

Original Article

Evaluation of Topical Antiseptics against Biofilm Formation of *Staphylococcus Aureus* and *Pseudomonas Aeruginosa* in Chronic Suppurative Otitis Media

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Abstract

Background & Objective(s): Chronic suppurative otitis media (CSOM) is a common chronic disease that is often difficult to treat. Biofilms have been demonstrated in bacteria causing CSOM infections contributing to its pathogenesis and resistance to treatment. Antiseptics have a nonspecific mode of action and this gives them the advantage of a broader spectrum of antibacterial activity and a lesser risk of resistance. The present study aimed to compare the effect of three antiseptics; 4% boric acid, Lugol's iodine and tetrasodium ethylenediaminetetraacetic acid (tEDTA) solutions against *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) and their biofilm forming ability.

Methods: Bactericidal effect of antiseptics was examined using disk diffusion method on Müller Hinton agar plates and the effect on biofilm formation was examined using biofilm-oriented antiseptics test.

Results: The best antiseptic agent for planktonic cells of both bacterial species was Lugol's iodine (mean±SD= 2.00±0.68), followed by tEDTA (mean±SD= 1.48±0.62) then boric acid (mean±SD= 0.20±0.47). The tested antiseptics had very close results when measuring the mean inhibition zones of *S. aureus* and *P. aeruginosa* isolates. All tested antiseptics reduced biofilm formation, but tEDTA was the most effective antiseptic in reducing the biofilm formation compared to Lugol's iodine and boric acid (Mean OD= 0.055±0.012 versus 0.145±0.137 and 0.122±0.071 respectively, $p < 0.001$).

Conclusion: Lugol's iodine had a better bactericidal effect on isolates, while tEDTA had a better effect on biofilm formation. Further in vivo studies are needed regarding both their efficacy and ototoxic effects to assess their possible use as local treatment of CSOM patients.

Keywords: Boric acid, Lugol's iodine, biofilm, EDTA, *Staphylococcus aureus*, *Pseudomonas aeruginosa*

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INTRODUCTION

Chronic suppurative otitis media (CSOM) is a common chronic disease than that is often difficult to treat and its persistence can lead to irreversible complications. Biofilms have been demonstrated in bacteria causing CSOM infections contributing to the pathogenesis of the disease and resistance to treatment.^(1, 2) It had also been related to recurrent otorrhea after tympanostomy tube insertion.⁽³⁾ Bacteria producing biofilms are different from those in their planktonic forms as they possess different metabolic and structural abilities. They start as planktonic cells that attach themselves to a surface and begin forming a multicellular bacterial structure. The bacteria forming the biofilm produce an extracellular polysaccharide matrix that protects pathogenic bacteria within that structure.^(4, 5) The biofilm

acts as a barrier that reduces diffusion of antimicrobials to individual cells and it can contain antimicrobial degrading enzymes that accumulate causing resistance. Furthermore efflux pumps are also activated in the biofilm structure that can expel several antimicrobials.⁽⁶⁾ Bacterial cells usually enter into a slowly growing or inactive metabolic state (persister cells) making them immune to antibiotics targeting active replicating cells.⁽⁷⁾ Also, biofilms are usually a heterogeneous population that contains a high genetic diversity thus promoting that the spread of resistance through horizontal gene transfer.⁽⁸⁾ These special features provide the bacteria within the biofilm with several mechanisms that make them resistant to antimicrobials. The resistance within these bacteria can reach up to 1000 folds its planktonic counterparts. That is why more options are urgently needed as treatment might be difficult with high rates of therapeutic failure. Unlike

antibiotics, antiseptics have a nonspecific mode of action as they attack several targets in the bacterial cell. This gives them the advantage of a broader spectrum of antibacterial activity and a lesser risk of development of resistance.⁽⁹⁾ In case of CSOM, they have an added advantage that they can be applied topically at higher concentrations.

Staphylococcus aureus (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are the most commonly isolated bacterial spp. from CSOM patients.^(10, 11) The present study aimed to compare the effect of three antiseptics; 4% boric acid, Lugol's iodine and ethylenediaminetetraacetic acid (EDTA) solutions against both these spp. and their biofilm forming ability.

METHODS

Collection of samples: Samples were collected from outpatient clinic in the Department of Otorhinolaryngology, Alexandria Main University Hospital, Egypt. The study protocol was approved by Ethics Committee at the High Institute of Public Health (HIPH) and an informed consent was obtained from all participants. Ear discharge was obtained from the diseased ear of each patient, using sterile swabs. Culture and identification of isolated of *S. aureus* and *P. aeruginosa* colonies were done according to standard methods.⁽¹²⁾ Clinical strains of *S. aureus* and *P. aeruginosa* were subcultured on blood agar plates for 24 hours at 37°C then used to perform disk diffusion and biofilm oriented antiseptic test.

Disk diffusion test: Isolated colonies from blood agar plates were picked and transferred into sterile saline. The suspension was measured adjusted to turbidity of McFarland 0.5 standard and the spread on Müller Hinton agar plates. Aliquots of 50µL of each of boric acid in ethanol (2% boric acid in 45% alcohol), Lugol's iodine (1% iodine-2% potassium iodide in sterile H₂O) and 40 mg/mL of tEDTA were applied to a diffusion disk that was applied to the Müller Hinton agar plates. The diameters of inhibition zones were measured after 18 hours of incubation at 37 °C.⁽¹³⁻¹⁶⁾

Biofilm-oriented antiseptics test (BOAT): Isolated colonies of each strain were suspended into 5mL of tryptic soy broth and incubated for 24 hours at 37 °C.⁽¹⁷⁾ Biofilm

formation was tested in 96-well microtiter plates, one plate as a control and an individual plate for each antiseptic used. Each strain was tested in duplicate wells. Fresh tryptic soy broth (180 µL) was added to all wells except for 2 blank wells. Overnight cultures from each strain were vortexed then 20 µL were added to each well. Microtiter plates were then incubated aerobically at 37 °C for 24 hours. The next day, plates were washed three times and dried at room temperature for 30 min.

The following antiseptics were added to subsequent wells in 200 µL amounts to each well: boric acid in ethanol (2% boric acid in 45% alcohol) for 30 minutes, Lugol's iodine (1% iodine-2% potassium iodide in sterile H₂O) for 1 minute and 40 mg/mL of tEDTA for 24 hours.^(15, 18, 19) Plates were then fixed by heating at 56 °C for 1 hour. After fixation, 200 µL of crystal violet (1%) were added and incubated at room temperature for 15 minutes. The wells were washed five times then 200 µL of 33% acetic acid were added to each well and incubated for another 15 minutes. A volume of 100 µL aliquot was removed from each well and placed in a new 96-well microtiter plate. The optical density was measured at 595nm using a microplate reader (Siemens BEP120 2000 Advance, Germany).⁽¹⁷⁾

RESULTS

Disk diffusion test: The antibacterial activity of the three tested antiseptics was measured as diameters of the zones of growth inhibition, in centimeters, presented in Table 1. The best antiseptic agent for planktonic cells of both bacterial species was Lugol's iodine, followed by tEDTA while boric acid gave the poorest results. Lugol's iodine showed the highest inhibition zones (mean±SD= 2.00±0.68) followed by tEDTA (mean±SD= 1.48±0.62) then boric acid (mean±SD= 0.20±0.47). The tested antiseptics had very close results when measuring the mean inhibition zones of *S. aureus* and *P. aeruginosa* isolates. While boric acid gave a slightly lower mean for *S. aureus* than *P. aeruginosa* (0.16±0.41 versus 0.25±0.53), EDTA and Lugol's iodine gave a slightly higher mean inhibition zones for *S. aureus* (1.65±0.40 versus 1.31±0.76 and 2.13±0.28 versus 1.86±0.92) The difference between the tested antiseptics was statistically significant (Kruskal Wallis test= 47.420 ($p < 0.001$) and 26.101 ($p < 0.001$) (Table 1).

Table (1): Comparison of inhibition zone diameter of tested antiseptics

Strain	Total (n = 40)			<i>S. aureus</i> (n = 20)			<i>P. aeruginosa</i> (n = 20)			
	Antiseptic	Mean	SD.	Max.-Min.	Mean	SD.	Max.-Min.	Mean	SD.	Max.-Min.
2% Boric acid		0.20	0.47	1.70-0.0	0.16	0.41	1.50-0.0	0.25	0.53	1.70-0.0
Tetrasodium EDTA		1.48	0.62	3.0-0.0	1.65	0.40	3.0-1.0	1.31	0.76	2.30-0.0
Lugol's iodine		2.0	0.68	3.50-0.0	2.13	0.28	2.50-1.50	1.86	0.92	3.50-0.0
H (p)						47.420* (<0.001*)				26.101* (<0.001*)

H: H for Kruskal Wallis test

p: p value for comparing between the studied groups

*: Statistically significant at $p \leq 0.05$

Biofilm assay: All tested antiseptics reduced biofilm formation. Results for Lugol's iodine and tEDTA were statistically significant for both *S. aureus* and *P. aeruginosa* strains. tEDTA was the most effective antiseptic in reducing the biofilm formation compared to

Lugol's iodine and boric acid (Mean OD= 0.055 ± 0.012 versus 0.145 ± 0.137 and 0.122 ± 0.071). It significantly reduced biofilm formation compared to both. (Post Hoc Test, Dunn's for multiple comparisons test, $p < 0.001$) (Table 2).

Table (2): Comparison of antibiofilm activity of tested antiseptics

	Antiseptic				H	p
	Untreated bacterial	2% Boric acid	Tetrasodium EDTA	Lugol's iodine		
<i>S. aureus</i>						
Min. – Max.	0.09 - 0.83	0.051-0.367	0.043-0.089	0.057-0.614	48.216*	<0.001*
Mean ± SD.	0.277±0.192	0.133±0.098	0.052±0.010	0.179±0.183		
Sig. with Untreated		0.005*	<0.001*	0.020*		
Sig. bet. antiseptics		$p_1 < 0.001^*$, $p = 0.603$, $p_3 < 0.001^*$				
<i>P. aeruginosa</i>						
Min. – Max.	0.094-0.562	0.06-0.152	0.048-0.106	0.062-0.246	52.669*	<0.001*
Mean ± SD.	0.224±0.114	0.111±0.025	0.059±0.013	0.111±0.052		
Sig. with Untreated		0.003*	<0.001*	0.001*		
Sig. bet. antiseptics		$p_1 < 0.001^*$, $p = 0.581$, $p_3 < 0.001^*$				
Total						
Min. – Max.	0.094-0.833	0.051-0.367	0.043-0.106	0.057-0.614		
Mean ± SD.	0.251±0.158	0.122±0.071	0.055±0.012	0.145±0.137		

H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 antiseptic was done using Post Hoc Test (Dunn's for multiple comparisons test)

Sig. with Untreated : p value for comparing between Untreated bacterial and each other antiseptic

p_1 : p value for comparing between 2% Boric acid and Tetrasodium EDTA

p_2 : p value for comparing between 2% Boric acid and Lugol's iodine

p_3 : p value for comparing between Tetrasodium EDTA and Lugol's iodine

*: Statistically significant at $p \leq 0.05$

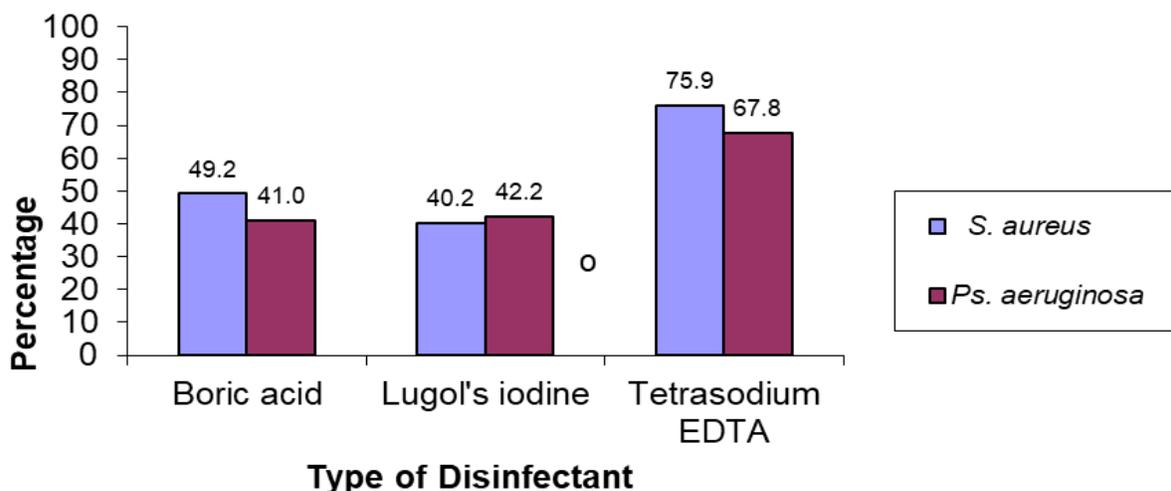


Figure 1: Antibiofilm activity of tested antiseptics against *S. aureus* and *Ps. aureginosa* compared to untreated samples

tEDTA and boric acid had a more inhibitory effect on *S. aureus* rather than *P. aeruginosa* biofilms (75.9%, 49.2% for *S. aureus* versus 67.8%, 41.0% for *P. aeruginosa*, respectively). On the other hand, Lugol's iodine had a slightly more potent inhibitory effect on biofilms of *P. aeruginosa* (42.2%) than those by *S. aureus* (40.2%) (Figure 1).

DISCUSSION

CSOM is a leading cause of preventable hearing loss especially in developing countries. Untreated cases can even lead to further complications and mortality in some cases. Management of cases can be expensive and unaffordable especially in countries with highest burden.^(20,21) Therefore search for effective cheaper options of treatment is highly needed. *S. aureus* and *P. aeruginosa* are among the most common causes of CSOM. Both are likely to form biofilm formation leading to further complicating treatment.⁽²²⁾

Several studies have examined the bactericidal effect of boric acid as well as its efficacy in treatment of CSOM patients with varying results. Boric acid exerts its bactericidal effect through multiple targets in the bacterial cell.⁽²³⁾ In the current study, 2% instead of 4% boric acid was used due to the toxic effect previously reported of the higher concentration.⁽²⁴⁾ This might be the reason why it was found to be less effective than Lugol's iodine and EDTA both on biofilm and as a bactericidal. Youn et al., reported that boric acid's antibacterial effect and inactivation of bacterial cells required a longer duration than other tested antiseptics.⁽²⁵⁾ Macfadyen et al., found that it was less effective than ciprofloxacin drops in treatment of CSOM cases.⁽¹⁴⁾ Both studies used the lower concentration of 2% boric acid. Grønseth Moshi et al., also reported lower efficacy of 4% boric acid in vitro.⁽²⁶⁾ On the contrary, Looek et al., used boric acid powder in a randomized control trial and reported superiority of boric acid than topical quinolone drops and⁽²⁷⁾ Youn et al., found 4% boric acid a potent biofilm inhibitor but after the longer time of exposure (24 - 72 hours).⁽²²⁾

The antibacterial action of iodine compounds is said to be through attacking nucleotides and fatty acids, cellular membrane proteins and respiratory enzymes.^(28, 29) Though it is more effective than EDTA and boric acid on planktonic cells, its effect on biofilm formation was less impressive. The studies describing the effect of iodine preparation on biofilm formation gave conflicting results. Some studies reported substantial inhibition of both planktonic and biofilm formation by iodine preparation.^(19, 30, 31) Results described by Oduwole et al., support these results as they found out that iodine inhibits that transcription of adhesions responsible for biofilm formation.⁽³²⁾ On the other hand, Presterl et al., showed that iodine was less effective on biofilm formation than other used antiseptics.⁽³³⁾ A possible explanation is that iodine may act as a stress factor that induces a stress response and activation of enzymes responsible for biofilm formation possibly counteracting its antibiofilm properties.⁽³⁴⁾ To our knowledge, only one other

study examined the effect of Lugol's iodine on CSOM isolates.⁽¹⁹⁾ It has the advantage though that it is not dissolved in alcohol which has ototoxic effects.⁽²⁴⁾ Lugol's iodine gave very close results for *S. aureus* and *P. aeruginosa* isolates. On the other hand, Junka et al., reported that iodine was more effective in complete eradication of biofilm of *S. aureus* while it only eradicated 66% of that of *P. aeruginosa* even after longer incubation periods.⁽³⁵⁾

EDTA, specifically tEDTA, has been recently demonstrated as a potent antibacterial and antibiofilm effect. Biofilm producing bacteria produce a substance called exopolymeric substance (EPS) and metal ions such as magnesium, calcium and iron are important to the development and integrity of this layer. EDTA acts as a metal ion chelator depriving bacteria of metal ions.⁽³⁶⁻³⁸⁾ In this study, EDTA was very effective in elimination of biofilm in both tested spp. It had the highest percentage reduction in biofilm production among the examined antiseptics. Several studies have reported the enhanced effect of tEDTA on biofilm formation especially in catheter associated infections.^(15, 39, 40) To our knowledge it has not been tested on isolates from CSOM patients. The pH of EDTA solutions is different according to its components. tEDTA solution has a higher pH^(8,50-10) than both disodium EDTA and trisodium EDTA.⁽³⁷⁾ This property could be used as a local treatment since local irrigation and change of pH in CSOM patients has been suggested to eliminate infection.

The results of this study can only determine the effect of the tested antiseptics in vitro. Further studies should examine their possible use in treatment of CSOM. Each antiseptic can be examined at different concentrations to determine the optimal concentration with the highest efficacy and lowest ototoxic effect. Also, further investigations can include their effect on other bacterial spp. that cause CSOM can be examined to determine its therapeutic effects.

CONCLUSION

Boric acid is frequently used in treatment of CSOM patients therefore more studies are needed to evaluate the optimum concentration, exposure time suitable for its use in treatment.

Despite that Lugol's iodine had the poorest effect on biofilm eradication, yet, it yielded the best results on the planktonic cells of both *S. aureus* and *P. aeruginosa*. tEDTA had a remarkable effect on biofilm formation and should be considered as a possible option for treatment.

Conflict of Interest: None to declare.

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