The in vitro efficacy of neutral electrolysed water and povidone-iodine against CRS-associated biofilms*

Christian A. Lux[1,2], Kristi Biswas[2], Michael W. Taylor[1], Richard G. Douglas[2]

[1] School of Biological Sciences, University of Auckland, Auckland, New Zealand

[2] Department of Surgery, School of Medicine, University of Auckland, Auckland, New Zealand

Rhinology 60: 1, 73 - 80, 2022

https://doi.org/10.4193/Rhin21.301

***Received for publication:**

August 22, 2021

Accepted: October 19, 2021

Abstract

Background: Despite best medical and surgical practice, some cases of chronic rhinosinusitis (CRS) can remain recalcitrant. Bacterial biofilms have been associated with the recalcitrance of sinonasal inflammation. Biofilms are highly resistant to commonly prescribed antibiotics. Accordingly, more effective antimicrobial treatment options are needed to treat refractory CRS. The aim of this study was to determine the in vitro efficacy of neutral electrolysed water (NEW) and povidone-iodine (PVI) against CRS-associated *Staphylococcus aureus* biofilms.

Methods: Mature *S. aureus* biofilms were grown in a Centre for Disease Control (CDC) biofilm reactor. The antimicrobial activity of NEW, PVI and doxycycline was determined for both planktonic and biofilm cultures of a clinical *S. aureus* isolate using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) assays.

Results: MICs and MBCs were determined for all antimicrobials. MBC values were similar to MICs for both antiseptics, but doxycycline MBCs were significantly higher than the associated MICs. Biofilms were highly resistant to NEW and doxycycline. The MBEC for doxycycline was between 500 and 1000 µg/mL. NEW was ineffective against biofilms and no MBEC could be determined. In contrast, a concentration of 10% of the commercial PVI solution (10 mg/mL PVI) led to effective eradication of mature biofilms.

Conclusion: In this study, only PVI showed promising antibiofilm activity at physiological concentrations. The in vivo efficacy of PVI warrants further investigation of its potential as a treatment for recalcitrant CRS.

Key words: chronic rhinosinusitis, biofilm, *Staphylococcus aureus*, povidone-iodine, hypochlorous acid

Introduction

Chronic rhinosinusitis (CRS) is a common and debilitating condition resulting from persistent inflammation of the nasal and paranasal sinus mucosal linings [(1)] and afflicting between 5 to 10% of the general population [(2, 3)]. Patients with medically recalcitrant CRS are generally treated surgically [(4)]. In some cases, symptoms can persist, and CRS remains refractory despite optimal medical and surgical treatment. Biofilms are complex clusters of microbial cells that adhere to the sinus mucosal epithelium and are embedded in a layer of extracellular polymers [(5)]. They can be observed in 29 – 72% of all cases [(6)] and may play an important role in the disease recalcitrance [(7)]. Biofilm formation is associated with more severe disease preoperatively as well as an increased likelihood of ongoing symptoms postoperatively [(8-10)]. Multiple bacterial species such as *S. aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Moraxella catarrhalis* have been implicated in CRS-associated biofilms [(11)]. Of these, *S. aureus* has been the most commonly found biofilm-forming organism in CRS patients [(12)]. *S. aureus* biofilms in particular have been linked to recurrent and recalcitrant cases of CRS and are associated with worse scores in patient outcome tests when compared with other biofilms [(13-15)].

The extracellular matrix that makes up an integral part of biofilms provides a physical barrier that impedes antibiotic penetration and diffusion into deeper layers of the biofilm structure [(16, 17)]. Bacteria in biofilms undergo phenotypical and behavioural

changes that manifest in a reduced metabolic activity which further reduces their susceptibility to antibiotics, most of which are more effective when there is a high rate of cell division [18, 19]. Bacteria in biofilms display a 10 to 1000-fold greater resistance to antimicrobial treatments than their planktonic counterparts [17, 20, 21].

Antiseptics or biocides provide a promising alternative to antibiotics for the treatment of CRS. In contrast to antibiotics, the actions of antiseptics are usually physicochemical mechanisms, and so are not dependent on interfering with cell metabolism or division, which can make them more effective against biofilms while also reducing the risk of developing resistance [22]. Neutral electrolysed water (NEW), which has hypochlorous acid (HOCL) as the main active ingredient, and povidone-iodine (PVI) are two examples of topical antiseptics with potential for treating biofilms. The bactericidal effects, as well as the efficacy and safety of NEW in CRS patients, have been demonstrated previously [23-26]. While the excellent antimicrobial properties of PVI are well documented [27, 28], there have been concerns regarding its safety profile due to potential cytotoxic effects [29]. However, recent studies showed no negative effects of newly developed formulations or low concentrations of PVI on nasal epithelial cells either in vitro or in vivo [30, 31].

In this study, we evaluate the antibiofilm activity of both NEW and PVI and compare their efficacy against mature CRS-associated *S. aureus* biofilms to an antibiotic commonly prescribed to CRS patients.

Materials and methods

Materials, reagents and antimicrobials

Tryptic soy broth (TSB), tryptic soy agar (TSA) plates and cation-adjusted Mueller Hinton broth (CAMHB) were purchased from Fort Richard Laboratories (Auckland, New Zealand). A CBR 90 CDC biofilm reactor and its components, including standard polypropylene coupon holder rods and 12.7 mm diameter polycarbonate sample coupons, were purchased from Biosurface Technologies Corp. (Bozeman, Montana). Both NEW (Nasocyn™, containing 0.006% HOCL) and PVI (Betadine®, containing 100 mg/mL PVI, active iodine compound = 1%) were obtained from commercial vendors and doxycycline hyclate was sourced from Abcam (Melbourne, Australia). Doxycycline was chosen as it is frequently prescribed for CRS, and because of the known susceptibility of the bacterial isolate used in this study.

Staphylococcus aureus clinical isolate

The *S. aureus* strain used in this study was cultured from a CRS patient with nasal polyps. The spa-type of this strain is t189 and it shows in vitro resistance to penicillin and susceptibility to erythromycin, flucloxacillin and doxycycline. Bacterial cultures in TSB were stored at -80°C in a 1:1 volume of 80% glycerol. An aliquot of 10 µL *S. aureus* stock was sub-cultured on TSA using the streak plate method and incubated for 24h at 37°C. Subsequently, single colonies from the streak plate were used to inoculate overnight cultures for antimicrobial activity assays and biofilm growth.

MIC and MBC assays

A modified version of the broth microdilution technique, as described in the Clinical and Laboratory Standards Institute guidelines (M07-A10), was used to determine the minimum inhibitory concentration (MIC) for all antimicrobial agents. In brief, a 10^{-5} dilution of a fresh overnight culture of the *staphylococcal* isolate was made with PBS to obtain a concentration of ~5 x 10^4 colony forming units (CFU)/mL for the initial inoculum. For the MIC assay, 100 µL of each of the three antimicrobial agents were added to row A of a 96-well flat bottom culture plate in replicates of four. Concentrations of antiseptics are reported as percentage of the commercial product and to facilitate comparability all antimicrobial agents were calculated in µg/mL of active ingredient in the original product. CAMHB (50 µL) was added to all wells of rows B to H and doubling dilutions were performed by transferring 50 µL from the first row of wells (A) to the next (B) and so on using a 12-channel micropipette. The last 50 µL taken from row G were discarded so that row H served as a positive control containing no antimicrobial agent. Lastly, 50 µL of bacterial inoculum were added to all wells resulting in a final volume of 100 µL with 50% CAMHB per well. Plates were covered and incubated in a humidity chamber (to prevent drying) at 37°C and 200 rpm for 24 h. At this time point, MICs were defined as the concentration of antimicrobial agent that inhibited turbidity or visible growth of bacteria.

After incubation, the minimum bactericidal concentration (MBC) assay was carried out by dispensing 10 µL from each well of the microdilution plate onto TSA using the spot-plating technique. Plates were allowed to air dry before incubation overnight at 37°C. The lowest concentration of antimicrobial agent that inhibited colony forming or confluent colony growth was considered the MBC. Both assays were repeated three times and median values are reported.

Biofilm growth

Mature biofilms were established using the CDC biofilm reactor with polycarbonate coupons according to the United States Environmental Protection Agency's (EPA) guidelines for antimicrobial testing methods and procedures (EPA MLB SOP MB-19) with modifications. One millilitre of fresh *S. aureus* overnight culture (10^9 CFU/mL) was inoculated into 350 mL of TSB in the reactor. The biofilm reactor was placed on a magnetic hotplate stirrer and incubated in batch mode at 34°C and a rotational speed of 120 rpm for 24 h to encourage cell adhesion. At this time point, a continuous flow of 20% TSB through the reactor was initiated and maintained at 10.5 mL/min for an additional 24 h to allow

Table 1. Minimum inhibitory and bactericidal concentrations of antiseptics and doxycycline against planktonic *S. aureus* cells.

Antimicrobial agent (active ingredient)	MIC		MBC	
	product (%)	active ingredient (µg/ml)	product (%)	active ingredient (µg/ml)
NEW (HOCL)	12.5	7.5	25	15
PVI	1.25	1250	1.25	1250
doxycycline	NA	0.05	NA	0.625

MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, HOCL = hypochlorous acid, PVI = povidone-iodine.

for biofilm expansion. Continuous flow was sustained for an extra 24 h with 10% TSB to stimulate maturation of the biofilm. After a total 72 h of biofilm growth, 5 L of saline were flushed through the reactor at maximum pump speed to wash out planktonic cells from the system. The presence of mature biofilm on the polycarbonate coupons was confirmed using scanning electron microscopy.

MBEC assays

Antibiofilm activity testing was performed based on a modified version of the single tube method for determining the efficacy of disinfectants against bacterial biofilm developed by the Environmental Protection Agency (EPA MLB SOP MB-20). Sample coupons were removed from the CDC biofilm reactor under aseptic conditions and individually distributed onto a 24-well cell culture plate. Each well contained a total of 2 mL treatment medium consisting of 50% CAMHB and different concentrations of the tested antimicrobial in PBS. Due to the experimental design, the maximum concentration of antiseptics that could be tested was 90% of the product. Two wells with 1 mL CAMHB and 1 mL PBS served as control treatment. After incubation at 37°C for 24 h, coupons were transferred from treatment wells into 50 mL tubes containing 5 mL PBS. Biofilms were retrieved from coupons by vortexing for 5 min, sonication for 10 min and an additional vortex for 5 min. The remaining bacterial solution was centrifuged, and the pellet washed two times to remove residual antimicrobials. A 10-fold dilution series of the washed bacterial solution was plated in triplicate on TSA using the spot-plating technique. TSA plates were incubated at 37°C overnight and the total amount of biofilm recovered from each coupon was determined by colony counts (CFU/coupon). All data presented in this study were gathered from four independent biofilm runs, each yielding 24 coupons that were split into treatment groups. Two coupons in each run underwent biofilm retrieval and bacterial enumeration immediately after being removed from the reactor to determine the baseline amount of biofilm per coupon (TP0 control). Coupons for which colony counts could not be determined due to experimental complications were removed from the dataset.

In addition to analysing the polycarbonate coupons, bacterial growth in the treatment medium was evaluated. The treatment medium in each well was homogenised before 1 mL was transferred to a 1 mL microfuge tube. Subsequently, the medium was washed and plated for CFU counting as described above.

Scanning electron microscopy

Scanning electron microscopy (SEM) imaging was carried out at the University of Auckland Biomedical Imaging Research Unit. Biofilms were grown as described above. Individual coupons were aseptically removed from the reactor and immediately fixed by submerging in Methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) for 7 days. Fixed samples were rinsed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 30 min and washed in water, 1% thiocarbohydrazide and 1% osmium tetroxide as previously described [32].

Subsequently, specimens were dehydrated by exposure to increasing alcohol concentrations (30 – 100%) and dried using a critical point dryer (Autosamdri-815, Tousimis). Dry coupons were mounted on an SEM chuck before gold coating using a DSR1 desk sputter coater (Nano-Structured Coating Co.). Prepared coupons were imaged with a Hitachi TM3030Plus Tabletop Microscope.

Statistical analyses

Statistical analysis was conducted in the R software environment (v4.0.2). Bacterial count data were analysed using pairwise Wilcoxon rank sum tests with the Benjamini-Hochberg correction for multiple comparisons. Differences were regarded as significant if p-values were below 0.05.

Results

Development of mature *S. aureus* biofilms

Selected baseline coupons underwent microscopic evaluation to confirm the presence of mature biofilm. *S. aureus* communities grown in the CDC-BR according to the protocol used in this study show distinct features of bacterial biofilms. Cells are adherent to each other as well as to the polycarbonate surface, forming large three-dimensional clusters (Figure 1A). Higher magnification highlights bacterial cells embedded in extracel-

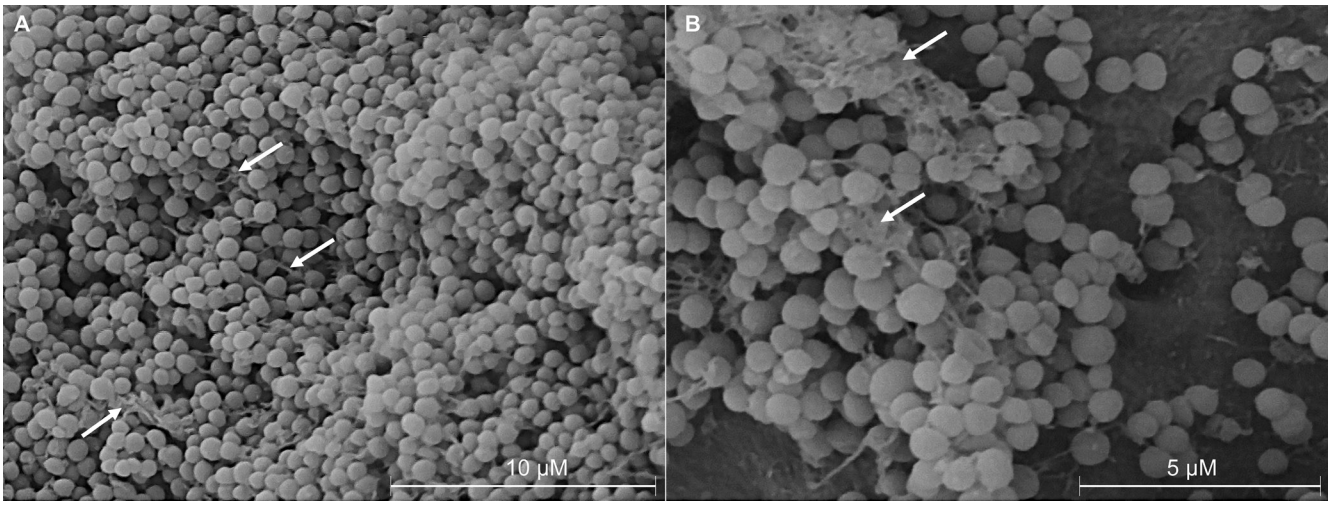

Figure 1. Scanning electron microscopy of *S. aureus* clinical isolate biofilm on a polycarbonate coupon at 10,000x (A) and 18,000x (B) magnification. Bacterial cells are embedded in extracellular matrix (white arrows).

lular matrix (Figure 1B).

Efficacy against planktonic bacterial cells

Minimum inhibitory concentrations and minimum bactericidal concentrations of NEW, PVI and doxycycline against planktonic *S. aureus* cells are summarised in Table 1. MICs were determined for all antimicrobials. Bacterial growth was inhibited at concentrations of 12.5% (7.5 µg/mL) NEW and 1.25% (1250 µg/mL) PVI. Notably, the amounts of active ingredients needed to achieve an antibacterial effect vary widely between all agents, with doxycycline showing the lowest MIC (0.05 µg/mL). The MBC for the antiseptic products is identical to the MIC in the case of PVI and approaches the MIC in the case of NEW (25%). In contrast, a concentration 12.5 times higher than the MIC of doxycycline is needed to exert a bactericidal effect.

Efficacy against bacterial biofilm

Antimicrobial agents were tested against well-established S. *aureus* biofilms to compare their efficacy against planktonic bacteria with that against their sessile counterparts. Concentrations higher than MBCs were required to achieve antibiofilm activity (Figure 2A-C). In all experiments, an up to two log10 reduction could be observed between baseline biofilms and control treatment (50% CAMHB) coupons. Compared to control treatment (i.e. 0% antimicrobial), the reduction of viable cells per coupon did not exceed 0.5 log for any of the tested NEW concentrations (1 – 90%). Thus, no minimum biofilm eradication concentration could be determined for NEW. A concentration of 10% PVI (10 mg/mL PVI) yielded a reduction of viable biofilm to below detectable limits. The MBEC for PVI was eight times higher than the observed MIC/MBC. Treatment with doxycycline resulted in a slight dose-dependent reduction of biofilm with an approximate four log reduction at 1000 µg/mL, which was the maximum dose tested (which is 1600 times the MBC determined in this study for this organism). *S. aureus* biofilms were strongly resistant to doxycycline. Statistical significances for total CFUs recovered from coupons between different concentrations within each group are provided as supplementary material (S1-S3). The treatment medium from each well was also analysed for microbial growth to investigate dispersion of biofilm or biofilm-like cells into the microenvironment. The number of CFU/mL in the control treatment medium was higher compared to control coupons and similar to baseline coupon counts in all experiments (Figure 2D-F). Overall, results from treatment media closely mirrored those from treatment coupons for all tested agents, which suggests biofilm dispersion from coupons into the media. Treatment with PVI resulted in a strong dose-dependent killing of biofilm with a five log reduction at a concentration of 10%. Statistical significances for total CFU counts of the treatment media between different concentrations within each group are shown in tables S4 - S5.

Discussion

This is the first study to compare the efficacy of NEW, PVI and doxycycline against planktonic and biofilm cultures of a CRS-associated *S. aureus* isolate. The in vitro antimicrobial activity of NEW has been investigated as part of several studies with varying results. One group reported an MBC of 3 - 6 µg/mL HOCL for clinical *S. aureus* isolates, which is around three times lower than the MBC found in the current study (15 µg/mL) [33]. Another study, using a maximum concentration of 1500 µg/mL HOCL, was unable to determine an MIC against any one of 17 bacterial isolates from the ocular surface of animals, including two *S. aureus* isolates [26]. A further study reported bactericidal effects against planktonic *P. aeruginosa* cells after treatment with an 80 µg/mL HOCL solution, which was the lowest concentration

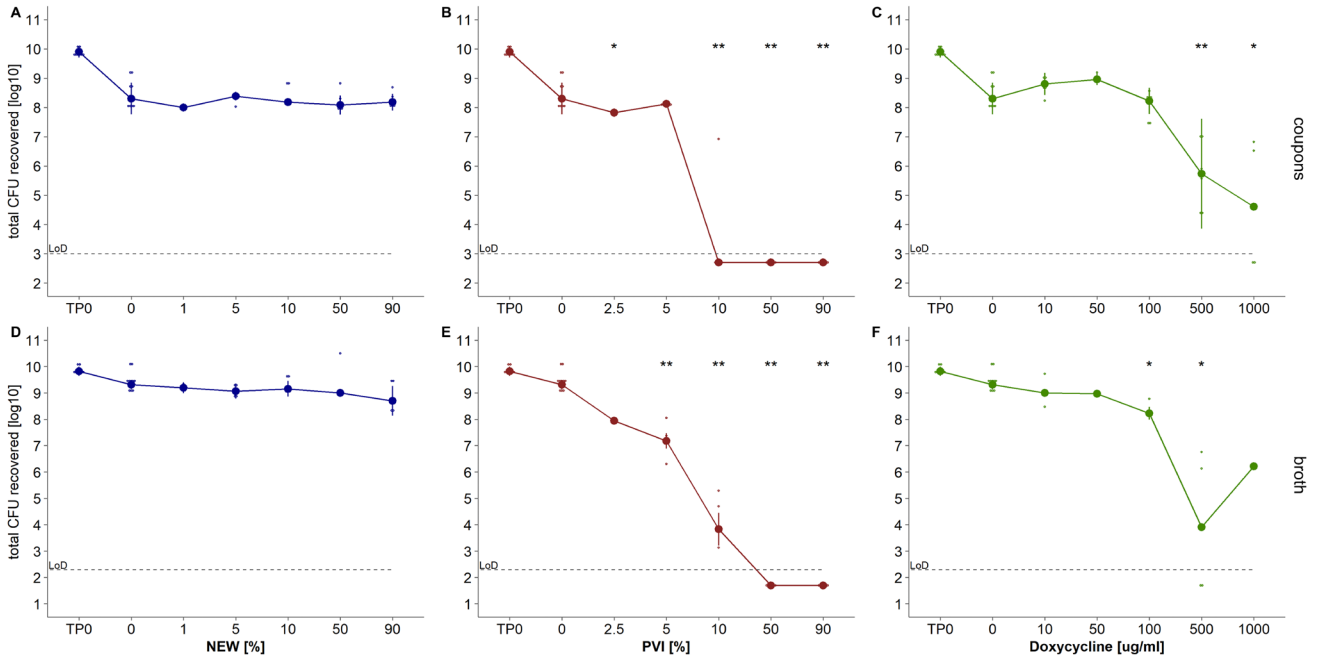

Figure 2. Total number of CFUs recovered from coupons (A-C) and treatment medium (broth, D-F) for each treatment group. LoD = Limit of Detection, TP0 = baseline counts. Statistical differences are indicated according to the following significance levels: $p < 0.05$ (*), $p < 0.01$ (**).

tested in that study [34].

The MIC and MBC for PVI (1250 µg/mL for both) determined in our study is similar to the range of previously reported values ranging between 800 - 1600 µg/mL for the MIC and 1320 – 2300 µg/mL for MBC against *S. aureus* [35-38]. The published MIC and MBC for doxycycline against *S. aureus* varies between 0.1 - 1 µg/mL and 5 – 100 µg/mL, respectively, with both found to be lower in this study (MIC: 0.05 µg/mL and MBC: 0.625 µg/mL). The relatively large difference between the MIC and MBC for doxycycline compared to both antiseptics may be due to doxycycline being a bacteriostatic rather than bactericidal antibiotic [39-41].

Testing against well-established biofilms

The antibiofilm activity of NEW in this study was low and no MBEC could be determined. In contrast, earlier studies observed substantial antibiofilm effects of different HOCL solutions on *S. aureus* biofilms grown in microtiter plates. One study reported impairment of mature biofilm at a concentration of 6 µg/mL HOCL [33]. More recently, two commercial HOCL solutions (Vache®, 0.033% HOCL and PhaseOne®, 0.025% HOCL) showed significant biocidal effects on 24 h matured biofilm [42]. *S. aureus* biofilm grown on contact lenses for 24 h was also effectively eradicated by another antiseptic product (Anenova®) containing 0.01% HOCL after 30 min exposure [43]. All antiseptic products used in these studies include at least twice the concentration of HOCL compared to the nasal spray used in the current study. NEW contains very dilute HOCL to optimise tolerance when applied to the sensitive sinonasal mucosa. However, the most pertinent difference between these studies and the current work lies in the development of the treated biofilms. Here we employ the CDC-BR in continuous stirred flow mode and follow a complex 72 h protocol that incorporates cell adhesion as well as biofilm expansion and maturation phases. The resulting biofilm is likely to be thicker, more complex and more resilient than the stationary phase-grown biofilms used in the earlier studies. Differences in the structure and maturity of biofilm between studies may contribute to the lower efficacy of the tested agents observed here.

Two recent studies have employed the CDC biofilm model to evaluate the efficacy of NEW and other antiseptics against microbial biofilms. One found that electrolysed water at 25% concentration reduced *S. aureus* biofilms grown in 48 h batch culture under sheer force by four logs [44]. While sodium hypochlorite and hypochlorous acid are mentioned as the two main constituents of the solution used in this study, the exact concentrations for these components are not stated but are probably higher than in our study. The second study tested a range of antiseptics, including Microcyn® (which is very similar in composition to Nasocyn™) on biofilms grown under shear force in batch mode for 24 and 48 h. These experiments showed complete eradication of mature *S. aureus* biofilms when exposed to pure Microcyn® solution for 24 h [45]. In contrast, treatments in our study were performed in the presence of MHB to be in accordance with international procedures for antimicrobial susceptibility testing. The availability of nutrients and other compounds such as proteins and starch may impact bacterial

susceptibility. Additionally, both studies utilised biofilm-forming ATCC reference strains in comparison to the clinically isolated *S. aureus* strain used in the current study. As a dominant bacterial strain from a sinus cavity of a CRS patient, this clinical isolate may have developed properties that increase bacterial persistence. CRS-associated bacteria have previously demonstrated their ability to form robust biofilms in vitro [46].

Johani and colleagues (2018) also demonstrated effective biofilm eradication by PVI, which was observed in an identical manner in our study. Similar results were shown in other studies [47, 48]. The greater efficacy of PVI in comparison to NEW may be partially due to the different concentrations of their respective active ingredients within each product.

Efficacy against static and dispersed biofilm

Higher CFU counts in the treatment medium containing antimicrobial agents at concentrations higher than the determined MICs suggest that the agents have caused a dispersion of biofilm or biofilm-like cells into the medium. We observed that bacterial reduction in the treatment medium was highly similar to that on the coupons. Only in the PVI group was bactericidal efficacy slightly increased at lower concentrations (Figure 2E). This may be due to the different mechanisms of action of PVI and doxycycline. The three-dimensional structure of static biofilm impedes the penetration of biocidal agents to deeper cell layers. The effectiveness of doxycycline is reliant on active bacterial metabolism. However, distinct phenotypes with altered metabolic activity can be observed in dispersed biofilm cells [49, 50]. In contrast, PVI acts via physical disruption of the cell wall, thus removing the three-dimensional structure of the static biofilm and facilitating better access to cells.

Clinical relevance and study limitations

Our results demonstrate antimicrobial activity of NEW for planktonic *S. aureus* cells but not for mature biofilm. Doxycycline only showed antimicrobial effects against the biofilms at very high concentrations that cannot be achieved at any target site in human patients. Interestingly, a recent study performed by our group determined the mean concentration of doxycycline in the sinonasal mucosa and its secretions in CRS patients (1.5 µg/mL and 0.3 µg/mL, respectively [51]). These concentrations are within the range of the determined MIC and MBC in this study, highlighting the potential for biofilms to limit the efficacy of antibiotic treatment in refractory CRS patients.

PVI showed promising antibiofilm activity at physiological concentrations. Typically used on external wounds, undiluted PVI cannot be applied to the sinus epithelium. However, nasal rinses with lower concentrations of PVI have been used in CRS patients without adverse effects on mucociliary clearance or olfaction [30]. Furthermore, a new and promising formulation has shown no negative effects on nasal epithelial cells at a concentration of 5% PVI, which is close to the MBEC found in our study [31].

In current clinical practice, xylitol is widely advised for biofilm associated CRS due to its demonstrated improvement of symptoms [52, 53]. However, it showed little activity against established *S. aureus* biofilms in vitro [54]. The observed in vivo benefits of xylitol may be attributable to its mucolytic properties and the ability to facilitate the host's native immune response [53, 55] while its effectiveness as an antibiofilm agent is limited in vitro.

Our study has several limitations. Bacterial colonies were grown in pristine conditions to encourage the establishment of maximally robust biofilms. It is likely that this in vitro model overestimates the MBEC for in vivo biofilms. In our experiments, biofilms were fully exposed to each agent for 24 h which does not mimic the real-world contact time of the rapidly cleared topical antimicrobials. Diluted bacterial nutrient medium was also part of the treatment medium and does not accurately represent the microenvironment of sinonasal cavities. Using a less nutrient-rich solution (e.g. PBS or saline) may have an effect on CFU counts particularly when analysing the treatment medium.

The development of antibiotic-resistant bacterial strains is of great concern, especially when antibiotics are administered over longer time periods (as can be the case in some CRS patients) and at doses that do not achieve effective concentrations [56, 57]. Antiseptics such as HOCL and particularly PVI as alternative treatment options may reduce the risk of resistance development [27, 28, 58].

Conclusion

In this study, PVI appeared to be effective against *S. aureus* biofilms, and its efficacy in vivo warrants further investigation. A future clinical trial with a povidone iodine-based antimicrobial compound is planned by our group.

Acknowledgements

The authors would like to thank Jillian Cornish and Stuart Irwin for their help with the biofilm reactor setup. Thanks also to Dr Brett Wagner Mackenzie for providing the *S. aureus* clinical isolate.

Authorship contribution

CL, KB, RD and MT conceived the study. CL designed and carried out the experiments and performed the data analysis. CL took the lead in writing the manuscript with support from RD and MT.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This study was supported by funding from the Garnett Passe and Rodney Williams Memorial Foundation (9107/3712790).

References

1. Orlandi RR, Kingdom TT, Hwang PH, et al. International consensus statement on allergy and rhinology: Rhinosinusitis. Int Forum Allergy Rhinol. 2016;6 (Suppl 1):22-209.
2. Dietz de Loos D, Lourijsen ES, Wildeman MAM, et al. Prevalence of chronic rhinosinusitis in the general population based on sinus radiology and symptomatology. J Allergy Clin Immunol. 2019;143(3):1207-14.
3. Hwang CS, Lee HS, Kim SN, Kim JH, Park DJ, Kim KS. Prevalence and risk factors of chronic rhinosinusitis in the elderly population of Korea. Am J Rhinol Allergy. 2019;33(3):240-6.
4. Rudmik L, Soler ZM, Hopkins C, et al. Defining appropriateness criteria for endoscopic sinus surgery during management of uncomplicated adult chronic rhinosinusitis: A RAND/UCLA appropriateness study. Int Forum Allergy Rhinol. 2016;6(6):557-67.
5. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2(2):95-108.
6. Fokkens WJ, Lund VJ, Hopkins C, et al. European position paper on rhinosinusitis and nasal polyps 2020. Rhinology. 2020;58(Suppl S29):1-464.
7. Fastenberg JH, Hsueh WD, Mustafa A, Akbar NA, Abuzeid WM. Biofilms in chronic rhinosinusitis: Pathophysiology and therapeutic strategies. World J Otorhinolaryngol Head Neck Surg. 2016;2(4):219-29.
8. Bendouah Z, Barbeau J, Hamad WA, Desrosiers M. Biofilm formation by Staphylococcus aureus and Pseudomonas aeruginosa is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. Otolaryngol Head Neck Surg. 2006;134(6):991-6.
9. Psaltis AJ, Weitzel EK, Ha KR, Wormald PJ. The effect of bacterial biofilms on post-sinus surgical outcomes. Am J Rhinol. 2008;22(1):1-6.
10. Singhal D, Psaltis AJ, Foreman A, Wormald PJ. The impact of biofilms on outcomes after endoscopic sinus surgery. Am J Rhinol Allergy. 2010;24(3):169-74.
11. Boase S, Foreman A, Cleland E, et al. The microbiome of chronic rhinosinusitis: Culture, molecular diagnostics and biofilm detection. BMC Infectious Diseases. 2013;13:210.
12. Foreman A, Psaltis AJ, Tan LW, Wormald P-J. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. Allergy Rhinol. 2010;1(1):10.
13. Foreman A, Wormald PJ. Different biofilms, different disease? A clinical outcomes study. Laryngoscope. 2010;120(8):1701-6.
14. Maina IW, Patel NN, Cohen NA. Understanding the role of biofilms and superantigens in chronic rhinosinusitis. Curr Otorhinolaryngol Rep. 2018;6(3):253-62.
15. Singhal D, Foreman A, Jervis-Bardy J, Wormald PJ. Staphylococcus aureus biofilms: Nemesis of endoscopic sinus surgery. Laryngoscope. 2011;121(7):1578-83.
16. Jefferson KK, Goldmann DA, Pier GB. Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus aureus biofilms. Antimicrob Agents Chemother. 2005;49(6):2467-73.
17. Singh R, Ray P, Das A, Sharma M. Penetration of antibiotics through Staphylococcus aureus and Staphylococcus epidermidis biofilms. J Antimicrob Chemother. 2010;65(9):1955-8.
18. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15(2):167-93.
19. Koo H, Allan RN, Howlin RP, Stoodley P, Hall-Stoodley L. Targeting microbial biofilms: Current and prospective therapeutic strategies. Nat Rev Microbiol. 2017;15(12):740-55.
20. Rasmussen TB, Givskov M. Quorum-sensing inhibitors as anti-pathogenic drugs. Int J Med Microbiol. 2006;296(2-3):149-61.
21. Stewart PS, Costerton WJ. Antibiotic resistance of bacteria in biofilms. Lancet. 2001;358(9276):135-8.
22. Del Rosso JQ, Bhatia N. Status report on topical hypochlorous acid: Clinical relevance of specific formulations, potential modes of action, and study outcomes. J Clin Aesthet Dermatol. 2018;11(11):36-9.
23. Cho HJ, Min HJ, Chung HJ, et al. Improved outcomes after low-concentration hypochlorous acid nasal irrigation in pediatric chronic sinusitis. Laryngoscope. 2016;126(4):791-5.
24. Lux CA, Johnston J, Biswas K, Douglas RG. Efficacy of neutral electrolyzed water in postoperative chronic rhinosinusitis patients-a pilot study. Int Forum Allergy Rhinol. 2020;11:81-3.
25. Yu MS, Kim BH, Kang SH, Lim DJ. Low-concentration hypochlorous acid nasal irrigation for chronic sinonasal symptoms: A prospective randomized placebo-controlled study. Eur Arch Otorhinolaryngol. 2017;274(3):1527-33.
26. Bergstrom BE, Abdelkhalek A, Younis W, Hammac GK, Townsend WM, Seleem MN. Antibacterial activity and safety of commercial veterinary cationic steroid antibiotics and neutral superoxidized water. PLoS One. 2018;13(3):e0193217.
27. Barreto R, Barrois B, Lambert J, Malhotra-Kumar S, Santos-Fernandes V, Monstrey S. Addressing the challenges in antisepsis: Focus on povidone iodine. Int J Antimicrob Agents. 2020;56(3):106064.
28. Eggers M. Infectious disease management and control with povidone iodine. Infect Dis Ther. 2019;8(4):581-93.
29. Martínez-De Jesús FR, Ramos-De la Medina A, Remes-Troche JM, et al. Efficacy and safety of neutral pH superoxidised solution in severe diabetic foot infections. Int Wound J. 2007;4(4):353-62.
30. Panchmatia R, Payandeh J, Al-Salman R, et al. The efficacy of diluted topical povidone-iodine rinses in the management of recalcitrant chronic rhinosinusitis: A prospective cohort study. Eur Arch Otorhinolaryngol. 2019;276(12):3373-81.
31. Ramezanpour M, Smith JLP, Psaltis AJ, Wormald PJ, Vreugde S. In vitro safety evaluation of a povidone-iodine solution applied to human nasal epithelial cells. Int Forum Allergy Rhinol. 2020;10(10):1141-48.
32. Dassanayake RP, Falkenberg SM, Stasko JA, Shircliff AL, Lippolis JD, Briggs RE. Identification of a reliable fixative solution to preserve the complex architecture of bacterial biofilms for scanning electron microscopy evaluation. PLoS One. 2020;15(5):e0233973.
33. Sakarya S, Gunay N, Karakulak M, Ozturk B, Ertugrul B. Hypochlorous acid: An ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. Wounds. 2014;26(12):342-50.
34. Sauer K, Thatcher E, Northey R, Gutierrez AA. Neutral super-oxidised solutions are effective in killing P. aeruginosa biofilms. Biofouling. 2009;25(1):45-54.
35. Cichos KH, Andrews RM, Wolschendorf F, Narmore W, Mabry SE, Ghanem ES. Efficacy of intraoperative antiseptic techniques in the prevention of periprosthetic joint infection: Superiority of betadine. J Arthroplasty. 2019;34(7S):312-S8.
36. Eggers M, Koburger-Janssen T, Eickmann M, Zorn J. In vitro bactericidal and virucidal efficacy of povidone-iodine gargle/mouthwash against respiratory and oral tract pathogens. Infect Dis Ther. 2018;7(2):249-59.
37. Oduwole KO, Glynn AA, Molony DC, et al. Anti-biofilm activity of sub-inhibitory povidone-iodine concentrations against Staphylococcus epidermidis and Staphylococcus aureus. J Orthop Res. 2010;28(9):1252-6.
38. van Meurs SJ, Gawlitta D, Heemstra KA, Poolman RW, Vogely HC, Kruyt MC. Selection of an optimal antiseptic solution for intraoperative irrigation: An in vitro study. J Bone Joint Surg Am. 2014;96(4):285-91.
39. Nemeth J, Oesch G, Kuster SP. Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: Systematic review and meta-analysis. J Antimicrob

Chemother. 2014;70(2):382-95.
40. Raval JP, Chejara DR, Ranch K, Joshi P. 6 - development of injectable in situ gelling systems of doxycycline hyclate for controlled drug delivery system. In: Inamuddin, Asiri AM, Mohammad A, editors. Applications of nanocomposite materials in drug delivery. Woodhead Publishing; 2018. p. 149-62.
41. Swayze EE, Griffey RH, Bennett CF. 2.26 - nucleic acids (deoxyribonucleic acid and ribonucleic acid). In: Taylor JB, Triggle DJ, editors. Comprehensive medicinal chemistry ii. Oxford: Elsevier; 2007. p. 1037-52.
42. Harriott MM, Bhindi N, Kassis S, et al. Comparative antimicrobial activity of commercial wound care solutions on bacterial and fungal biofilms. Ann Plast Surg. 2019;83(4):404-10.
43. Romanowski EG, Stella NA, Yates KA, Brothers KM, Kowalski RP, Shanks RMQ. In vitro evaluation of a hypochlorous acid hygiene solution on established biofilms. Eye Contact Lens. 2018;44(Suppl 2):187-S91.
44. Salisbury AM, Percival SL. The efficacy of an electrolysed water formulation on biofilms. Adv Exp Med Biol. 2019;1214:1-8.
45. Johani K, Malone M, Jensen SO, et al. Evaluation of short exposure times of antimicrobial wound solutions against microbial biofilms: From in vitro to in vivo. J Antimicrob Chemother. 2018;73(2):494-502.
46. Shields RC, Mokhtar N, Ford M, et al. Efficacy of a marine bacterial nuclease against biofilm forming microorganisms isolated from chronic rhinosinusitis. PLoS One. 2013;8(2):e55339.
47. Herruzo R, Herruzo I. Antimicrobial efficacy of a very stable hypochlorous acid formula compared with other antiseptics used in treating wounds: In-vitro study on microorganisms with or without biofilm. J Hosp Infect. 2020;105(2):289-94.
48. Hoekstra MJ, Westgate SJ, Mueller S. Povidone-iodine ointment demonstrates in vitro efficacy against biofilm formation. Int Wound J. 2017;14(1):172-9.
49. Fleming D, Rumbaugh K. The consequences of biofilm dispersal on the host. Scientific Reports. 2018;8(1):10738.
50. Rumbaugh KP, Sauer K. Biofilm dispersion. Nat Rev Microbiol. 2020;18(10):571-86.
51. Siu J, Klingler L, Wang Y, et al. Oral antibiotics used in the treatment of chronic rhinosinusitis have limited penetration into the sinonasal mucosa: A randomized trial. Xenobiotica. 2020;50(12):1443-50.
52. Weissman JD, Fernandez F, Hwang PH. Xylitol nasal irrigation in the management of chronic rhinosinusitis: A pilot study. Laryngoscope. 2011;121(11):2468-72.
53. Lin L, Tang X, Wei J, Dai F, Sun G. Xylitol nasal irrigation in the treatment of chronic rhinosinusitis. Am J Otolaryngol. 2017;38(4):383-9.
54. Jain R, Lee T, Hardcastle T, Biswas K, Radcliff F, Douglas R. The in vitro effect of xylitol on chronic rhinosinusitis biofilms. Rhinology. 2016;54(4):323-8.
55. Hardcastle T, Jain R, Radcliff F, et al. The in vitro mucolytic effect of xylitol and dornase alfa on chronic rhinosinusitis mucus. Int Forum Allergy Rhinol. 2017;7(9):889-96.
56. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol. 2014;12(7):465-78.
57. Schwartz JS, Tajudeen BA, Cohen NA. Medical management of chronic rhinosinusitis - an update. Expert Rev Clin Pharmacol. 2016;9(5):695-704.
58. Punjataewakupt A, Napavichayanun S, Aramwit P. The downside of antimicrobial agents for wound healing. Eur J Clin Microbiol Infect Dis. 2019;38(1):39-54.

Richard G. Douglas, MD, FRACS
Department of Surgery
The University of Auckland
Private Bag 92019
Auckland 1142
New Zealand

Tel: +64 9 923 1658
E-mail:
richard.douglas@auckland.ac.nz

This manuscript contains online supplementary material

SUPPLEMENTARY MATERIAL

Table S1. p-values for pairwise comparisons of CFU counts from coupons treated with NEW.

coupons	baseline	control	NEW1%	NEW10%	NEW5%	NEW50%
control	0.008	-	-	-	-	-
NEW1%	0.014	0.267	-	-	-	-
NEW10%	0.008	0.938	0.191	-	-	-
NEW5%	0.014	0.938	0.267	0.938	-	-
NEW50%	0.008	0.667	0.769	0.591	0.667	-
NEW90%	0.008	0.769	0.400	0.769	0.769	0.806

Table S2. p-values for pairwise comparisons of CFU counts from coupons treated with PVI

coupons	baseline	PVI10%	PVI2.5%	PVI5%	PVI50%	PVI90%
PVI10%	0.005	-	-	-	-	-
PVI2.5%	0.017	0.022	-	-	-	-
PVI5%	0.005	0.007	0.030	-	-	-
PVI50%	0.005	0.405	0.014	0.006	-	-
PVI90%	0.005	0.405	0.014	0.006	-	-
control	0.005	0.005	0.031	0.240	0.005	0.005

Table S3. p-values for pairwise comparisons of CFU counts from coupons treated with doxycycline.

coupons	baseline	control	Doxy 1000ug/ml	Doxy 100ug/ml	Doxy 10ug/ml	Doxy 500ug/ml
control	0.005	-	-	-	-	-
Doxy1000 μg/ml	0.018	0.018	-	-	-	-
Doxy100 μg/ml	0.005	0.349	0.023	-	-	-
Doxy10 μg/ml	0.007	0.368	0.029	0.040	-	-
Doxy500 μg/ml	0.005	0.007	0.454	0.008	0.013	-
Doxy50 μg/ml	0.018	0.109	0.040	0.023	0.205	0.023

Table S4. p-values for pairwise comparisons of CFU counts from treatment medium with NEW™.

broth	baseline	control	NEW1%	NEW10%	NEW5%	NEW50%
control	0.112	-	-	-	-	-
NEW1%	0. 016	0. 298	-	-	-	-
NEW10%	0. 044	0. 589	1	-	-	-
NEW5%	0.028	0.298	0.614	0.614	-	-
NEW50%	0.293	0.298	0.589	0.589	1	-
NEW90%	0.016	0.293	0.608	0.589	0.614	0.589

Table S5. p-values for pairwise comparisons of CFU counts from treatment medium with PVI.

broth	baseline	PVI10%	PVI2.5%	PVI5%	PVI50%	PVI90%
PVI10%	0.005	-	-	-	-	-
PVI2.5%	0.022	0.028	-	-	-	-
PVI5%	0.005	0.008	0.154	-	-	-
PVI50%	0.005	0.006	0.012	0.005	-	-
PVI90%	0.005	0.006	0.012	0.005	-	-
control	0.028	0.005	0.018	0.005	0.005	0.005

Table S6. p-values for pairwise comparisons of CFU counts from treatment medium with doxycycline.

broth	baseline	control	Doxy 1000ug/ml	Doxy 100ug/ml	Doxy 10ug/ml	Doxy 500ug/ml
control	0.077	-	-	-	-	-
Doxy1000 μg/ml	0.087	0.085	-	-	-	-
Doxy100 μg/ml	0.040	0.040	0.124	-	-	-
Doxy10 μg/ml	0.066	0.124	0.114	0.085	-	-
Doxy500 μg/ml	0.044	0.040	0.498	0.077	0.066	-
Doxy50 μg/ml	0.085	0.098	0.244	0.156	0.845	0.124