-2}$) and afterwards incubated at 35 $^\circ$C for 18 h. After incubation the number of surviving bacteria, or CFUs, was counted. Bacteria that survive the plasma treatment and afterwards form CFUs can exist ‘accidentally’ (e.g. due to shielding, etc) or they may be resistant. To determine the cause of survival quantitatively and to test for resistance, reasonable numbers of bacteria, which survive the plasma treatment, are needed. To obtain ‘reasonable numbers’ of surviving CFUs (~10) the plasma treatment time for E. coli was found to be optimal for a plasma treatment time of 60 s. In the case of E. mundtii, the treatment time was 30 s. We note in passing that E. coli is a very good candidate for resistance tests.
It is a very common bacterium and from the many EHEC mutations known to be very adaptable.

For the production of the next generation, the CFUs from one of the three agar plates were collected and in turn dissolved in phosphate-buffered saline (PBS) to a bacterial density of $10^8$ cells ml$^{-1}$. The implicit assumption here is that, among the surviving bacteria, there would be some that possess resistance against the treatment and that this property is passed on during multiplication. Then, $3 \times 200 \mu l$ of this suspension were again smeared out on three agar plates, which were afterwards treated with plasma. This experiment was repeated four times for *E. coli* and *E. mundtii*, so that four generations of bacteria were produced and, in addition, treated with plasma (figure 5).

As described earlier for the performance tests with MRSA, controls were produced for every generation by diluting the bacteria suspension of the respective generation by a factor of $10^5$. Then, $200 \mu l$ of the respective diluted control suspension was then smeared out on agar and incubated at 35 °C for 18 h so that the number of CFUs could be counted.

As described previously we tested four generations of bacteria for each strain—the first generation from a bacteria stock solution, the second generation from the survivors of the first generation of bacteria and so on. In short, the survivors of the respective generation were used for the production of the next generation. The experimental results obtained for *E. coli* are depicted in figures 6(a) and (b). The first column in figure 6(a) functions as a control and shows agar plates, which contained a $10^5$ bacteria dilution of the respective generation. The agar plates in columns 2, 3 and 4 (samples 1–3 for all four generations) originally contained the undiluted bacteria suspension of the respective generation and were treated with plasma for 60 s. All agar plates were incubated overnight to allow the formation of CFUs, which were afterwards counted (white and red numbers depicted on the agar plates in figure 6(a)). The number of CFUs on the control agar plates in the first column (each containing $200 \mu l$ of the $10^5$ dilution of the original bacteria suspension of the respective generation) can be used to recalculate the number of bacteria, which were originally spread out on samples 1–3 of the respective generation, before
Figure 6. Experimental results of the bacterial resistance tests with *E. coli*. (a) Photographs of agar plates containing *E. coli* colonies. The agar plates in the first column represent the control and contain the $10^5$ bacteria dilution of the respective generation. In columns 2, 3 and 4 the agar plates (samples 1–3 for all four generations), which contained the undiluted bacteria suspension of the respective generation, were treated with plasma for 60 s. All agar plates were incubated for 18 h to allow the formation of colonies. The numbers on the agar plates refer to the number of CFUs. The colonies whose numbers are marked in red were collected (as described earlier) and used for the production of the next generation. (b) The number of bacteria (i.e. the number of *E. coli* colonies) that survived, plotted against the respective generation (red data points). For comparison, the $10^5$ diluted control samples are shown additionally (blue data points). No increase in the number of surviving bacteria for higher generations was detected.

the plasma treatment. The CFUs counted on the plasma-treated agar plates correspond to the individual bacteria, which survived the plasma treatment (survivors). Using this information the reduction factor for every plasma-treated sample can be calculated. For example, in generation 2, 115 CFUs were counted in the $10^5$ diluted control sample. From this, one can conclude that
Figure 7. Experimental results of the bacterial resistance tests with \textit{E. mundtii}. (a) Photographs of agar plates containing \textit{E. mundtii} colonies. The agar plates in the first column again represent the control and contain the $10^5$ bacteria dilution of the respective generation. In columns 2, 3 and 4, the agar plates (samples 1–3 for all four generations) that contained the undiluted bacteria suspension of the respective generation were treated with plasma for 30 s. All agar plates were incubated for 18 h to allow the formation of colonies. The numbers on the agar plates refer to the number of CFU. The colonies whose numbers are marked in red were collected and used for the production of the next bacteria generation. (b) The number of bacteria (i.e. the number of \textit{Enterococcus} colonies) that survived the plasma treatment, plotted against the respective generation (red data points). For comparison, the $10^5$ diluted control samples are shown (blue data points). No increase in the number of surviving bacteria for higher generations was detected.

originally $\sim 1 \times 10^7$ bacteria were spread out on sample 1 (and on samples 2 and 3). After plasma irradiation and incubation, the agar plates (samples 1–3) contained between 4 and 22 CFUs. This leads to a reduction factor of $\sim 3 \times 10^6$ for a plasma treatment time of 60 s (sample 1).
Figure 6(b) shows the experimental results for *E. coli*. In this graph, the number of bacteria (i.e. the number of *E. coli* colonies) that survived is plotted against the respective generation (red data points). For comparison, the $10^5$ diluted control samples are also plotted (blue data points). This graph clearly shows that a bacterial reduction larger than $10^5$ is achieved for every generation after a 60 s plasma treatment. Furthermore, no increase in the number of surviving bacteria for higher generations was detected.

For illustration, if in a given generation with e.g. 10 CFUs there was one that had developed resistance, then the next generation would show about $10^6$ survivors! This is a very clear signal. We will discuss this in detail in section 6.

For the bacterial resistance experiments for *E. mundtii*, similar results as those for *E. coli* were obtained (figures 7(a) and (b)). As can be seen in figure 7(a), on average the number of survivors, i.e. the number of bacteria that survived the plasma treatment, is larger for *E. mundtii* compared with *E. coli* (figure 6(a)). This is due to the lower plasma exposure time (30 s instead of 60 s). From figure 7(b) we see that a bacterial reduction of $\sim 10^5$ is achieved for every generation. Again no increase in the number of surviving bacteria for higher generations was detected.

### 6. Analysis and interpretation

Resistance once acquired becomes a major hazard if it can be transmitted by the pathogens on cell division. ‘Spurious resistance’ of individual bacteria may in principle also exist, but can in general be ignored since it is only large numbers that make an infection hazardous. Our test series was designed to investigate primary and acquired transmittable resistance.

1. **Primary resistance**

   We first illustrate the principle of the measurements and then present the results. If we start off with a sample of $N_1$ (e.g. $10^3$) bacteria and plasma-treat this, then after incubation we observe $n_1$ (e.g. 20) CFUs, i.e. $n_1$ survivors. These survivors could be primary resistant or accidental. We then regrow all these $n_1$ colonies until we have $N_2$ (e.g. $10^5$) bacteria, which are distributed on another agar plate. If there were primary resistant bacteria among the survivors, each individual one would on average produce $N_2/n_1$ (e.g. $5 \times 10^5$ using the above numbers) resistant bacteria in this second batch. The $N_2$ bacteria are again plasma treated and after incubation we count $n_2$ (e.g. 20) CFUs, i.e. $n_2$ survivors. If $n_2 \ll N_2/n_1$ we know that there were no primary resistant bacteria. We took great care to collect all bacteria, but there is always a slight chance that one CFU (of the $n_1$ present) might have been overlooked. The probability that this one originates from a resistant bacterium is (statistically) given by $1/n$. There is also a test (statistically) uncertainty in the result of $P_{11} \ll 1/N_1$ (the first subscript signifies the one possible missed CFU and the second subscript the tested generation).

2. **Acquired (secondary) resistance**

   The basic idea is to see if bacteria develop some defence strategy or not. To test for this, the procedure used for determining the primary resistance (see above) is repeated to the fourth generation. This gives three generations (including the first one if there was no primary resistance detected) with which to build up the statistics for the acquired resistance. Since we may treat each generation as statistically uncorrelated, the same argument as before holds:

   If $n_{i+1} \ll N_{i+1}/n_i$, where $i = 1, 2, 3$, then we know that no secondary (acquired) resistance has developed. By arbitrarily assuming that at most one CFU has been overlooked in each
In each case the number of CFUs was counted after plasma treatment and incubation. Assuming an equal likelihood of overlooking a single CFU gives a total probability of $O_i = 1/n_i a n_i b n_i c$ (e.g. $1/20 \times 20 \times 20 = 1/8000$ for the above numerical example of 20 CFUs per plate) of that CFU being due to a resistant bacterium (the 1 again stands for one overlooked CFU). For the three generations tested, the combined (uncorrelated) probability is

$$O_s \leq \prod_i O_{1i} \quad \text{and} \quad O_3 = O_{11} \cdot O_{12} \cdot O_{13}.$$ 

The probability of acquired (secondary) resistance, $P_S$, is then altogether $P_S \leq P_3 \cdot O_3$. We note in passing that the total probability of primary resistance is $P_P = P_{11} \cdot P_{12} \cdot P_{13}$ since for the first generation we only had two samples.

**Quantitative estimates and uncertainties.** The measured primary resistance to plasma treatment is consistent with zero—not a single bacterium was resistant. The (statistical) measurement uncertainty for *E. coli* is

$$P_P \leq \frac{10^{-7}}{2 \times 49} \cdot \frac{1}{38} \cdot \frac{1}{21} = 1.5 \times 10^{-12}.$$ 

The measured secondary resistance to plasma treatment is also consistent with zero. The (statistical) measurement uncertainty for *E. coli* is

$$P_S \leq \frac{10^{-21}}{2 \times 49 \times 1.15 \times 1.47} \left( \frac{1}{38 \times 21 \times 4 \times 18 \times 22 \times 1 \times 9 \times 13} \right) = 4.0 \times 10^{-32}.$$ 

Similarly, for the primary resistance of *E. mundtii* we have a result consistent with zero (no primary resistance) and an uncertainty of

$$P_P \leq \frac{10^{-7}}{0.82} \cdot \frac{1}{87 \times 23} = 6.1 \times 10^{-11}.$$ 

For the secondary resistance of *E. mundtii*, we have again a result of

$$P_S \leq \frac{10^{-18}}{8.2 \times 1.5 \times 0.8} \cdot \left( \frac{1}{87 \times 23 \times 12 \times 45 \times 26 \times 22 \times 46 \times 6} \right) = 6.0 \times 10^{-31}.$$ 

**7. Conclusion**

We have performed resistance tests for Gram-positive and Gram-negative bacterial strains against plasma treatment. Primary resistance or acquired resistance to some bacterial strains would constitute a severe restriction for the envisaged usefulness of plasmas in all areas of hygiene. Obviously, with the available techniques there has to be some limit regarding quantitative results. Our measurements are consistent with no primary resistance and no acquired resistance. The purely statistical upper limits (based on accidentally ‘missing’ a resistant bacterium in collection) are in the range of

$$P_P \leq 10^{-10},$$



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i.e. we cannot rule out on statistical grounds that 1 in 10 billion bacteria may be primary resistant.

For the acquired (secondary) resistance the upper limit is much more stringent, since we used four generations of bacterial growth. It is in the range of

\[ P_S \leq 10^{-30} \]

To put these numbers into some perspective, \(10^{12}\) bacteria weigh roughly 1 g (given a typical bacterial mass of \(10^{-12}\) g). Hence, the upper limit for acquired resistance corresponds to 1 bacterium out of \(10^{15}\) kg of bacterial biomass to (perhaps) become resistant to plasma treatment. (Just for comparison, \(10^{15}\) kg of biomass corresponds to roughly one millionth the mass of the Earth!) Note that our test for acquired resistance does not admit back-evolution regarding the primary resistance, since all the bacteria grown were (by definition) descendants of bacteria that showed no primary resistance. The primary upper limit estimate is therefore the weakest link, one that will be difficult to better dramatically in the types of tests performed here.

However, since the best-known resistant bacterium, MRSA, also showed no primary resistance against plasma treatment, we feel confident that we have a bactericidal process here that may be very difficult to overcome. The reasons were outlined at the beginning—the diverse paths available to CAP for membrane permeabilization, reactive species generation and bacterial inactivation.

For future work it will be important to investigate a whole range of bacteria in this way, to improve the statistics, to identify and pinpoint the bacterial mechanisms of plasma treatment and to optimize the plasma design, tailoring it (perhaps) to the specifics of the targets (bacteria, spores, virus and fungi).

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References

[1] 2011 Der Spiegel (Hamburg: Spiegel)
[2] EARSS (European Antimicrobial Resistance Surveillance System) 2008 Annual Report Period 1999–2007 ISBN: 978-90-6960-214-1
[3] Grundmann H et al 2006 Lancet 368 874–85
[4] McDonnell G E 2007 Antisepsis, Disinfection and Sterilization (Washington, DC: ASM)
[5] Isbary G et al 2010 Br. J. Dermatol. 163 78
[6] Isbary G, Morfill G, Zimmermann J, Shimizu T and Stolz W 2011 Arch. Dermatol. 147 388
[7] Morfill G E, Shimizu T, Steffes B and Schmidt H-U 2009 New J. Phys. 11 115019
[8] Kong M G, Kroesen G, Morfill G E, Nosenko T, Shimizu T, van Dijk J and Zimmermann J L 2009 New J. Phys. 11 115012
[9] Przybylska M, Bryszewska M and Chapman I V 1993 Int. J. Rad. Biol. 63 419
[10] Lövenklav M, Arunder P and Borch E 2010 SIK-Swedish Institute for Food and Biotechnology, Goteburg unpublished report

New Journal of Physics 14 (2012) 073037 (http://www.njp.org/)
[11] Babaeva N Y and Kushner M J 2010 J. Phys. D: Appl. Phys. 43 185206
[12] Steel B, Bilek M M, dos Remedios C G and McKenzie D R 2004 Eur. Biophys. J. 33 117
[13] Wilson K R, Zou S, Shu J, Rühl E, Leone S R, Schatz G C and Ahmed M 2007 Nano Lett. 7 2014
[14] Liburdyana R P and Vanek P Jr 1985 Rad. Res. 102 190
[15] Ivanov I T and Boytcheva S 1994 J. Therm. Biol. 19 199
[16] Leduc M, Guay D, Leask R L and Coulombe S 2009 New J. Phys. 11 115021
[17] Halliwell B and Gutteridge J M C 2007 Free Radicals in Biology and Medicine (New York: Oxford University Press)
[18] Yonson S, Coulombe S, Léveillé V and Leask R L 2006 J. Phys. D: Appl. Phys. 39 3508
[19] Stoffels E, Sakiyama Y and Graves D B 2008 IEEE Trans. Plasma Sci. 36 1441
[20] Evans E and Rawioz W 1990 Phys. Rev. Lett. 64 2094
[21] Sakiyama Y, Graves D B, Shimizu T and Morfill G E 2010 63rd GEC and 7th ICRP (Paris, France)
[22] Sakiyama Y, Graves D B, Jarrige J and Laroussi M 2010 Appl. Phys. Lett. 96 041501
[23] Sakiyama Y and Graves D B 2009 Plasma Sources Sci. Technol. 18 025022
[24] Nosenko T, Shimizu T and Morfill G E 2009 New J. Phys. 11 115013
[25] Yoshioka Y, Kitao T, Kishino T, Yamamuro A and Maeda S 2006 J. Immunol. 176 4675
[26] Wink D A, Hanbauer I, Krishna M C, DeGraff W, Gamson J and Mitchell J B 1993 Proc. Natl Acad. Sci. USA 90 9813–7
[27] Kotamraju S, Tampo Y, Keszler A, Chitambar C R, Joseph J, Haas A L and Kalyanaraman B 2003 Proc. Natl Acad. Sci. USA 100 10653
[28] Chae H J, Kim H R, Kwak Y G, Ko J K, Joo C U and Chae S W 2003 Immunopharmacol. Immunotoxicol. 23 187
[29] CLSI 2006 Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard 9th edn, vol 26 (Wayne, PA: CLSI)