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Comparative efficacies of topical antiseptic eardrops against biofilms from methicillin-resistant *Staphylococcus aureus* and quinolone-resistant *Pseudomonas aeruginosa*

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Main Article

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Abstract

Objective. The present study aimed to compare the anti-biofilm activities of four commonly available antiseptic eardrops against biofilms from methicillin-resistant *Staphylococcus aureus* and quinolone-resistant *Pseudomonas aeruginosa in vitro*.

Methods. The anti-biofilm activities of 50 per cent Burow's solution, vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution were evaluated using biofilm assays. Additionally, the anti-biofilm activities of the four antiseptic solutions against tympanostomy tube biofilms were compared using a scanning electron microscope.

Results. The inhibition of biofilm formation from methicillin-resistant *S aureus* and quinolone-resistant *P aeruginosa* occurred after treatment with 4 per cent boric acid solution, 2 per cent acetic acid solution, and vinegar with water (1:1). However, 50 per cent Burow's solution did not exhibit effective anti-biofilm activity.

Conclusion. The results indicate that 4 per cent boric acid solution and vinegar with water (1:1) are potent inhibitors of biofilms from methicillin-resistant *S aureus* and quinolone-resistant *P aeruginosa*, and provide safe pH levels for avoiding ototoxicity.

Introduction

Otorrhoea is a common problem in children with tympanostomy tubes.[1] Bacterial biofilm formation has been related to the high rate of refractory otorrhoea after tympanostomy tube insertion.[2] In particular, biofilm formation from antibiotic-resistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and quinolone-resistant *Pseudomonas aeruginosa* has become a serious therapeutic problem.

Adequate antibiotic treatment based on antimicrobial susceptibility tests does not always result in therapeutic success because of the intrinsic antimicrobial tolerance of biofilms.[3] Antiseptics are effective against bacterial biofilms from antibiotic-resistant micro-organisms, and the topical administration of antiseptic eardrops provides a high concentration directly to the site of the biofilm.[4] The present study aimed to compare the anti-biofilm activities of four commonly available antiseptic solutions against biofilms from MRSA and quinolone-resistant *P aeruginosa*, to guide the selection of topical antiseptics.

Materials and methods

Solution preparation

In order to evaluate the anti-biofilm effects of antiseptic solutions, 50 per cent Burow's solution, 2 per cent acetic acid solution, vinegar with water (1:1), and 4 per cent boric acid solution were prepared. Burow's solution (50 per cent) was prepared according to the protocol described in the *British Pharmacopoeia 2009*.[5] Acetic acid (Junsei Chemical, Tokyo, Japan) was diluted to 2 per cent. Commercially available vinegar (Ottogi, Seoul, South Korea) was diluted with water in a 1:1 ratio. Boric acid (Sigma, Saint Louis, Missouri, USA) was diluted to 4 per cent.

Biofilm assay

For the anti-biofilm assay, MRSA and quinolone-resistant *P aeruginosa* clinical isolates taken from patients with otorrhoea for antibiotic susceptibility testing were used.

Methicillin-resistant *S aureus* and quinolone-resistant *P aeruginosa* were grown in Mueller–Hinton agar (Difco Laboratories, Sparks, Maryland, USA) at 37 °C for 24 hours. The colonies were then suspended in Mueller–Hinton broth (Difco Laboratories) and cultured at 37 °C for 24 hours. Thereafter, the cultures were diluted in phosphate-buffered saline to a density of 0.063 Au at an absorbance of 600 nm.

In order to detect the production of biofilm, sterile, flat-bottomed 96-well polystyrene plates were used, and tryptic soy broth containing 1 per cent glucose (180 µl) was poured in each well. The prepared bacterial suspensions (20 µl) were then added to each well. After incubation for 24 hours at 37 °C, the plates were decanted and washed three times with phosphate-buffered saline (200 µl). Subsequently, 200 µl of the antiseptic solutions (Burow's solution (50 per cent), acetic acid (2 per cent), vinegar to water (1:1), boric acid (4 per cent)) was added to each well. The plates were then incubated for 24, 48 or 72 hours at 37 °C.

The wells were washed three times with phosphate-buffered saline, and then air dried at room temperature. The plate was fixed with 100 per cent methanol (200 µl per well) for 30 minutes, dried and stained with 200 µl of 1 per cent crystal violet solution (Sigma) per well for 15 minutes.

The wells were washed three times with phosphate-buffered saline and air dried. The dye bound to the biofilm in the wells was then released using 95 per cent ethanol (200 µl per well) for 20 minutes. The absorbance was measured at 570 nm using a plate reader (BioTek, Winooski, Vermont, USA). Each test was performed six times.

Scanning electron microscope analysis

In order to observe bacterial biofilm formation on the surface of the tympanostomy tubes, the tympanostomy tubes were placed in 96-well plates, with tryptic soy broth containing 1 per cent glucose (180 µl) in each well. Thereafter, the previously prepared MRSA and quinolone-resistant *P aeruginosa* suspensions (20 µl) were added to each well.

After incubation for 72 hours at 37 °C, the plates with tympanostomy tubes were decanted and washed three times with phosphate-buffered saline (200 µl). The antiseptic solutions (200 µl) were added to each well and the plates were incubated for 48 hours at 37 °C.

Thereafter, the plates were washed three times with phosphate-buffered saline and air dried at room temperature. The tympanostomy tubes were then transferred to 12-well plates and fixed with 2 per cent osmium tetroxide (Sigma) in phosphate-buffered saline (2 ml per well) for 4 hours, and then washed three times with phosphate-buffered saline.

The plates with tympanostomy tubes were immersed in 50, 60, 70, 80, 90 or 100 per cent ethanol for 10 minutes. They were then air dried, and coated using an ion sputtering coating machine E-1030 (Hitachi High-Technologies, Tokyo, Japan). A Field Emission Scanning Electron Microscope S-4800 (Hitachi High-Technologies) was used to observe the plates for 30 minutes.

Statistical analysis

The results were statistically analysed using SPSS 19.0 software (SPSS, Chicago, Illinois, USA). The Mann–Whitney U test was used to analyse the data. A p value of less than 0.05 was considered statistically significant.

Results

Effects on methicillin-resistant S aureus *biofilm*

The anti-biofilm activities of the four antiseptic solutions – 50 per cent Burow's solution, vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution – against biofilms from MRSA were compared with the untreated control. Vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution significantly inhibited the rate of biofilm formation from MRSA. The 2 per cent acetic acid and 4 per cent boric acid solutions exhibited the highest anti-biofilm activities. The rate of biofilm formation from MRSA decreased as the exposure time to each solution increased. However, 50 per cent Burow's solution did not inhibit the rate of biofilm formation from MRSA at these time periods (Figure 1).

Effects on quinolone-resistant P aeruginosa *biofilm*

The anti-biofilm activities of 50 per cent Burow's solution, vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution against biofilms from quinolone-resistant *P aeruginosa* were compared with the untreated control. Vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution significantly inhibited the rate of biofilm formation from quinolone-resistant *P aeruginosa*. The rate of biofilm formation from quinolone-resistant *P aeruginosa* decreased after 48 hours of exposure to each solution. The rates were not changed at 72 hours. The rate of biofilm formation from quinolone-resistant *P aeruginosa* was not inhibited by 50 per cent Burow's solution at 48 hours, but it had decreased at 72 hours (Figure 2).

Effects on tympanostomy tube biofilm

The anti-biofilm activities of 50 per cent Burow's solution, vinegar with water (1:1), 2 per cent acetic acid, and 4 per cent boric acid solution against tympanostomy tube biofilms from MRSA and quinolone-resistant *P aeruginosa* were compared using a scanning electron microscope after 48 hours of treatment with each solution.

Biofilm formation and many MRSA and quinolone-resistant *P aeruginosa* colonies were observed on the surface of the tympanostomy tubes in the control group. A marked reduction of MRSA biofilms and colonies was seen in the 2 per cent acetic acid and 4 per cent boric acid solution treated groups. A reduction of MRSA biofilm was also observed in the vinegar with water (1:1) treated group. A marked reduction of quinolone-resistant *P aeruginosa* biofilms and colonies was seen in the vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution treated groups. No reduction of MRSA or quinolone-resistant *P aeruginosa* biofilms was seen in the 50 per cent Burow's solution treated group (Figure 3).

Discussion

Post-tympanostomy tube otorrhoea is common as a result of upper respiratory tract infection and contamination secondary to the surgical procedure.[6] The predominant micro-organisms isolated from otorrhoea are *S aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *P aeruginosa*.[7] Refractory otorrhoea commonly develops in *S aureus* and *P aeruginosa* infections because these two pathogens are likely to form biofilms on the tympanostomy tube.[8] A biofilm is a group of micro-organisms that exists in a matrix of an extracellular polysaccharide substance. Bacteria in biofilms have a different susceptibility and increased tolerance to antimicrobial agents because biofilms provide structural protection for the bacteria.[9,10] Therefore, biofilms can cause chronic and refractory infections.

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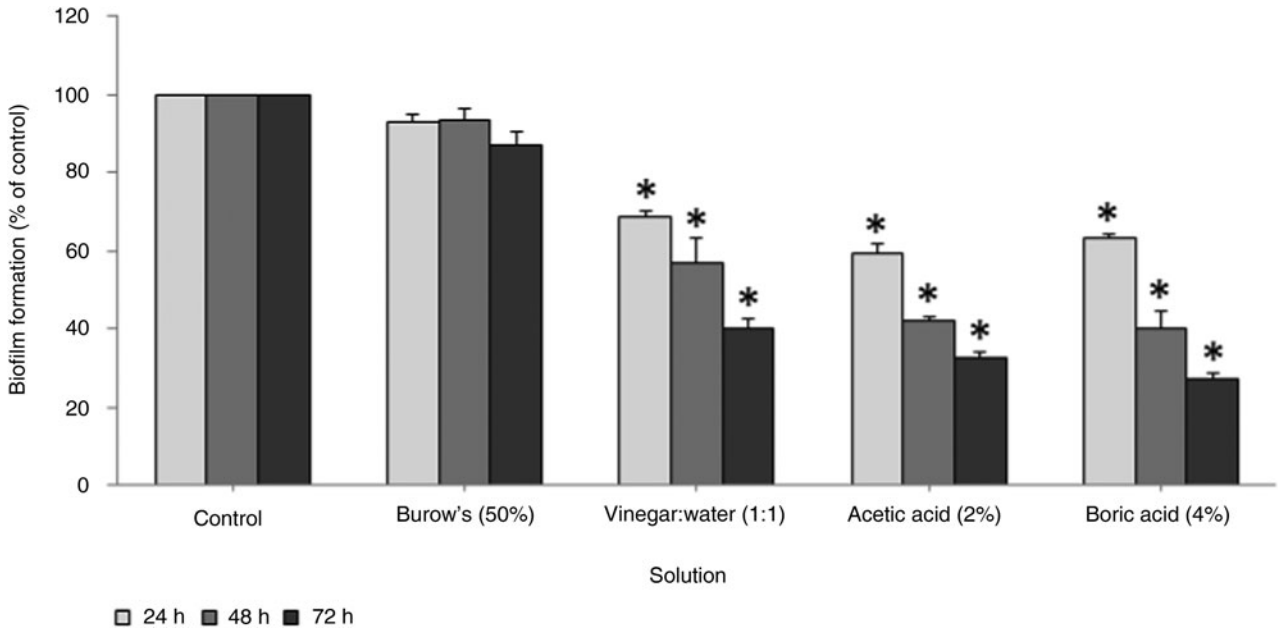

Fig. 1. Anti-biofilm activities of four solutions against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. Vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution significantly inhibited the rate of biofilm formation from MRSA. *$p < 0.05$. h = hours

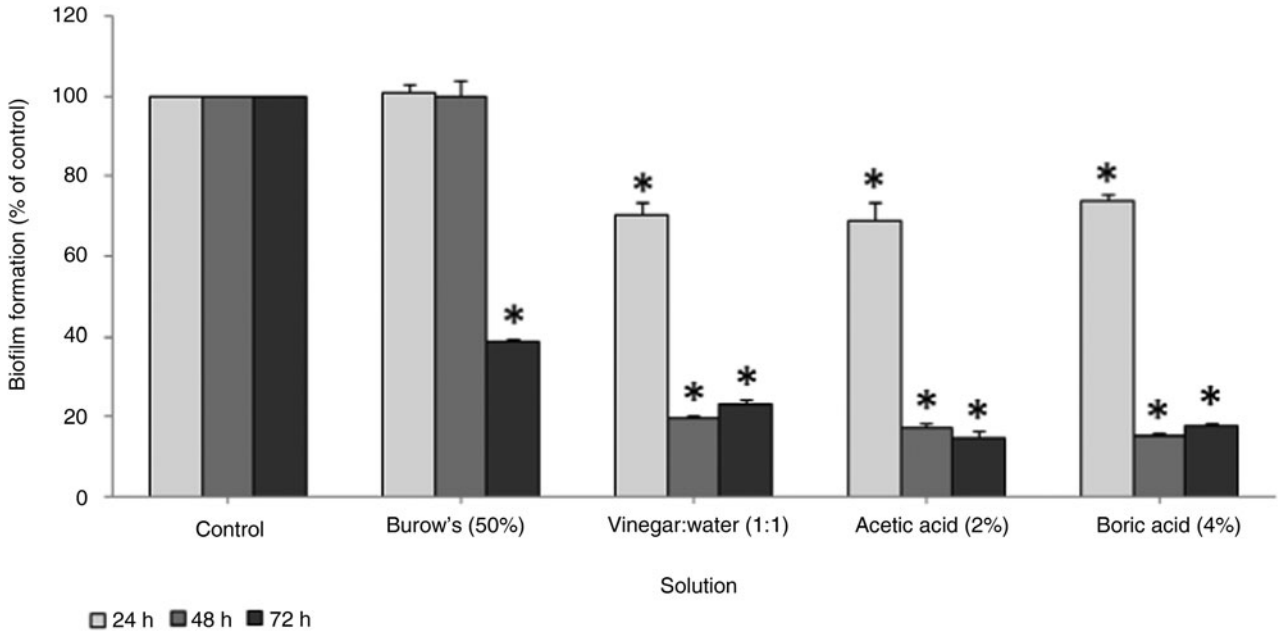

Fig. 2. Anti-biofilm activities of four solutions against quinolone-resistant *Pseudomonas aeruginosa* biofilms. Vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution significantly inhibited the rate of biofilm formation from quinolone-resistant *P aeruginosa*. The 50 per cent Burow's solution inhibited the rate of biofilm formation from quinolone-resistant *P aeruginosa* only at 72 hours. *$p < 0.05$. h = hours

The emergence of MRSA and quinolone-resistant *P aeruginosa* has created a serious therapeutic problem for post-tympanostomy tube otorrhoea.[11,12] Surgical removal of the infected tubes is an effective treatment for biofilm on tympanostomy tubes, but it is associated with antibiotic-resistant infections such as MRSA and quinolone-resistant *P aeruginosa*.[13] However, previous reports indicated that antiseptic solutions are effective against refractory infections due to biofilm formation from antibiotic-resistant micro-organisms.[4,14]

A high pH environment promotes biofilm formation by bacteria in the ear, but a lower pH environment (below pH 5.5) inhibits biofilm formation and the viability of mature biofilms.[15,16] The topical administration of commonly available antiseptic eardrops results in lower pH conditions in the ear because of their acidity. This leads to acid–base imbalance, changes in proteins and the degradation of the biofilm membrane.[17]

In the present study, biofilm assays and a scanning electron microscope confirmed that vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution were potent inhibitors of biofilms formed from MRSA and quinolone-resistant *P aeruginosa*.

However, the application of solutions with a pH lower than 4 in the middle ear have been shown to induce significant ototoxicity and changes in endocochlear potential.[18,19] The topical application of 2 per cent acetic acid solution (pH 2.6) in the middle ear can be ototoxic because of its stronger acidity compared to other antiseptic solutions. Vinegar with water

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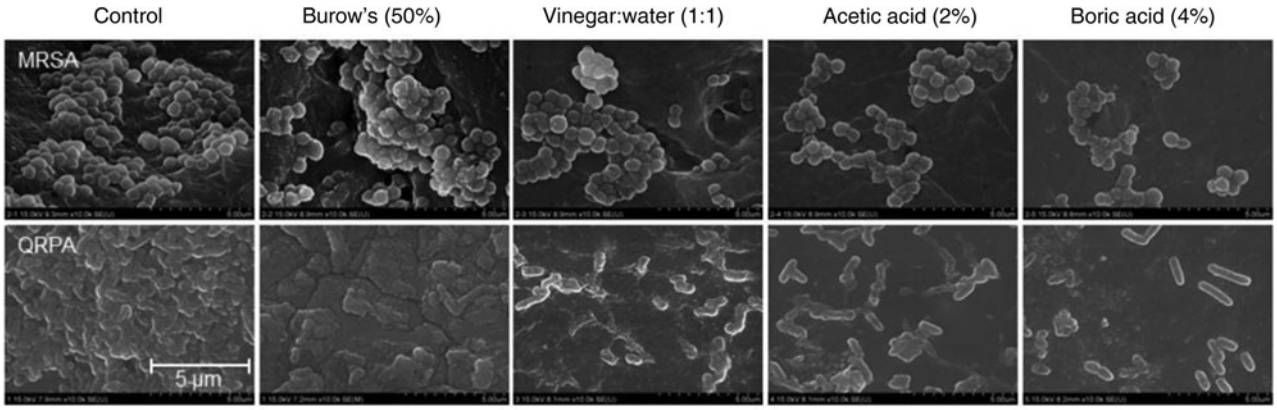

Fig. 3. Scanning electron microscope images showing the representative anti-biofilm activities of four solutions against methicillin-resistant *Staphylococcus aureus* (MRSA) and quinolone-resistant *Pseudomonas aeruginosa* ('QRPA') biofilms on the surface of the tympanostomy tubes at 48 hours. Vinegar with water (1:1), 2 per cent acetic acid solution, and 4 per cent boric acid solution caused a greater reduction in biofilm formation than 50 per cent Burow's solution.

(1:1, pH 4) and 4 per cent boric acid solution (pH 4.7) have pH levels that are relatively safe for the ear.[20]

This study provides the first comparison of the anti-biofilm activities of various antiseptic solutions. Four per cent boric acid solution and vinegar with water (1:1) were potent inhibitors of biofilms from MRSA and quinolone-resistant *P aeruginosa*, and provided safe pH levels for the ear.

- Antiseptic eardrops result in lower pH ear conditions because of acidity and lead to biofilm membrane degradation
- Four per cent boric acid solution and vinegar with water (1:1) were potent inhibitors of methicillin-resistant *S aureus* and quinolone-resistant *P aeruginosa* biofilms
- Four per cent boric acid solution and vinegar with water (1:1) provided safe pH levels for the ear

Conclusion

Four per cent boric acid solution and vinegar with water (1:1) are alternatives to antibiotics for the management of biofilms and refractory post-tympanostomy tube otorrhoea from antibiotic-resistant strains. Further clinical studies in patients are necessary to support this conclusion.

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Competing interests. None declared.

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