



Evaluation of Bacterial Biofilm Removal Properties of MEDSTER 2000 Cold Sterilant on Different Materials

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Abstract

We studied the antibacterial and anti-biofilm properties of MEDSTER 2000, a pH neutral biodegradable mixed acidic peroxide disinfectant belonging to the class I Ib medical device which has been designed for decontamination and cold sterilization of hospital instruments. The broth microdilution method was used to define the antibacterial activity against planktonic form of both classified bacteria and antibiotic resistant strains of clinical source, whereas effectiveness toward their biofilm was determined on mature biofilm, grown both on plastic and stainless steel surfaces. The results showed that for the planktonic form the antibacterial activity of MEDSTER 2000 was already observed after 10 min at the lowest concentration (0.1%), and this effect was not exposure-and/or concentration-dependent. After the same time of exposure at the concentration of 2% the disinfectant was able to completely eradicate all tested bacteria

grown in sessile form on both surfaces, with a greater than 6 log CFU/cm² reduction in viable cells. This result is supported by the microscope observation by crystal violet and live/dead assays. For the high antibacterial and anti-biofilm ability emerged, MEDSTER 2000 could represent a new and more effective approach for semicritical devices that need a high-level disinfection and could not sustain the process of heat sterilization.

Keywords

Antimicrobial activity · Biocide · Biofilm · High-level disinfection · Medical device · Pathogens

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1 Introduction

Biofilm is a structured community of microorganisms, enclosed in a self-produced polymeric matrix (mainly polysaccharides), variously stratified and containing bacterial cells of different living and dormant species inside. Biofilm, in fact, has been defined by some as “a city for microbes”, while by others it is equated with an analogue of a multicellular organism. The formation of biofilms offers ecological advantages to the resident microorganisms, including protection from the environment (eg temperature, pH and osmotic extremes, exposure UV light, drying),

increased availability of nutrients, metabolic enhancement and facilitation of genetic material transfers (Davey and O'Toole 2000; Watnick and Kolter 2000; Assere et al. 2008; Messi 2013).

Unlike the more commonly studied and well-understood planktonic (fluctuating) form, biofilms represent the predominant form of bacterial growth, and it is estimated that 80% of all human infections are of biofilm origin (Hu et al. 2015; Percival et al. 2015; Costa et al. 2019). Various inert substrates such as Teflon™, stainless steel, rubber and polyurethane can even support the adherence and growth of biofilm, which is regulated by various environmental conditions such as pH, temperature and concentration of dissolved mineral salts. This microbial consortium is a complex system that protects microbes from environmental stress and allows its "inhabitants" to better resist, providing a real physical barrier against antimicrobial substances such as antibiotics, disinfectants and bacteriocins produced by competing microorganisms.

This increased resistance to antibiotics and disinfectants can be estimated from 10 to 1000 times, depending on the studies (Davies 2003; Otter et al. 2015). This represents a real problem and a potential health hazard, especially in health facilities and hospitals where these wild strains can establish themselves and spread as resident surface environmental flora on surgical and diagnostic equipment.

Most of the products used for the disinfection of hospital's surfaces and instruments include quaternary ammonium salts (QACs), aldehydes, chlorine-based products, hydrogen peroxide and peracetic acid. Many of these, however, show serious limits when tested against biofilms. In fact, in order to be registered, all disinfectants must be tested on bacteria in suspension tests, but only few compounds have been tested on consolidated biofilms. QACs have long been shown to be ineffective on *Staphylococcus aureus* and *Pseudomonas aeruginosa* hospital isolated strains (Guerin-Mechin et al. 1999; Méchin et al. 1999). The limited penetration of chlorine-based products into the biofilm matrix

can explain the reduced killing action towards microorganisms living inside (de Beer et al. 1994; Jang et al. 2006). More interesting data have been reported on oxidizing agents, especially on peracetic acid which has been demonstrated to have a better performance on consolidated biofilm on different materials and surgical instruments than aldehydes (Neves et al. 2016), chlorine-based products, alcohol and others (Tote et al. 2010; Ledwoch and Maillard 2018; Skowron et al. 2018; Chowdhury et al. 2019).

Given all these data from literature, we tested the anti-biofilm property of MEDSTER 2000 – cold sterilant (Euro Medical Center srl – Firenze – Italy), a class IIb medical device in a sophisticated powder form used to reprocess medical and odontoiatric instruments. Once dissolved into water, this compound shows a synergistic activity of peracetic acid (1500 ppm a 1% concentration) with hydrogen peroxide and organic acids (acetic acid and citric acid), thus improving its biocidal action at a balanced neutral pH environment (around 7). Unlike other similar products, technical data confirm that MEDSTER 2000 solutions have a more stable peracetic acid titration even several days after its preparation. Its bactericidal, virucidal, mycobactericidal, fungicidal and sporicidal activity has been largely demonstrated in standard UNI EN protocols since 0,1% dilution and 5 min contact time. The peracetyl ions released in such a neutral pH environment have been recently described as a crucial and as a further advantage in the reduction of viable count of biofilms over conventional peracetic acid acting at pH value lower than 4 (Meyer et al. 2019).

2 Materials and Methods

2.1 Bacteria, Culture Conditions and Biocide

Reference strains (ATCC-American Type Culture Collection) and antibiotic resistant clinical isolates were used. Gram positive *Staphylococcus aureus* ATCC 6538 and methicillin-resistant *Staphylococ-*

cus aureus (MRSA), Gram negative *Escherichia coli* ATCC 25922 and extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 9027 and *Clostridium difficile* ATCC 9689 were grown in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (TSB-YE) (Difco), and kept at 30 °C for 18 h. All cultures were centrifuged at 2000g for 20 min. After discarding the supernatant fluid, the pellets were re-suspended in 5 ml sterile deionized water. Centrifugation, supernatant discard and re-suspension were repeated three times. The density of the final suspensions was measured on selective media (Mannitol Salt Agar for Gram-positive bacteria, MacConkey Agar for Gram-negative strains and Clostridium Difficile Agar with 7% Sheep Blood for *Clostridium difficile*), all from Difco Laboratories, Detroit, MI) by the plate count method.

The obtained suspensions were stored until required in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g Na₂HPO₄·12H₂O, 0.2 g KH₂PO₄ with 1 l of distilled water) supplemented with 30% (vol./vol.) glycerine at -80 °C.

MEDSTER 2000 (Euro Medical Center srl, Firenze, Italy) is a class IIb medical device which has been designed for decontamination and cold sterilization of invasive and non-invasive odontoiatric, surgical, hospital and laboratory surfaces and instruments which cannot be processed through heat sterilization or autoclaving.

MEDSTER 2000 can be defined as a pH neutral biodegradable mixed acidic peroxide disinfectant in powder form. Once activated into water it can release active oxygen in a neutral pH solution. The formulation is enhanced by the presence of surfactant, corrosion inhibitors and a mixture of organic acids with their own specific biocidal properties offering a rapid killing action against microbial contaminants within a few minutes of contact.

Its cold sterilizing profile has been well documented with all European Standard tests to obtain the CE mark registration as a medical device but no tests against biofilms have been done before. The aim of this study is to verify the efficacy profile of this product against a mature biofilm on different materials.

2.2 Antibacterial Activity of Medster 2000

The study was divided into three set of experiments: treatment of bacteria in planktonic form, eradication of preformed (mature) biofilm on plastic surface, eradication of preformed (mature) biofilm on stainless steel surface.

All the experiments were carried out in triplicate and the bacterial count was performed on three plates. The arithmetic means of the three determinations, expressed as log bacterial count, was plotted against biocide concentrations. The results were analysed statistically with the Student's *t*-test and differences were considered significant when $p < 0.05$.

2.2.1 Treatment of Bacteria in Planktonic Form

The antibacterial activity of MEDSTER 2000 was assessed by the broth microdilution method. The washed suspensions were diluted up to 10⁸ CFU/ml (colony forming units) and 100 μ L inoculated into on 96-well polystyrene microtiter plates and the biocide solution (100 μ L) was added to the suspensions at various concentrations: 1%, 0.5%, 0.25% and 0.1%. After 10- and 20-min incubation at room temperature, the viable cells were measured by the plate count method, performed spreading 100 μ L of samples on plates added with selective media, incubated at 37 °C for 24 h.

2.2.2 Eradication of Preformed (Mature) Biofilm on Plastic Surface

The effectiveness of biocidal treatments was tested on '2 day-old' pre-formed biofilm using Gram-positive *Staphylococcus aureus* ATCC 6538 and MRSA *Staphylococcus aureus*, Gram-negative *Escherichia coli* ATCC 25922 and ESBL *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 9027 as single-inoculated cultures. The assay was performed using a modified 96-well microtiter-plates method (Stepanović et al. 2007; Condò et al. 2020) under static conditions. The mature biofilm was obtained adding 180 μ l of TSB and 20 μ l of washed suspensions (10⁵ CFU/ml) to each wells

of a 96-well polystyrene microtiter plates. After incubation for 48 h at 30 °C, to allow for cell attachment, the bottoms of the 96 well-plates were washed three times with sterile *PBS* in order to remove planktonic cells and 100 µL of MEDSTER 2000 at 2% concentration was added. Following a contact time of 10 min at room temperature, the possible residual biofilm was determined removing biofilm by scraping the entire surface of each well bottom with a sterile plastic loop. Serial tenfold dilutions of the obtained re-suspensions were spreaded onto appropriate agar plates for the viable cell count (CFU cm⁻²). The colonies were counted following incubation at 37 °C for 24 h. Controls were performed by plate count method, adding in the wells bacterial culture only.

2.2.3 Eradication of Preformed (Mature) Biofilm on Stainless Steel Surface

Biofilms produced by all the species as above were grown on stainless steel AISI 316 coupons (25 cm²), previously treated with HCl 5 N for 10 min and washed in a detergent solution (ethanol 70%) with sonication (model Elma Transonic T570. Elma GmbH & Co KG, Elma GmbH & Co KG Kolpingstr. 1-7 D-78224 Singen/Germany) for 20 min to detach debris, rinsed in distilled water, and sterilized by autoclaving at 121 °C for 15 min. The mature biofilm was obtained introducing the coupons in a 50-ml tube containing TSB broth added with 0.1 ml of washed bacterial suspensions (10⁵ CFU/ml). After incubation at 25 °C for 48 h, to allow for cell attachment, each coupon was rinsed in 500 ml of sterile *PBS* and the anti-biofilm activity determined after 10 min contact, both immersing the coupon in a 2% solution or sprinkling the surfaces with the same solution. After this time, the coupons were washed three times with sterile saline solution to remove unattached cells, sonicated for 15 min, and vortexed. Serial tenfold dilutions of the obtained re-suspensions were spreaded onto appropriate agar plates for the viable cell count (CFU cm⁻²). The colonies were counted following incubation at 37 °C for 24 h. Controls were performed by plate count method, adding in the wells bacterial culture only.

2.3 Evaluation of Anti-biofilm Activity by Light Microscopy

The effectiveness of biocidal treatments on pre-formed biofilm on plastic and stainless steel surfaces was also evaluated with a morphological study using a Light Microscope. Both the mature biofilms formation and the anti-biofilm activity of MEDSTER 2000 at 2% were tested as described above. Concerning biofilm on plastic surface the assay was performed using 6-well polystyrene microtiter plates, to have a larger optical field to observe under microscope.

2.3.1 Anti-biofilm Evaluation by Crystal Violet (CV) Assay

Biofilms grown on polystyrene wells, both treated with MEDSTER 2000 at 2% and untreated control, were washed three times with sterile *PBS* and fixed with 150 µL of methanol for 5 min. Then, the supernatant was removed again and 150 µL of CV solution at 0.1% was added to each well. After incubation at room temperature for 30 min, the excess of CV was removed by washing three times with sterile *PBS*. The biofilm on the stainless steel coupons treated and untreated with biocide solution at 2% was washed three times with sterile *PBS* and the attached cells were fixed with paraformaldehyde for 1 h at 4 °C. Subsequently, the stainless steel surfaces were washed twice with sterile *PBS* and 150 µL of CV solution at 0.1% was added. After incubation for 30 min at room temperature, the unbound dye was removed by washing three times with sterile *PBS* and a Scotch tape was stucked by the adhesive side to the stainless steel coupon for 15 min at room temperature. Then, the tape with the adherent CV stained biofilms was removed and put on a glass slide.

Both the glass slides and the polystyrene wells were analyzed by Light Microscope Nikon Eclipse 90i imaging system equipped with Nomarski DIC optics (Nikon Instruments, Melville, NY, USA). DS-2Mv Nikon digital camera was employed to obtained images.

2.3.2 Anti-biofilm Evaluation by Live/Dead Assay

Both biofilm formation (used as control) and biocidal activity evaluation of MEDSTER 2000 at 2% was performed as described above. Biofilms treated with MEDSTER 2000 at 2% and untreated control were washed twice with sterile PBS and stained by the “live/dead cells stain kit” (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer’s instructions. The method is based on the use of propidium iodide (PI) as marker of dead cells and 5(6)- carboxyfluorescein diacetate (cFDA) to detect alive cells. After incubation in the dark at room temperature for 30 min, the samples were analyzed using the same light instrument as above.

biocide. MEDSTER 2000 inhibited the growth of all the strains and this effect was not exposure-and/or concentration-dependent. In fact, already after treatment for 10 and at all the biocide concentrations MEDSTER 2000 was able to eliminate the viable cells of all tested bacteria (range of p-value from 0.00014 to 0.0011) as no cells grew, as revealed by plate count method.

3 Results

3.1 Antibacterial Activity of Medster 2000

3.1.1 Treatment of Bacteria in Planktonic Form

The activity of MEDSTER 2000 against planktonic bacteria was determined by viable count following 10 and 20 min of exposure to 1%, 0.5%, 0.25% and 0.1% concentrations of the

3.1.2 Eradication of Preformed (Mature) Biofilm on Plastic Surface

Biofilm eradication is considered the strictest measure of the efficacy of a biocide rather than bacterial viable count reduction. Biofilm is a structured community of microorganisms that offers ecological advantages, including protection from the antibiotics and disinfectants. After 10 min of exposure at the concentration of 2% MEDSTER 2000 was able to eradicate all tested bacteria grown on plastic surface in sessile form, with a greater than 6 log CFU/cm² reduction in viable cells of biofilm (range of p-value from 0.0004 to 0.0009) (Fig. 1).

3.1.3 Eradication of Preformed (Mature) Biofilm on Stainless Steel Surface

The anti-biofilm activity of MEDSTER 2000 at 2% was also determined on the stainless steel AISI 316, a surface usually employed for devices

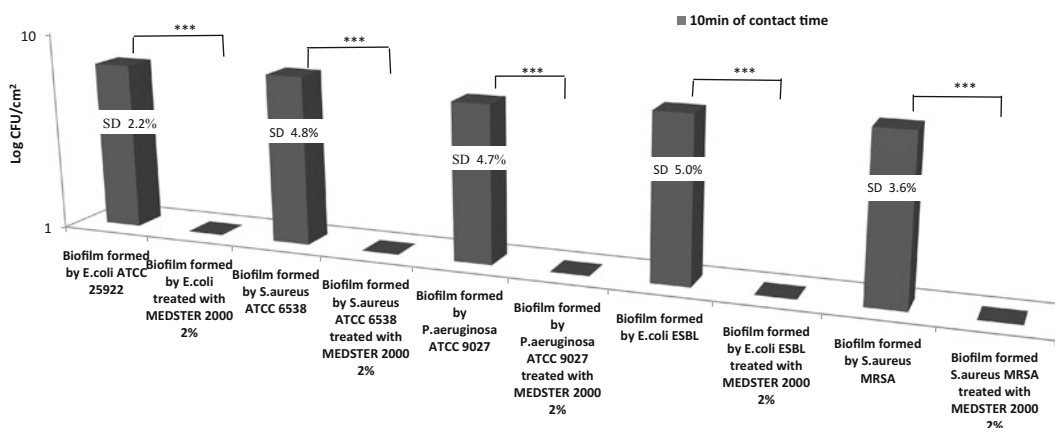


Fig. 1 The anti-biofilm activity of MEDSTER 2000 at 2% on the plastic surface after 10 min of exposure. Results were expressed in log₁₀ CFU/cm² as the arithmetic mean of the three determinations. The standard deviation

(SD) presented a range from 2,2% to 5% for the controls and 0% for the samples. p- value of <0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****) were considered significant

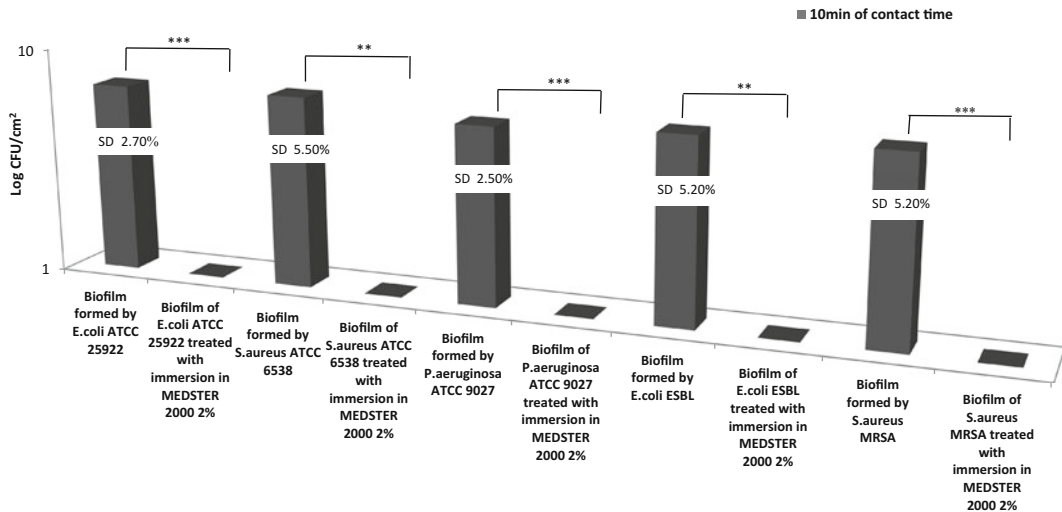


Fig. 2 The anti-biofilm activity of MEDSTER 2000 at 2% on the stainless steel AISI 316 surface after 10 min of exposure by immersing the coupon in a 2% solution. Results were expressed in \log_{10} CFU/cm² as the arithmetic mean of the three determinations. The standard deviation

(SD) presented a range from 2,7% to 5,5% for the controls and 0% for the samples. p -value of <0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) were considered significant

and implant materials in the hospital setting. The ‘2 day-old’ biofilms on stainless steel were treated with the biocide solution for 10 min both immersing the coupon in a 2% solution and sprinkling the surfaces with the same solution. Even on this surface MEDSTER 2000 was able to eradicate biofilms formed by all strains examined, with cell counts reduction greater than 6 log CFU/cm². Furthermore, no differences were found between the two methods used (range of p -value from 0.00012 to 0.0014). The Fig. 2 shows, as example, of the data obtained with the method of immersion. Lastly, the anti-biofilm activity of MEDSTER 2000 at 2% was equal both on plastic and on stainless steel surfaces, so its effect was not typology surface-dependent.

3.2 Evaluation of Anti-biofilm Activity by Light Microscopy and by Live/Dead Assay

The anti-biofilm activity of MEDSTER 2000 at 2% on pre-formed biofilm on plastic and stainless

steel surface was evaluated using a Light Microscope.

3.2.1 Anti-biofilm Evaluation by Crystal Violet (CV) Assay

The microscopic observations showed a meaningful eradication in the structures of biofilm and an evident decrease of the number and the adherent cells on both surfaces (Figs. 3 and 4).

3.2.2 Anti-Biofilm Evaluation by Live/Dead Assay

To verify the viability of bacteria observed with the Light Microscope, after the treatment with MEDSTER 2000 at 2% on the different surfaces, the cells were stained with PI and CFDA to discriminate live from dead cells. As shown in Figs. 5 and 6, the treatment with the biocide solution for 10 min led to the decrease in the amount of cells embedded in the polymer matrix of biofilm. Moreover, the few remaining cells were red colored, which makes them definable as dead cells.

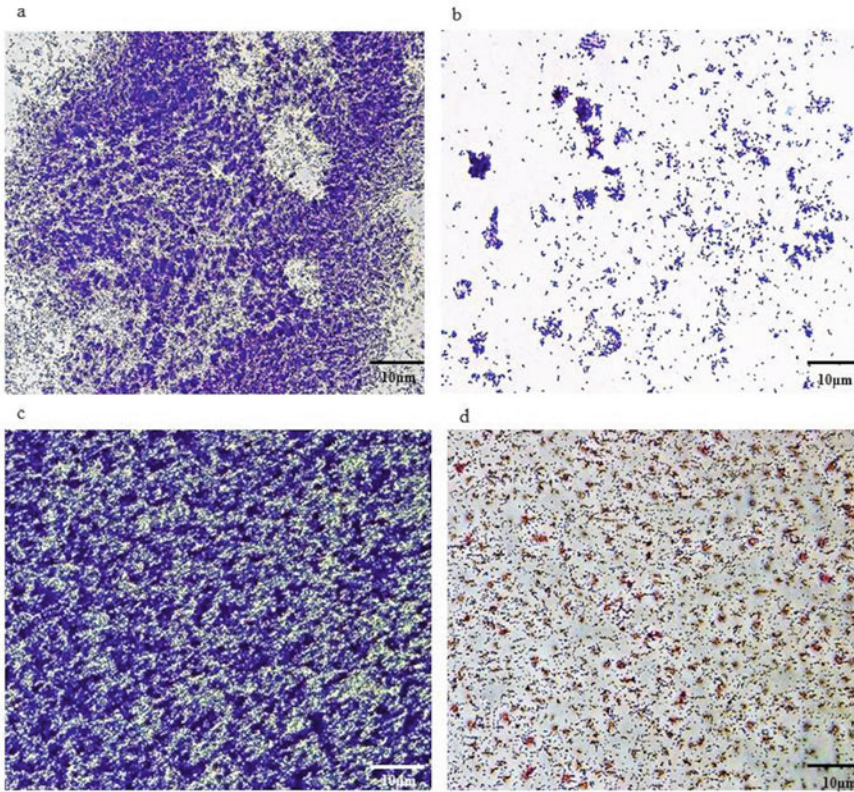


Fig. 3 *Escherichia coli* ATCC 25922 (a and b) and *Staphylococcus aureus* ATCC 6538 (c and d) ‘2 day-old’ mature biofilm on plastic surface. Images of light

microscopy obtained by using Crystal Violet (CV) Assay before (a) (c) and after (b) (d) 10 min disinfection with MEDSTER 2000 at 2%. The scale bars indicate 10 µm

4 Discussion

The constant increase of hospital acquired infections (HAI) is a cause of concern, particularly when they are due to multidrug-resistant (MDR) bacteria. The risk of acquiring methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), extended spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae*, *Clostridium difficile* infections is increased over time (ECDC 2019) and it contributes to morbidity and is considered a major risk factor for mortality. The environment has long been recognized for having an important role in dissemination of microbial pathogens, and biofilm in particular represents an important reservoir of the involved bacteria. Biofilm represents a functional ecological niche for pathogenic and

opportunistic strains, and there is now proof that some HAI outbreaks are related to the presence of biofilms (Hall-Stoodley and Stoodley 2005; Percival et al. 2015). The ability to form biofilm, is a critical feature already reported in a lot of studies and represents an evolutionary advantage for the microorganisms living within because this microbial consortium offers protection against different adverse conditions, including disinfection treatments.

In these scenarios the best studied biofilm infections are those related to the colonization of central venous catheters which lead to a mortality ranging from 12% to 25% with an additional cost for the healthcare facility estimated at \$ 33,000–35,000 per event (El-Azizi et al. 2016).

In instruments with a complex structure, such as gastroscopes and endoscopic fibers in general,

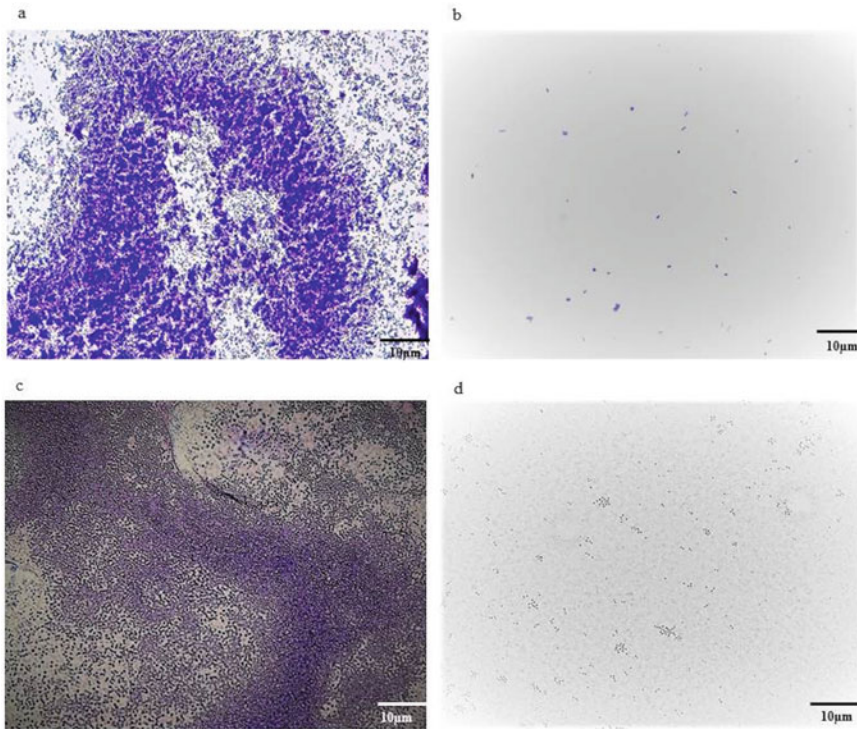


Fig. 4 *Escherichia coli* ATCC 25922 (**a** and **b**) and *Staphylococcus aureus* ATCC 6538 (**c** and **d**) '2 day-old' mature biofilm on stainless steel. Images of light

microscopy obtained by using Crystal Violet (CV) Assay before (**a**) (**c**) and after (**b**) (**d**) 10 min disinfection with MEDSTER 2000 at 2%. The scale bars indicate 10 µm

the operating channels are the perfect site for the colonization by a resistant biofilm (Alfa and Singh 2020). The presence of stagnant water, contamination from organic dirt and high initial microbial load are all factors favoring the biofilm formation. In simulation studies of contamination of the working canals with high microbial charges it was shown how decontamination and prewashing procedures are fundamental in the reduction of the initial microbial load and how the use of common disinfectants does not totally eliminate the microbial risk (about 5–18% of endoscopes remained contaminated). With regard the time factor, the fast anti-biofilm activity shown by MEDSTER 2000 allows to speed up the disinfection practices and the ability to completely eliminate any viable cells observed in the present investigation represents an important feature for an optimal infective risk management because the bacterial growth in sessile form (biofilm) begins immediately after the high

disinfection procedure, when the instrument is stored (Neves et al. 2016). MEDSTER 2000 inhibited the growth of all the strains, both in planktonic form and organized in biofilm. For the planktonic form the antibacterial activity of MEDSTER 2000 was already observed after 10 min at the lowest concentration (0.1%), and after the same time of exposure at the concentration of 2% the disinfectant was able to totally eradicate all tested bacteria grown in sessile form on both plastic and stainless steel surfaces, with a greater than 6 log CFU/cm² reduction in viable cells. This result is supported by the light microscopy observation of the dead cells marked in red with the live/dead assay. Furthermore, the resistance to sterilization processes of dry surface biofilms is not only to chemical disinfectants but has also been described to 121C° autoclaving for 30 min (Almatroudi et al. 2018). Acid peroxidic systems are more effective than glutaraldehyde and orthophthaldehyde (Chino et al. 2017), and in

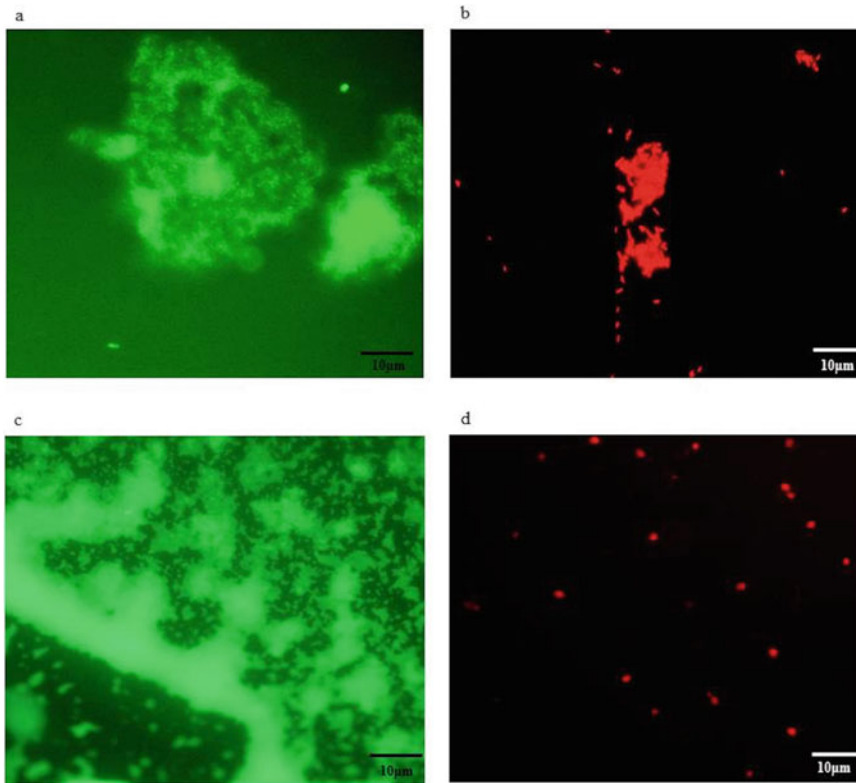


Fig. 5 *Escherichia coli* ATCC 25922 (a and b) and *Staphylococcus aureus* ATCC 6538 (c and d) ‘2 day-old’ mature biofilm on plastic surface. Images of light microscopy obtained by using “live/dead cells stain kit” before

(a) (c) and after (b) (d) 10 min disinfection with MEDSTER 2000 at 2%. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The scale bars indicate 10 µm

conditions of dry surface *Candida auris* biofilm they showed again a significant higher efficacy than other chlorine based products, such as chlorine dioxide (Ledwoch and Maillard 2018). This higher activity has also been demonstrated in presence of organic load and for relatively short exposure times (Chowdhury et al. 2019). Moreover, for some chemical compounds the removal of the biofilm can be achieved with the increase of the temperature only, but the biofilm reduction obtained in this way is detrimental to the integrity of the materials (just think of the aggressive action on the chlorine materials). On the other hand, a direct or catalyzing action of corrosion of metals and degradation of prosthesis and medical instrumentation materials has been demonstrated precisely for some types of biofilms (Beech et al. 2006; Procópio 2019). Under our

study conditions we demonstrated that a complex mixture of precursors of reactive oxygen and peracids, working in a neutral pH solution and at room temperature, is able to remove biofilm from different materials such as plastic or surgical stainless steel AISI 316 both through spraying or immersion test. These results are consistent with other recent studies confirming a higher activity in biofilm removal for peroxyacetic acid under neutral buffered pH than in acid conditions (Meyer et al. 2019).

In conclusion, instead of conventional instrument processing through decontamination, precleaning and final high level disinfection with 3 different products that could interfere with each other, in the present study an “overkill approach” based on a unique antibacterial compound MEDSTER 2000 is proposed. Based on

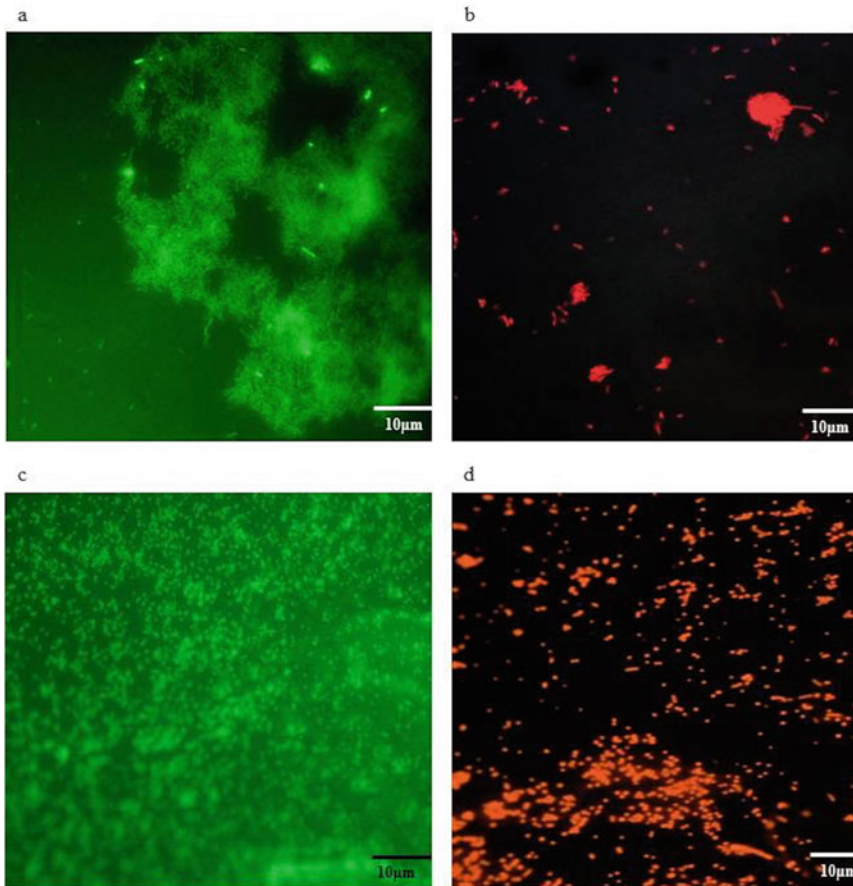


Fig. 6 *Escherichia coli* ATCC 25922 (a and b) and *Staphylococcus aureus* ATCC 6538 (c and d) ‘2 day-old’ mature biofilm on stainless steel. Images of light microscopy obtained by using “live/dead cells stain kit” before

(a) (c) and after (b) (d) 10 min disinfection with MEDSTER 2000 at 2%. Green fluorescence labels live cells, whereas red fluorescence labels dead cells. The scale bars indicate 10 µm

the results obtained, this disinfectant highly effective against biofilm and endowed with biosafety margins can be employed at different concentrations to perform the 3 conventional steps (decontamination, precleaning, disinfection). This treatment could be a new a more effective approach for semicritical devices that need of a high-level disinfection than any other reusable medical device.

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