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Efficacy of Dilute Povidone-Iodine against Multi-Drug Resistant Bacterial Biofilms, Fungal Biofilms and Fungal Spores

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Abstract

Objective: To study the *in vitro* effect of novel low-dose povidone-iodine formulations against established biofilms of multi-drug resistant *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* and to test the Minimal Inhibitory Concentration of the same formulations against *Candida auris, Trichophton mentagrophytes, Microsporum canis, Candida albicans and Aspergillus fumigatus.*

Methods: Biofilms of multi-drug resistant *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* were developed on solid surfaces using the Calgary Biofilm Device plate. Minimum biofilm eradication concentration was then determined for each test drug and for control samples of known antimicrobials, ciprofloxacin and itraconazole. Quality control fungal strains of *Candida auris, Trichophton mentagrophytes, Microsporum canis, Candida albicans* and *Aspergillus fumigatus* were grown on Sabouraud's Dextrose Agar plates as the growth medium for the anti-fungal susceptibility. Minimal Inhibitory Concentration was then determined for each test drug and for control samples of know antifungals, fluconazole and amphotericin B.

Results: The low-dose povidone-iodine formulations completely eliminated all biofilms of bacterial and fungal species in the test systems. Ciprofloxacin was able to eradicate one bacterial biofilm only at concentrations greater than 0.25 ug/mL. Fluconazole was ineffective against *C. albicans, A. fumigatus* and *C. auris.* Amphotericin B had good anti-fungal activity against fungal strains.

Conclusions: These novel dilute PVP-I formulations are effective anti-biofilm and anti-fungal /sporicidal agents *in vitro*. Further evaluation in living models is warranted.

Keywords: Biofilm; Povidone-Iodine; Wounds;

Introduction

Biofilms are pathogen agglomerations attached to a surface and embedded in an extracellular matrix [1]. Formation of biofilm is a successful strategy that protects the bacteria, fungi and yeasts from environmental danger, antimicrobial peptides, antibiotics and phagocytosis enabling a state of chronic persistence in the host. Biofilms are not just bacterial slime layers but rather represent complex biological systems. The comprising microorganisms are organized into a coordinated functional community. Biofilms are often attached to a surface and may include a single species or a diverse group of sessile microorganisms. These specialized heterogeneous communities are strengthened and protected by the biofilm in a manner that eventually leads to downstream deleterious gene expression and the manifestation of pathogenic factors. Chronic wounds have been shown to involve biofilm formation of both bacterial and fungal species [2,3]. It is in such a setting that biofilm microorganisms are able to share nutrients and are sheltered from harmful factors in the environment, such as desiccation, antibiotics, and the body's immune system [4,5].

More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds [6]. Chronic wounds are often a result of cutaneous surgery, vascular insufficiency or trauma, frequently requiring management by primary care physicians, dermatologists, plastic surgeons and wound care specialists. During normal wound healing, the process that leads to tissue regeneration results from a series of tightly regulated sequential events [7,8]. In the case of chronic, non-healing wounds, this process is disrupted, leading to a prolonged inflammatory response and stalled healing. It is hypothesized that microbial colonization and the formation of a biofilm within the wound bed is positively associated with the transition from the acute to chronic state even without classical signs of infection [9]. When a biofilm forms, the penetration of some antimicrobials is reduced, while some individual members shift their metabolism to a more dormant state that can render other antibiotics ineffective. Treatment strategies are further complicated by co-morbidities that affect circulation such as diabetes, poor perfusion, and malnutrition, increasing the risk of infection and reducing the success of orally administered antibiotics [9]. It follows therefore, that early detection of biofilms in wounds is crucial to successful chronic wound management.

Many of the treatments currently available are designed to treat acute wound infections, which, unlike chronic infections, tend to appear quickly and run their course over a short period of time. Planktonic bacteria typically respond to antibiotics and are easily exterminated by a healthy immune system. In contrast, chronic wounds are normally characterized by a tenacious and excessive inflammatory response when compared with acute wounds and are less susceptible to antibiotics [10]. Hence, the lack of antimicrobial effectiveness may be related to reduced or incomplete penetration of antimicrobials into the biofilm. A novel approach to the aforementioned problem is presented that employs the common antiseptic povidone-iodine at very low concentrations in a gel formulation to chronic wounds. Similar formulations have already been successfully used for indications for dermatology and ophthalmology [11,12]. An *in vitro* assay was performed to further characterize the anti-biofilm effect of this clinically successful treatment against organisms known to form biofilms in cutaneous wound infections.

Materials and Methods

Preparation of PVP-I Formulation

The test formulations for biofilms consist of povidone-iodine at or below 2% (w/w) in solvent systems that can be tailored to form solutions or cellulosic gels incorporating dimethylsulfoxide as a co-solvent. These dilute povidone-iodine systems allow the preparation and room-temperature stabilization of povidone-iodine concentrations from 1% to as low as 0.15% in solutions and gel systems. For these experiments povidone-iodine solutions were prepared at 2% (w/w) and povidone-iodine gels were prepared at 0.25% (w/w). Subsequent dilutions were prepared of the solutions and gels to achieve lower concentration solutions for evaluation.

Challenge Bacterial Organism Preparation

Test organisms were obtained from the American Type Culture Collection (Manassas, VA) or Eurofins Medinet, Inc. (Herndon, VA) (Table 1). The strains were maintained as a frozen glycerol stocks at -80°C. The bacterial strains or C. albicans were prepared by thawing a glycerol stock, streaking and growing onto Tryptic Soy Agar (TSA) or Sabouraud's Dextrose Agar (SDA) plates, respectively and incubating overnight at 35 ± 2°C in aerobic conditions and then used for the assay. The completely synthetic medium RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) was buffered with 0.165 mol/L. MOPS (3-[N-morpholino] propanesulfonic acid) buffer was used as the growth medium for the fungal strain in the MBEC assay.

Challenge Fungal Organism Preparation

The fungal strains were obtained from the American Type Culture Collection (Manassas, VA), and Center for Disease Control and Prevention (CDC) (Atlanta, GA) (Table 1). The strains were maintained as a frozen glycerol stocks at -80°C. The fungal strains were prepared by thawing a glycerol stock, streaking and growing onto Sabouraud's Dextrose Agar (SDA) plates with an overnight incubation at 27-30 ± 2°C in aerobic conditions and then used for the assay. The completely synthetic medium RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as a pH indicator) was buffered with 0.165 mol/L MOPS (3-[N-morpholino] propanesulfonic acid) to obtain a pH of 6.9 - 7.1 at room temperature and was used as the growth medium for the anti-fungal susceptibility MIC assay.

Table 1: Organism Details

#	Organism	Isolate #	Phenotype	Source
1	*Staphylococcus aureus*	ATCC 33591	Multi-drug resistant	ATCC
2	*Klebsiella pneumoniae*	ATCC BAA-2473™	Multi-drug resistant	ATCC
3	*Pseudomonas aeruginosa*	1674623	Multi-drug resistant	Eurofins
4	*Candida albicans**	3288194	Multi-drug resistant	Eurofins
5	*Candida auris*	CDC 0389	Multi-drug resistant	ATCC
6	*Trichophton mentagrophytes*	MYA4439	Quality Control	ATCC
7	*Microsporum canis*	ATCC 26299	Quality Control	ATCC
8	*Candida albicans***	ATCC 90028	Quality Control	ATCC
9	*Aspergillus fumigatus*	MYA3626	Quality Control	ATCC

*Strain tested for Minimum Biofilm Eradication Concentration
**Strain tested for Minimal Inhibitory Concentration

Comparator antimicrobials and controls

Minimum Biofilm Eradication Concentration (MBEC) for bacteria was measured using ciprofloxacin (Cat # 17850, Lot # 456829, Potency 98.0 µg/mL) and itraconazole (Cat # I6657, Lot # 087K1322) were obtained from Sigma (St. Louis, MO, USA). Antibiotic stocks were prepared in appropriate solvents following the CLSI guidelines [13].

Comparator antifungals and controls

Minimal Inhibitory Concentration (MIC) for fungi was measured using amphotericin B (Cat # A9528-100 mg, Lot # 076M4136V) and fluconazole (F8929-100 mg, Lot # 036MA709V) were obtained from Sigma (St. Louis, MO, USA). Antifungal stocks were prepared in appropriate solvents following the CLSI guidelines [13,14].

Minimum Biofilm Eradication Concentration

Minimum biofilm eradication concentration values provide estimates on the concentration of an antimicrobial product required to kill/disrupt the bacterial biofilm. The Calgary Biofilm Device (CBD) plate allows for biofilm formation on a lid containing 96 pegs. The inoculum was diluted to 1 x 10^7 CFU/mL in Tryptic Soy Broth (TSB) for bacterial strains and RPMI media

for *C. albicans* before inoculating the CBD plate. The CBD plate was incubated with test microorganism for 24 hours at 35°C on a shaker at 150 rpm. The MBEC assay was conducted as described in Ceri, *et al* [15].

Biofilm Eradication Assay

A treatment plate was made with the three test articles and comparator antibiotics. The 0.25% PVP-I gel test article was highly viscous; hence only one concentration (full strength at 100%) was tested. Two percent (w/w) PVP-I solutions were serially diluted 2-fold and the resulting diluted test articles were tested down to 0.00018% (v/v). Ciprofloxacin and itraconazole were used as positive controls for bacterial strains and *C. albicans*, respectively. Ciprofloxacin was tested at the following concentrations (µg/mL):128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/mL. Itraconazole was tested at the following concentrations (µg/mL): 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/mL.

The cation-adjusted Mueller Hinton Broth (CAMHB)/ RPMI (for *C. albicans*) were used as negative control. The CBD plate with the pegs containing a robust biofilm was first rinsed in PBS and then transferred to the treatment plate containing the test articles and control. The plate was incubated for 24 hours at 35°C and then read for determination of MIC values. After incubation, the pegs were rinsed in PBS twice and transferred to a recovery plate containing fresh culture media. The pegs were sonicated in a water bath sonicator for 30 minutes to detach any remaining adherent biofilm. The plate was incubated overnight at 35°C to evaluate growth and the MBEC values were determined.

Anti-fungal Susceptibility Minimal Inhibitory Concentration (MIC) Testing

Each MIC assay included eleven 2-fold serial dilutions of the antifungal agents. Test concentrations for the test article were tested at the following concentrations: 50%, 25%, 12.5%, 6.250%, 3.125%, 1.563%, 0.781%, 0.391%, 0.195%, 0.098% and 0.049% v/v. Amphotericin B and Fluconazole were tested at 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/mL.

MIC – Broth microdilution assays were performed according to the procedures detailed in CLSI document M38-A2 and document M27-A3 [1 – 2]. Briefly, 2X stock solutions of test articles were prepared at 128 µg/mL for Amphotericin B and Fluconazole. Two-fold serial dilutions of the stock solutions were performed. Diluted stock solutions of the fungal spores were adjusted (to be equivalent to 8 x 10^4 CFU/mL). 1X final concentration of the antifungals or antibiotics was obtained when they were combined with the fungal spore broth in the microtiter plate. The final concentration of the fungal spores in the microtiter plate was 4 x 10^4 CFU/mL. Plates were incubated for 7 days at 30±2°C for *M. canis* and 48 h at 27 – 30°C for all the other strains in an ambient air incubator. MIC values recorded are the minimum concentration of the test articles that inhibited the visible growth as observed by unaided eye. The MIC values were expressed in v/v % for the test article and in µg/mL for the control antibiotics (Table 2). The assay was performed in duplicates.

Table 2: MBEC assay results

Strains	Isolate ID	Phenotype	MBEC (% PVP-I and µg/mL Abx)			
			1% PVP-I Solution	0.25% PVP-I Gel	Ciprofloxacin	Itraconazole
S. aureus	ATCC 33591	MDR	25	100	0.25	NA
K. pneumoniae	BAA-2473	MDR	25	100	>128	NA
P. aeruginosa	1674623	MDR	25	100	>128	NA
C. albicans	3288194	MDR	25	100	NA	>1024

Results

MBEC assay was carried to determine if the low-dose PVP-I test articles can disrupt a pre-existing robust biofilm of the microorganisms that were grown on the pegs for 24 hours. PVP-I solutions at concentration as low as 0.25% (w/w) and PVP-I gel at 0.25% (w/w) completely eradicated the biofilms of all the test microorganisms (Table 2). Comparator antibiotics were ineffective in eradicating biofilms of test microorganism except ciprofloxacin which had a MBEC value of 0.25 µg/mL against S. aureus ATCC 33591 (Table 2).

MIC assay was carried to determine if the the lose dose PVP-I test article had anti-fungal activity. At serially diluted concentrations as low as 6.25% and 3.125%, PVP-I solutions showed anti-fungal activity against all the test strains. Fluconazole was ineffective against *C. albicans, A. fumigatus and C. auris.* Amphotericin B had good anti-fungal activity against all the strains tested (Table 3).

Discussion

Povidone-iodine is one of the few topical antimicrobials shown to be effective against bacteria, viruses, fungi, spores, protozoa, and amoebic cysts [16]. In PVP-I solutions, iodine forms a complex with the synthetic carrier polymer polyvinypyrrolidone, which itself has no microbiocidal activity [17]. In an aqueous medium, free iodine is released into solution from the povidone-iodine complex and equilibrium is established, with more free iodine being released from the povidone-iodine reservoir as iodine-consuming germicidal activity proceeds [18,19]. As surgical antiseptics for routine medical practice, PVP-I products are supplied as 5% (w/w) or 10% (w/w) solutions, however in vitro analysis paradoxically suggests that solutions with a lower concentration may be more effective than those with higher concentrations [20]. This unique PVP-I chemistry allowed efficacy testing at extremely low concentrations in this study,

Table 3: MIC testing results

Strains	Isolate ID	Phenotype	MIC (% PVP-I and µg/mL Antifungal)					
			1% PVP-I Solution		Amphotericin B		Fluconazole	
C. albicans	ATCC 90028	QC	6.25	6.25	0.5	0.5	>64	>64
A. fumigatus	ATCC MYA 3626	QC	6.25	6.25	1	1	>64	>64
M. canis	ATCC 36299	QC	3.125	3.125	0.125	0.125	8	8
T. mentagrophytes	ATCC MYA 4439	QC	3.125	3.125	0.25	0.25	16	16
C. auris	CDC 0389	QC	3.125	3.125	2	2	>64	>64

which yielded complete eradication of all tested biofilm-forming microorganisms. At serially diluted concentrations as low as 6.25% and 3.125%, PVP-I solutions showed anti-fungal activity against all the test strains. In contrast to other antiseptics (with the apparent exception of an absence of cross-resistance to silver), no acquired resistance or cross-resistance has been reported for molecular iodine in over 150 years of use [21].

Biofilms are found in approximately 60% of chronic wounds and 6% of acute wounds. Eradication of the resident bacteria is an ongoing challenge in the treatment of these cases. Effective antiseptics for wound healing should ideally address both inflammation and biofilm formation associated with chronic wounds [22]. In vitro evidence suggests that iodine not only has broad spectrum antibacterial effects, but also counteracts inflammation elicited by both pathogens and the host response. These anti-inflammatory effects appear to be multifactorial and have been shown to be clinically relevant [23]. Antiseptics, as an alternative for topical wound treatment, tend to be microbiocidal and have a broader spectrum of antimicrobial activity than antibiotics. Furthermore, in comparison to most antibiotics and antifungals, antiseptics reduce the likelihood of resistance emerging due to their multiple mechanisms of action targeting various aspects of cell biology in microbes; hence the use of topical antibiotics and antifungals should be discouraged if appropriate antiseptics are available [24]. Although amphotericin B was effective against all fungal strains, and the other control antibiotics and antifungals in this study were partially effective, they all share to propensity for this development of resistance as none are broad spectrum antimicrobials, nor are they produced in topical formulations. Comparison of the antimicrobial spectra of the most commonly used antiseptics (Povidone-iodine 10%, Polihexanide, Chlorhexidine, Octenidine, Ethanol 70%) against Gram-positive, Gram-negative, Actinobacteria, spores, fungi and viruses demonstrates the strong microbiocidal activity of Povidone-Iodine 10% against all classes of microorganisms. The remaining categories of antiseptics were not strongly biocidal across all categories [24].

The use of PVP-I for chronic wounds has been well documented in the literature. *In vivo* human studies conducted in varying settings have established the efficacy of PVP-I in reducing the bacterial load in both acute and chronic wounds [25-31]. There have been concerns about perceived cytotoxicity with PVP-I, and the potential detrimental effect of PVP-I on wound healing, and therefore biofilms, has been widely argued, with several *in vitro* studies demonstrating the dose-dependent cytotoxicity of PVP-I on cultures of granulocytes, monocytes, keratinocytes, and fibroblasts [32-34]. *In vivo* studies have failed to demonstrate detrimental effects on wound healing and the much lower concentrations of PVP-I used in this study reduce this potential effect greatly. Low dose PVP-I formulations for a variety of diseases have recently gained interest in the eye, ear and skin without any reported toxicity [11,12,35].

Also of note is the efficacy of low dose PVP-I against a biofilm comprised of C. auris, which has proven to be multi-drug resistant and difficult to culture emerging pathogen which can be causative in both systemic and wound infections. To our knowledge, this is the first topical approach to C. auris. The limitations of this study include utilization of the MIC assay for fungal organisms, rather than the MBEC assay. The MBEC is a more comprehensive test and better simulator of in vivo biofilm conditions; however this assay is not currently developed for fungi as they are much less prevalent as causative agents for biofilms. Another limitation is the study performed was *in vitro* and may not mimic *in vivo* conditions.

Conclusion

Established biofilms of multi-drug resistant *S. aureus, P. aeruginosa, K. pneumoniae* and *C. albicans* can be successfully disrupted *in vitro* by treatment with a dilute PVP-I solution or gel. Concentrations as low as 6.25% and 3.125% PVP-I serially diluted solutions showed anti-fungal activity against Candida auris, Trichophton mentagrophytes, Microsporum canis, Candida albicans and Aspergillus fumigatus strains in the MIC assay. As the search for potent, non-toxic anti-biofilm agents continues to evolve, further investigation of dilute PVP-I-containing systems *in vitro* and *in vivo* could yield a new class of therapeutic agents with important efficacy for recalcitrant, chronic wounds.

Declarations

Conflict of Interest: All authors listed are employees and equity holders in Veloce BioPharma.

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