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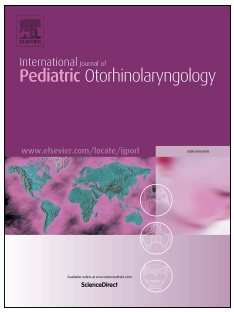

Lugol's solution eradicates *Staphylococcus aureus* biofilm in vitro

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ABSTRACT

Objectives

The aim of the study was to evaluate the antibacterial efficacy of Lugol's solution, acetic acid, and boric acid against *Staphylococcus aureus* biofilm.

Methods

The efficacy of Lugol´s solution 1%, 0.1%, and 0.05%, acetic acid 5% or boric acid 4.7% for treatment of *Staphylococcus aureus* biofilm in vitro was tested using 30 clinical strains. Susceptibility in the planktonic state was assessed by disk diffusion test. Antiseptic effect on bacteria in biofilm was evaluated by using a Biofilm-oriented antiseptic test (BOAT) based on metabolic activity, a biofilm bactericidal test based on culturing of surviving bacteria and confocal laser scanning microscopy combined with LIVE/DEAD staining.

Results

In the planktonic state, all tested *S. aureus* strains were susceptible to Lugol's solution and acetic acid, while 27 out of 30 tested strains were susceptible to boric acid. In biofilm the metabolic activity was significantly reduced following exposure to Lugol's solution and 5% acetic acid, while boric acid exposure led to no significant changes in metabolic activities. In biofilm, biocidal activity was observed for Lugol`s solution 1% (30/30), 0.1% (30/30), and 0.05% (26/30). Acetic acid and boric acid showed no bactericidal activity in this test. Confocal laser scanning microscopy, assessed in 4/30 strains, revealed significantly fewer viable biofilm bacteria with Lugol`s solution (1% $p<0.001$, 0.1% $p=0.001$ or 0.05% $p=0.001$), acetic acid 5% for 10 minutes ($p=0.001$) or 30 minutes ($p=0.015$), but not for acetic acid for 1 minute or boric acid.

Conclusion

Lugol`s solution 1.0% and 0.1% effectively eradicated *S. aureus* in biofilm and could be an alternative to conventional topical antibiotics where *S. aureus* biofilm is suspected such as external otitis, pharyngitis and wounds.

Keywords: Boric acid, Lugol`s solution, acetic acid, biofilm, Staphylococcus aureus, Confocal laser scanning microscopy.

1. INTRODUCTION

Staphylococcus aureus is commonly identified in the secretion of purulent draining ears, pharynx and chronic suppurating wounds and is known to be a potential biofilm producer [1-4]. Bacteriological analysis and antimicrobial treatments have traditionally focused on bacteria in their planktonic state without considering them as biofilm. In recent years biofilm formation has received more attention. The bacteria within the biofilm exhibit altered metabolism, gene expression and protein production compared to their planktonic counterparts [5]. The biofilm can also serve as a protected reservoir for pathogenic bacteria [6-8].

The altered characteristics of biofilm bacteria cause antimicrobial resistance through several mechanisms such as, a dormant phenotype, or a high proportion of persister cells [6, 9]. The metabolic quiescent state inactivates antimicrobial targets or reduces the requirements for their cellular function [6]. The biofilm can also act as a diffusion barrier, with reduced antimicrobial permeability through the biofilm matrix, or by deactivation of the antimicrobial substances in the surface layer of the biofilm [7, 10, 11]. Furthermore, the biofilm prevents immune cells and components from engulfing and eradicating the bacteria [12, 13]. The MIC values of biofilm can reach 500-1000 times that of their planktonic counterparts [7, 14]. Mature biofilms can shed planktonic bacteria or micro colonies into the local environment, or migrate and attach to other parts of the body, causing relapsing infections if not treated appropriately [10].

Because of these biofilm defense mechanisms and growing antimicrobial resistance [15, 16], we urgently need new treatment options. Antiseptics have many advantages over antibiotics, such as generally acting on several targets in the microorganism instead of one specific site only, and demonstrating less risk of antimicrobial resistance and a broader spectrum of antimicrobial activity. Antiseptics have proven efficacy against different groups of bacteria,

fungi, viruses, and protozoa [17]. However, the use of antibiotics has reduced the scientific attention to antiseptics.

Lugol´s solution and boric acid have been used as antiseptics in medical practice since the 19th century. In 1829 the French physician J.G.A. Lugol created the disinfectant Lugol´s solution, which consists of 5g iodine (I_2) and 10g potassium iodide (KI) mixed with 85mL distilled water [18]. The effect of boric acid in modern medical practice was first described by Lord Lister in 1875 [19, 20]. Acetic acid was used by Hippocrates to treat wounds [21].

The present study aims to evaluate the efficacy of Lugol's solution, boric and acetic acid on biofilms produced by *S. aureus*, and thereby, potential candidates for topical treatment of diseases with *S. aureus* biofilm, such as external otitis, pharyngitis and wounds.

2. MATERIALS AND METHODS

A relatively large number of clinical wildtype strains were tested, since the susceptibility to antimicrobials may differ between clones [22, 23]. The *S. aureus* strains were obtained at Oslo University Hospital, an academic tertiary referral center, in the period from April 2014 to October 2014. The strains were collected consecutively from 29 unique; 15 strains from blood culture and 14 strains from draining ears. *S. aureus* 1378-1, a previously described strain known for its biofilm-producing capabilities, was used as a positive control [24]. The strains from the draining ears were obtained by using an otomicroscope and a sterile swab (VWR transport swabs, Copan, Breschia, Italy). The identification and antibiotic susceptibility testing did not reveal any MRSA strains. (MALDI-TOF-MS, Bruker Daltonik GmbH, Bremen, Germany, VITEK® 2, bioMérieux S.A. France). The bacteria were stored in a freezing storing broth at -70˚C (Frysebuljong, Oslo University Hospital, Oslo, Norway)

before being plated on to blood agar plates for amplification and verification of purity. The blood agar plates were incubated for 24 hours at 37 ±1°C.

2.1 Disk diffusion test. Each of the strains was tested in its planktonic state to evaluate the efficacy of the antiseptics by a disk diffusion test according to the EUCAST disk diffusion method, version 5. Single colonies from a fresh overnight bacterial culture on blood agar were picked and transferred into sterile saline. The suspension was measured to McFarland 0.5 and the spread on Müller Hinton agar plates using an automated plate spreader. Aliquots of 50µL of antiseptic were applied to a diffusion disk (6mm Blank Paper Discs, Becton, Dickinson and Company, Sparks, MD, USA) that was applied to the agar plates. Inhibition zones were evaluated after 18 hours of incubation at 36 ±1°C with calipers.

2.1 Biofilm assay. The ability of the *S. aureus* strains to form biofilm was tested in a 96-well microtiter plate (Nunclon Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark) according to a previously published method [25]. One colony of each bacterial strain was inoculated in 5mL of tryptic soy broth (TSB) which was cultured over night at 37 ±1°C. The next day, 180µL of TSB w/ 1% glucose/ 1% NaCl was transferred to each of the wells on the microtiter plate, except for the first three blank control wells to which 200µL were transferred. The overnight cultures were then vortexed at 222 rpm for 40 secs and 20µL were transferred to all the wells, except for the blank control. Each strain of the *S. aureus* was tested in three parallel wells. The microtiter plate was incubated at 37 ±1°C for 24 hours. The wells were then washed three times with 220µL of tap water and left to dry at room temperature for 30 min. After drying, 220µL of crystal violet (1% solution, Sigma Aldrich, St. Louis, MO, USA) was added and incubated for 30 min. The wells were washed five times with 220µL of tap water. To extract the crystal violet from the biofilm, 220µL of ethanol:acetone (70:30 w:w) was added to the wells. The results were then calculated by measuring the optical density at 595nm (Multiscan MS, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3 Antiseptics. Antiseptics and exposure times are shown in Table 1. The exposure times were chosen from a pilot test and after considering what would be a practical duration in a clinical setting.

2.4 Biofilm-oriented antiseptics test (BOAT). To test the efficacy of the antiseptics on the bacterial strains in biofilm, the Biofilm-oriented antiseptics test (BOAT) was applied [26], with some modifications. The same 96-well microtiter plate was used as in the biofilm assay and the biofilm was produced as described above with six parallel wells for each strain. After 24 hours of incubation, the wells were washed with 220µL sterile 0.85 % NaCl, before adding the undiluted antiseptics and sterile 0.85% NaCl for the selected contact time. For each strain three parallel wells were exposed to antiseptics and three were controls. The antiseptic and 0.85% NaCl were then removed and Dey Engley neutralizing broth was added for 5 minutes. The wells were filled with 200µL of TSB:tetrazolium chloride (TSB:TTC) in the ratio of 20:1. The microtiter plate was incubated at 37 ±1 °C for 12 hours. The results were evaluated visually by color change and measured calorimetrically. The amount of formazan produced was calculated calorimetrically by measuring the optical density at 492nm (Siemens BEP 2000 Advance, Germany). In the presence of viable metabolic active bacteria, TTC is reduced from a colorless compound to red formazan, which correlates to the number of viable cells [27-29]. The experiment was repeated three times.

2.5 Biofilm bactericidal test. To confirm the eradication effects of antiseptics on *S. aureus* biofilm, a model described by T. Mah was used, modified for *S. aureus* [23]. All 30 strains were tested. The first steps of establishing a biofilm, and applying antiseptics, sterile 0.85% NaCl, and neutralizing broth was identical to the BOAT method described above. However, instead of then adding TSB:TTC, 200µL of TSB was added to each well and incubated at 37 ±1 °C for 24 hours. Of the overnight culture 5µL was transferred from each well onto a blood

agar plate and incubated at 37 ˚C for 24 hours before the results were evaluated visually. If there was no growth, the antiseptic was considered bactericidal.

2.6 Confocal laser scanning microscopy. Three strains were chosen randomly from the previous experiment among those which were susceptible to Lugol`s solution 0.05%, and one random strain from those which were not susceptible to Lugol`s solution 0.05%. The tested strains were; 14BA 010 492, 14BA 010 425, 14BA 020 489 and 14BA 020 499. The first steps of establishing a biofilm and applying antiseptics, sterile 0.85% NaCl and neutralizing broth was identical to the BOAT method described above, except that a Lab-Tek II Chambered Coverglass with cover 8-wells, (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used instead of a microtiter plate. Each strain was exposed to the antiseptics or to sterile 0.85% NaCl as a control. The slides were stained with Filmtracer™ LIVE/DEAD® Biofilm Viability Kit, (Molecular Probes, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's specifications. Images of the stained biofilm were generated on a confocal laser scanning microscope (Zeiss LSM 710, Germany), employing a 488 nm argon laser line for the SYTO® 9 and a 561 nm DPSS laser line for the propidium iodide. The ratio of dead or dying cells to the total number of cells in the biofilm was determined by ImageJ software (open source, public domain). Four scans were performed per strain per antiseptic and control. In order to ensure that the antiseptics had penetrated the whole biofilm, scans were performed to the bottom layers of biofilm.

2.7 Statistical analysis. All statistical analyses were performed using SPSS statistical software (release 22.0 SPSS Inc., Chicago, Il, USA)

When comparing inhibition zone diameter between the antiseptics, a paired t-test was performed.

To compare the amount of biofilm produced by *S. aureus* strains taken from ear cultures and blood cultures an independent t-test was applied.

In the BOAT test, each strain was tested in three parallel wells for both the antiseptics and the controls. The median value was calculated for the antiseptic and the control in order to reduce the possibility of one well distorting the results. The experiment was repeated three times and the average of the medians for each strain was calculated. A paired t-test was performed to identify any significant difference between the antiseptic-treated groups compared to controls.

In the biofilm bactericidal test, the bactericidal activity of each antiseptic, was tested in three parallels for both the antiseptics and the controls. If there was no growth, the antiseptic was considered bactericidal. For the antiseptics that were bactericidal for only some strains, the McNemar test was applied to determine statistical significance.

The effect of antiseptics displayed in confocal laser scanning microscopy was measured by comparing the ratio of compromised cells in the antiseptic-treated groups to the control group. For statistical significance, an average ratio for each group was calculated and a paired t-test used.

2.8 Approval. The collection of specimens from human subjects was approved by REK, the regional ethical committee.

3. RESULTS

3.1 Disk diffusion test. All antiseptics at all concentrations showed clear inhibition zones in the disk diffusion tests, with the exception of three strains for boric acid (Fig. 1, Table 2). The difference in inhibition zone diameters between all the different antiseptics and concentrations were significant ($p<0.02$), with the one exception between boric acid 4.7% and Lugol's solution 0.05% ($p=0.35$).

3.2 Biofilm assay. All strains of *S. aureus* grew biofilm within 24 hours. The amount of biofilm measured varied between the strains (Table 3). There were no statistical differences in the amount of biofilm produced between the group of strains taken from blood cultures and the group of strains from ear cultures. ($p=0.534$).

3.3 Biofilm-oriented antiseptics test (BOAT). The reduction in metabolic activity was significant for all concentrations of Lugol's solution and for all three exposure times of acetic acid compared with controls (Table 4). Although acetic acid and Lugol's solution showed significant reduction in metabolic activity, there were important differences in efficacy. The optical densities of all three concentrations of Lugol's solution were close to the blank control while acetic acid was not (Table 4). This was also observed visually where all wells treated with Lugol's solution appeared blank (Fig. 2) while the wells exposed to acetic acid produced different shades of red, indicating surviving metabolic active bacteria (Fig. 3). Exposure to boric acid for 30 min did not significantly reduce the metabolic activity compared with control strains (Table 4), visualized by no clear difference in color intensity between the antiseptic and control groups.

3.4 Biofilm bactericidal test. Only Lugol's solution 1.0% and 0.1% fully eradicated all 30 strains of *S. aureus* biofilm (Table 4). Lugol's solution 0.05% eradicated 26 out of the 30

strains, which is statistically significant ($p<0.001$). Acetic acid and boric acid did not display any bactericidal effect (Table 4).

3.5 Confocal laser scanning microscopy. Lugol's solution 1.0%, 0.1% and 0.05%, and acetic acid with 10 and 30 min exposure showed significant reduction in viable cells (Table 4). For Lugol's solution the ratios of compromised cells to the total number of cells were close to 1, indicating that all bacteria were dead or dying. Boric acid and acetic acid did not reach a ratio of 1, indicating there were surviving bacteria (Table 4). The results suggest that only Lugol's solution effectively eradicated the biofilm bacteria (Fig. 4).

4. DISCUSSION

The results show significant differences in the bactericidal effect of antiseptics on *S. aureus* in biofilm in all three test systems. For stronger evidence of antiseptic efficacy, a large number of different clinical strains were tested, since previous studies indicated that different antimicrobial effects were found in laboratory and wild strains [23, 30, 31]. The risk of confounding factors was reduced by diluting the antiseptics in sterile H_2O and not combined with other possible substances. This measure, combined with the use of three different evaluation methods, make us more confident in drawing conclusions about the effect of antiseptics on *S. aureus* biofilm.

When tested using relevant concentrations and exposure times, Lugol's solution was by far the most effective antiseptic, whereas acetic acid and boric acid were less successful. The strains used are from patients with no known epidemiological relationships, and the results are therefore believed to be representative for clinical isolates from ear and blood. The results from this study indicate that Lugol's solution could be potential supplement to antibiotic topical treatment of diseases with *S. aureus* biofilm, such as external otitis, pharyngitis and wounds. If the efficacy and safety regarding ototoxicity is established *in vivo*, Lugol's

solution could become a supplement in the treatment arsenal and thereby reduce the need for topical application of antibiotics.

. The heterogeneity in biofilm-producing capabilities among our strains, combined with the use of three different evaluation methods, make us more confident in drawing conclusions about the effect of antiseptics on *S. aureus* biofilm. To reduce the risk of confounding factors, Lugol´s solution, boric acid and acetic acid were diluted in sterile H_2O and not combined with other possible substances.

4.1 Lugol´s solution. The results from the present study showed that Lugol's solution was effective in eradicating *S. aureus* in biofilm. To our knowledge, there are no previous studies describing the effect of Lugol's solution, and only a few previous studies exploring the effect of iodine-containing antiseptics on *S. aureus* biofilm. In those studies, the tested iodine concentration was higher or iodine was combined with other substances, such as ethanol, or in a combination with carrier molecules. Apart from one other study [32], previous studies found different iodine combinations to be effective against *S. aureus* biofilm [24, 26, 33]. This is in line with our findings of elemental iodine's effectiveness in the eradication of biofilm bacteria, even at concentrations as low as 0.01% of Lugol's solution.

The exact antimicrobial action of iodine is unknown. It has been suggested that iodine attacks proteins, nucleotides and fatty acids [17], which are key components of the extracellular protective matrix of *S. aureus* [34]. A disturbance of these components may disrupt the biofilm matrix, leaving the bacterial cells less protected against the antiseptic. The promising results of Lugol's solution need to be confirmed in *in vivo* studies.

4.2 Acetic acid. A concentration of 5% acetic acid was used since it is widely available in many commercial products. Several previous studies have found acetic acid effective in

241 treating chronic suppurative otitis media (CSOM) [35] and venous leg ulcers [36], and in
242 inhibiting [37] and eradicating [38] *S. aureus* biofilm formation. Contrary to these findings,
243 acetic acid 5% did not eradicate any of the bacterial strains in biofilm in our study.

244 There are several possible explanations for acetic acid being less effective in killing biofilm
245 bacteria in vitro. It could be that the pH of the extra cellular matrix is too high. The
246 bactericidal effect of acetic acid results from the undissociated form of the acid that freely
247 crosses the cell membrane, dissociates and acidifies the cytoplasm. This leads to a strong
248 reduction of metabolic activity and disruption in the electrochemical gradient across the cell
249 membrane causing cell death. A strong inorganic acid, HCl, mainly acidifies the growth
250 medium and not microbial cytosol, as protons diffuse poorly through the cell envelope [39,
251 40], and is therefore less effective in reducing the biofilm of *S. aureus* at the same pH
252 compared with acetic acid [41]. The variance in metabolic activity reduction between strains
253 could be explained by a difference in extracellular matrix (Figure 3). One reason for better
254 results in clinical studies may be mechanical rinsing which is important for the outcome [42].
255 Another possible reason is longer exposure time, though we did not see any additional effect
256 in the reduction of metabolic activity when increasing the exposure time from 10 to 30
257 minutes. The present and previous studies show the importance of evaluating antiseptics by
258 different measuring methods and on several bacterial strains before drawing any definitive
259 conclusions.

260 **4.3 Boric acid**. The efficacy of boric acid in the treatment of draining ears has been reported
261 [43] as well as its bactericidal effects on *S. aureus* [44]. Like many antiseptics, boric acid is
262 thought to exert its action on multiple targets in the microbial cell, but the exact mechanism is
263 unknown[45]. Boric acid is a non-polar molecule and only the undissociated form is believed
264 to be capable of crossing the microbial cell membrane[45]. The tested concentration of 4.7%
265 is close to the maximum concentration possible to dissolve in H_2O at room temperature [46].

We did not obtain a significant reduction in metabolic activity, bactericidal effect and a significant increase in the ratio of dead to viable bacterial cells. One possible explanation could be that boric acid is often dissolved in ethanol, which in itself has been shown to have a bactericidal effect on *S. aureus* in biofilm [24, 47]. Another possible explanation is that the exposure time was too short to kill all the bacteria [45]. This could explain why boric acid powder is described as effective in draining ears, where it may be present for a longer and thus more effective time [43].

4.4 Side effects of antiseptic. Studies regarding ototoxicity in humans are quite scarce, and most studies are performed on animals. In animal experiments ototoxicity from iodine solutions seems to be related to the iodine concentration and additives such as ethanol [48-50]. Based on the available studies, it seems likely that the concentrations of iodine in Lugol's solution in our study are safe in regards to ototoxicity, however, own experiments with Lugol's solution are needed before concluding. Although documentation is limited, there is some concern about ototoxicity caused by acetic acid [51, 52], but boric acid diluted in sterile H_2O has been reported as safe [53, 54].

Another concern is wound healing and wound strength after application of antiseptics [55-58]. Some studies report povidone-iodine and acetic acid as having no effect on reepithelization [59, 60], while others report delayed reepithelization [55]. Some reports have found that povidone-iodine reduces tensile strength [55, 61], some no effect, while others show increased strength [62]. Numerous clinical studies have evaluated the effect of povidone-iodine on wound healing, and most of them conclude that there is no decrease in wound healing effects [63, 64].

Unjustified fear of allergic reactions has prevented wide-scale use of iodine-containing products. One possible reason for this unfavorable reputation may be hypersensitive-type

reactions experienced by some with iodine-containing contrast media. These reactions were more commonly experienced in earlier years, when the contrast media were hypertonic and ionic solutions. Lugol´s solution contains only H_2O, potassium iodine and elementary iodine, which can be found in the body, and allergic reactions should therefore not be of any concern [65, 66].

5. CONCLUSION

Lugol's solution 1.0% and 0.1% was bactericidal for all clinical wild type strains of *S. aureus* when in biofilm, while 0.05% was bactericidal for 26 out of the 30 strains. Acetic acid 5% and boric acid 4.7% did not eradicate any of the biofilm strains in vitro. We therefore conclude that Lugol´s solution could be an alternative to antibiotics for topical applications in diseases such as external otitis, pharyngitis and wounds where a *S. aureus* biofilm is considered part of the pathogenesis. Further in vivo studies are required, regarding its efficacy, as well as ototoxicity.

REFERENCES:

[1] DeAntonio R, Yarzabal JP, Cruz JP, Schmidt JE, Kleijnen J., Epidemiology of otitis media in children from developing countries: A systematic review, Int. J. Pediatr. Otorhinolaryngol. 2016; 85: 65-74.

[2] Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW., A review of the scientific evidence for biofilms in wounds., Wound. Repair Regen 2012; 20: 647-657.

[3] Burmolle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homoe P, Tvede M, Nyvad B, Tolker-Nielsen T, Givskov M, Moser C, Kirketerp-Moller K, Johansen HK, Hoiby N, Jensen PO, Sorensen SJ,

Bjarnsholt T., Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections., 2010. FEMS Immunol. Med. Microbiol 2010; 59: 324-336.

[4] Lowy FD., Staphylococcus aureus Infections., N. Engl. J. Med. 1998; 339: 520-532.

[5] Donlan RM, Costerton JW., Biofilms: survival mechanisms of clinically relevant microorganisms., Clin. Microbiol. Rev 2002; 15: 167-193.

[6] Lewis K., Persister cells., Annu. Rev. Microbiol 2010; 64: 357-372.

[7] Nickel JC, Ruseska I, Wright JB, Costerton JW., Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm on urinary catheter material., Antimicrob. Agents Chemother 1985; 27: 619-624.

[8] Stewart PS, Costerton JW., Antibiotic resistance of bacteria in biofilms., Lancet 2001; 358: 135-138.

[9] Hall-Stoodley L, Stoodley P., Evolving concepts in biofilm infections, Cell. Microbiol. 2009; 11: 1034-1043.

[10] Hall-Stoodley L, Costerton JW, Stoodley P., Bacterial biofilms: from the natural environment to infectious diseases., Nat. Rev. Microbiol 2004; 2: 95-108.

[11] Singh R, Ray P, Das A, Sharma M., Penetration of antibiotics through Staphylococcus aureus and Staphylococcus epidermidis biofilms., J. Antimicrob. Chemother 2010; 65: 1955-1958.

[12] Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M., Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system., Cell. Microbiol. 2004; 6: 269-275.

[13] Leid JG, Shirtliff ME, Costerton JW, Stoodley P., Human leukocytes adhere to, penetrate, and respond to Staphylococcus aureus biofilms., Infect. Immun 2002; 70: 6339-6345.

[14] Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM., Microbial biofilms., Annu. Rev. Microbiol 1995; 49: 711-745.

[15] Linares J, Ardanuy C, Pallares R, Fenoll A., Changes in antimicrobial resistance, serotypes and genotypes in Streptococcus pneumoniae over a 30-year period., Clin. Microbiol. Infect 2010; 16: 402-410.

[16] Moxnes JF, de Blasio BF, Leegaard TM, Moen AE., Methicillin-resistant Staphylococcus aureus (MRSA) is increasing in Norway: a time series analysis of reported MRSA and methicillin-sensitive S. aureus cases, PLoS. One 2013; 8: e70499.

[17] McDonnell G, Russell AD., Antiseptics and disinfectants: activity, action, and resistance., Clin. Microbiol. Rev 1999; 12: 147-179.

[18] Petruzzi M, Lucchese A, Baldoni E, Grassi FR, Serpico R., Use of Lugol's iodine in oral cancer diagnosis: an overview., Oral Oncol. 2010; 46: 811-813.

[19] Woods WG., An introduction to boron: history, sources, uses, and chemistry., Environ. Health Perspect 1994; 102 Suppl 7: 5-11.

[20] JORDAN JW, CRISSEY JT., Boric acid poisoning; a report of fatal adult case from cutaneous use; a critical evaluation of the use of this drug in dermatologic practice., AMA. Arch. Derm 1957; 75: 720-728.

[21] Johnston CS, Gaas CA., Vinegar: medicinal uses and antiglycemic effect., MedGenMed 2006; 8: 61.

[22] Atshan SS, Nor SM, Lung LT, Sekawi Z, Pei PC, Karunanidhi A, Jeevajothi NJ, Mateg AA, Ghaznavi-Rad E, Abduljaleel SA, Awang HR., Genotypically different clones of Staphylococcus aureus are diverse in the antimicrobial susceptibility patterns and biofilm formations., Biomed. Res. Int 2013; 2013: 515712.

[23] Mah TF., Establishing the minimal bactericidal concentration of an antimicrobial agent for planktonic cells (MBC-P) and biofilm cells (MBC-B)., J. Vis. Exp 2014: e50854.

[24] Vestby LK, Nesse LL., Wound care antiseptics - performance differences against Staphylococcus aureus in biofilm., Acta Vet. Scand 2015; 57: 22.

[25] Stepanovic S, Vukovic D, Hola V, Di BG, Djukic S, Cirkovic I, Ruzicka F., Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci., APMIS 2007; 115: 891-899.

[26] Junka A, Bartoszewicz M, Smutnicka D, Secewicz A, Szymczyk P., Efficacy of antiseptics containing povidone-iodine, octenidine dihydrochloride and ethacridine lactate against biofilm formed by Pseudomonas aeruginosa and Staphylococcus aureus measured with the novel biofilm-oriented antiseptics test., Int. Wound. J 2013; 11: 730-734.

[27] Moussa SM TA, Al-Hassan AA, Farouk A., Tetrazolium/Formazan Test as an Efficient Method to Determine Fungal Chitosan Antimicrobial Activity., Journal of Mycology 2013; 7536922013.

[28] Tengerdy RP, Nagy NJ, Martin B., Quantitative Measurement of Bacterial Growth by the Reduction of Tetrazolium Salts. Appl Microbiology 1967; 954-955.

[29] Perez LM, Alvarez BL, Codony F, Fittipaldi M, Adrados B, Penuela G, Morato J., A new microtitre plate screening method for evaluating the viability of aerobic respiring bacteria in high surface biofilms., Lett. Appl. Microbiol 2010; 51: 331-337.

[30] Zenga J, Gagnon PM, Vogel J, Chole RA., Biofilm formation by otopathogenic strains of Pseudomonas aeruginosa is not consistently inhibited by ethylenediaminetetraacetic acid., Otol. Neurotol 2012; 33: 1007-1012.

[31] Wang EW, Agostini G, Olomu O, Runco D, Jung JY, Chole RA., Gentian violet and ferric ammonium citrate disrupt Pseudomonas aeruginosa biofilms., Laryngoscope 2008; 118: 2050-2056.

[32] Akiyama H, Huh WK, Yamasaki O, Oono T, Iwatsuki K., Confocal laser scanning microscopic observation of glycocalyx production by Staphylococcus aureus in mouse skin: does S. aureus generally produce a biofilm on damaged skin?, Br. J. Dermatol 2002; 147: 879-885.

[33] Akiyama H, Oono T, Saito M, Iwatsuki K., Assessment of cadexomer iodine against Staphylococcus aureus biofilm in vivo and in vitro using confocal laser scanning microscopy., J. Dermatol 2004; 31: 529-534.

[34] Kiedrowski MR, Horswill AR., New approaches for treating staphylococcal biofilm infections., Ann. N. Y. Acad. Sci 2011; 1241: 104-121.

[35] Aminifarshidmehr N., The management of chronic suppurative otitis media with acid media solution., Am. J. Otol 1996; 17: 24-25.

[36] Hansson C, Faergemann J., The effect of antiseptic solutions on microorganisms in venous leg ulcers., Acta Derm. Venereol 1995; 75: 31-33.

[37] Nostro A, Cellini L, Ginestra G, D'Arrigo M, di GM, Marino A, Blanco AR, Favaloro A, Bisignano G., Staphylococcal biofilm formation as affected by type acidulant., APMIS 2013; 122: 648-653.

[38] Bjarnsholt T, Alhede M, Jensen PO, Nielsen AK, Johansen HK, Homoe P, Hoiby N, Givskov M, Kirketerp-Moller K., Antibiofilm Properties of Acetic Acid., Adv. Wound. Care (New Rochelle.) 2015; 4: 363-372.

[39] Trcek J, Mira NP, Jarboe LR., Adaptation and tolerance of bacteria against acetic acid., Appl. Microbiol. Biotechnol 2015; 99: 6215-6229.

[40] Cherrington CA, Hinton M, Mead GC, Chopra I., Organic acids: chemistry, antibacterial activity and practical applications., Adv. Microb. Physiol 1991; 32: 87-108.

[41] Akiyama H, Yamasaki O, Tada J, Arata J., Effects of acetic acid on biofilms formed by Staphylococcus aureus., Arch. Dermatol. Res 1999; 291: 570-573.

[42] Paranhos HF, Silva-Lovato CH, Souza RF, Cruz PC, Freitas KM, Peracini A., Effects of mechanical and chemical methods on denture biofilm accumulation., J. Oral Rehabil 2007; 34: 606-612.

[43] Loock JW., A randomised controlled trial of active chronic otitis media comparing courses of eardrops versus one-off topical treatments suitable for primary, secondary and tertiary healthcare settings., Clin. Otolaryngol 2012; 37: 261-270.

[44] Yilmaz MT, Minimum inhibitory and minimum bactericidal concentrations of boron compounds against several bacterial strains. Turk J Med Sci 2012: 1423-1429.

[45] Borokhov O, Schubert D., Antimicrobial properties of boron derivatives, In Zhu PC (ed), New Biocides Development. The Combined Approach of Chemistry and Microbiology. New Biocides Development, ACS Symposium Series: ACS Publications, Washington, DC., 2007; 418-419.

[46] National Pesticide Information Center, http://npic.orst.edu/factsheets/archive/borictech.html#prop. Accessed 9th July 2017.

[47] Macfadyen C, Gamble C, Garner P, Macharia I, Mackenzie I, Mugwe P, Oburra H, Otwombe K, Taylor S, Williamson P., Topical quinolone vs. antiseptic for treating chronic suppurative otitis media: a randomized controlled trial., Trop. Med. Int. Health 2005; 10: 190-197.

[48] Ichibangase T, Yamano T, Miyagi M, Nakagawa T, Morizono T., Ototoxicity of Povidone-Iodine applied to the middle ear cavity of guinea pigs., Int. J. Pediatr. Otorhinolaryngol 2011; 75: 1078-1081.

[49] Perez R, Freeman S, Sohmer H, Sichel JY., Vestibular and cochlear ototoxicity of topical antiseptics assessed by evoked potentials., Laryngoscope 2000; 110: 1522-1527.

[50] Aursnes J., Ototoxic effect of iodine disinfectants., Acta Otolaryngol. 1982; 93: 219-226.

[51] Ikeda K, Morizono T., The preparation of acetic acid for use in otic drops and its effect on endocochlear potential and pH in inner ear fluid., Am. J. Otolaryngol 1989; 10: 382-385.

[52] Haynes DS, Rutka J, Hawke M, Roland PS., Ototoxicity of ototopical drops--an update., Otolaryngol. Clin. North Am 2007; 40: 669-683, xi.

[53] Ozturkcan S, Dundar R, Katilmis H, Ilknur AE, Aktas S, Haciomeroglu S., The ototoxic effect of boric acid solutions applied into the middle ear of guinea pigs. Eur. Arch. Otorhinolaryngol 2009; 266: 663-667.

[54] Ozdemir S, Tuncer U, Tarkan O, Akar F, Surmelioglu O., Effects of topical oxiconazole and boric acid in alcohol solutions to rat inner ears., Otolaryngol. Head Neck Surg 2013; 148: 1023-1027.

[55] Lineaweaver W, Howard R, Soucy D, McMorris S, Freeman J, Crain C, Robertson J, Rumley T., Topical antimicrobial toxicity., Arch. Surg 1985; 120: 267-270.

[56] Goldenheim PD., An appraisal of povidone-iodine and wound healing., Postgrad. Med. J 1993; 69 Suppl 3: S97-105.

[57] Burks RI., Povidone-iodine solution in wound treatment., Phys. Ther 1998; 78: 212-218.

[58] Leaper DJ, Durani P., Topical antimicrobial therapy of chronic wounds healing by secondary intention using iodine products., Int. Wound. J 2008; 5: 361-368.

[59] Geronemus RG, Mertz PM, Eaglstein WH., Wound healing. The effects of topical antimicrobial agents., Arch. Dermatol 1979; 115: 1311-1314.

[60] Gruber RP, Vistnes L, Pardoe R., The effect of commonly used antiseptics on wound healing., Plast. Reconstr. Surg 1975; 55: 472-476.

[61] Kashyap A, Beezhold D, Wiseman J, Beck WC., Effect of povidone iodine dermatologic ointment on wound healing., Am. Surg 1995; 61: 486-491.

[62] Kramer SA., Effect of povidone-iodine on wound healing: a review. J. Vasc. Nurs 1999; 17: 17-23.

449 **[63] Niedner R., Cytotoxicity and sensitization of povidone-iodine and other frequently used anti-**

450 **infective agents., Dermatology 1997; 195 Suppl 2: 89-92.**

451 **[64] Drosou A, Falabella A, Kirsner RS., Antiseptics on Wounds: An Area of Controversy. Wounds**

452 **2003:149-66.**

453 **[65] Schabelman E, Witting M., The relationship of radiocontrast, iodine, and seafood allergies: a**

454 **medical myth exposed., J. Emerg. Med 2010; 39: 701-707.**

455 **[66] Katelaris CH, Smith WB, 'Iodine allergy' label is misleading. Australian Prescriber 2009; 125-128.**

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Figure legends

Fig. 1. Disk diffusion test: a; 0.85% saline, b; acetic acid 5%, c; Lugol´s solution 0.1%, d; Lugol´s solution 0.05%, e; Lugol´s solution 0.005%, f; boric acid 4.7%. *Mueller Hinton Agar*

Fig. 2. Biofilm-oriented antiseptics test (BOAT), Lugol´s solution 1min exposure. First 6 wells are control, 6 continuous wells per *Staphylococcus aureus* strain. The three lateral wells on each side treated with 0.85% saline. The 6 middle wells treated with antiseptic. Red formazan is a sign of viable cells. *96 well microtiter plate (Nunclon Delta Surface, Thermo)*

Fig. 3. Biofilm-oriented antiseptics test (BOAT), acetic acid 5% 30 min exposure. First 6 wells are control, 6 continuous wells per *Staphylococcus aureus* strain. The three lateral wells on each side treated with 0.85% saline. The 6 middle wells treated with antiseptic. Red formazan is a sign of viable cells. *96 well microtiter plate (Nunclon Delta Surface, Thermo Fischer Scientific)*

Fig. 4. CLSM stacks of *Staphylococcus aureus* biofilm exposed to antiseptics and a control; **a** Control, **b** Acetic acid 1min, **c** Acetic acid 10min, **d** Acetic acid 30min, **e** Boric acid, **f** Lugol's solution 0.05%, **g** Lugol's solution 0.1%, **h** Lugol´s solution 1.0%. The units are in µm.

Lugol´s solution eradicates Staphylococcus aureus biofilm in vitro

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Running title: Lugol´s solution as treatment of *S. aureus* biofilm

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Table 1. Antiseptics and exposure time

Antiseptic	Exposure time
Acetic acid	
Acetic acid 5%	1 minute
Acetic acid 5%	10 minutes
Acetic acid 5%	30 minutes
Lugol's solution	
Lugol's solution 1.0% (1% iodine-2% potasiumiodide in sterile H2O)	1 minute
Lugol's solution 0.1% (by diluting 1.0% Lugol's solution in sterile H2O)	1 minute
Lugol's solution 0.05% (by diluting 1.0% Lugol's solution in sterile H_2O)	1 minute
Boric acid	
Boric acid 4.7%	30 minutes

All antiseptics were dissolved in sterile H_2O and not ethanol. All antiseptics were from Oslo University Hospital, Oslo, Norway

Table 2. A comparison of antiseptic inhibition zone diameter

Inhibition zone diameter			
Antiseptic	**n**	**Mean**	**± 1 SD**
Lugol's solution 1%,	30	30	1,9
Lugol's solution 0.1%,	30	16	1,1
Lugol's solution 0.05%,	30	13	0,6
Acetic acid 5%,	30	17	2,2
Boric acid 4.7%,	30	13	4,9
NaCl 0.85%	30	0	0

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Table 3. Study comparing the amount of biofilm produced by *Staphylococcus aureus* from ear and blood cultures

	Blood culture samples	**Ear culture samples**
Number of strains	15	14
Mean optical density	1.202	1.364
Standard deviation	0.584	0.789
Minimum	0.613	0.639
Maximum	2.921	3.100

Table 4. A comparison of antiseptic effects versus control

		BOAT test			Bactericidal biofilm test	Confocal laser scanning microscopy with LIVE/DEAD staining,			
Antiseptic	**n**	**Mean**	**± 1 SD**	**p-value**	**Number of strains with bactericidal effect**	**n**	**Dead to total cell number**	**± 1 SD**	**p-value**
Untreated bacterial strains	30	2.756	0.37	-	0/30	4	0.27	0.11	-
Lugol's solution 1%, 1 min exposure	30	0.152	0.05	<0.001	30/30	4	1.03	0.10	<0.001
Lugol's solution 0.1%, 1 min exposure	30	0.232	0.05	<0.001	30/30	4	1.06	0.11	0.001
Lugol's solution 0.05%, 1 min exposure	30	0.243	0.06	<0.001	26/30	4	1.00	0.07	0.001
Acetic acid 5%, 1 min exposure	30	2.304	0.66	0.002	0/30	4	0.37	0.10	0.093
Acetic acid 5%, 10 min exposure	30	1.140	0.67	<0.001	0/30	4	0.62	0.11	0.001
Acetic acid 5%, 30 min exposure	30	1.324	0.68	<0.001	0/30	4	0.75	0.14	0.015
Boric acid 4.7%, 30 min exposure	30	2.745	0.40	0.117	0/30	4	0.57	0.35	0.172
Blank control	6	0.131	0.05	-	-	-	-	-	-

Significance calculated by paired t-test