Journal of Global Antimicrobial Resistance 17 (2019) 160–167

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Evaluation of activity and potential for development of antimicrobial resistance to a new tinted 2% chlorhexidine gluconate/70% isopropyl alcohol film-forming sterile preoperative skin preparation

Kathryn Dormstetter[a], Linda K.M. Olson[b], Assumpta Bennaars-Eiden[b], Stéphanie F. Bernatchez[b,*]

[a] Microbac Laboratories, Inc., Sterling, VA, USA
[b] 3 M Health Care, 3 M Company,St. Paul, MN, USA

ARTICLE INFO

Article history:
Received 2 October 2018
Received in revised form 4 December 2018
Accepted 16 December 2018
Available online 21 December 2018

Keywords:
Skin antisepsis
Surgical skin prep
Chlorhexidine gluconate
Antiseptic resistance
In vitro

ABSTRACT

Objectives: Chlorhexidine gluconate (CHG) is routinely used for skin antisepsis before surgery. Its activity may be affected by formulation ingredients and the presence of organic matter such as blood and proteins. This in vitro study was designed to evaluate the antimicrobial activity of a new CHG skin prep containing a film-forming copolymer, and detect its potential for developing resistance and the potential for cross-resistance to antibiotics after CHG exposure.

Methods: Antimicrobial activity was evaluated in the presence and absence of serum in an in vitro time-kill study. Emergence of resistance to CHG and cross-resistance with antibiotic procedures were performed in vitro using 10 repository isolates from eight species and eight clinical isolate strains equal to the repository isolate strains (four isolates, two resistant and two non-resistant per species).

Results: A 5 $\log_{10}$ reduction (99.999%) for all organisms was observed using the copolymer formulation. The activity remained unchanged in the presence of serum. The minimum inhibitory concentration (MIC) did not increase for any of the strains evaluated for emergence of resistance. In addition, there was no change in MIC related to cross-resistance observed for any of the organism/antibiotic combinations tested.

Conclusions: These results suggest that the film-forming copolymer and the tint in the new CHG skin prep did not interfere with antimicrobial efficacy, even in the presence of an organic soil load, and that the tested formulations showed no potential for developing resistance or cross-resistance with antibiotics.

1. Introduction

Skin antisepsis is performed before surgery to minimize the risk of surgical site infections. Several agents are available for this procedure, including alcohols, iodine and iodophors, chlorhexidine gluconate (CHG), and combinations of these agents [1]. Recent meta-analyses of the literature indicate that preparations containing CHG and alcohol provide the best preoperative antimicrobial efficacy, combining the fast action of alcohol and residual activity of CHG [2,3].

Chlorhexidine gluconate has been used for over 50 years in preparations for hand hygiene and skin decolonization. The use of this antiseptic has considerably increased in recent years, following efforts to decolonize individuals who are carriers of methicillin-resistant *Staphylococcus aureus* (MRSA). The various uses of CHG for decolonization, including bathing intensive care unit (ICU) patients to reduce healthcare-associated infections (HAIs) and universal bathing of hospitalized patients to decrease the acquisition of multidrug-resistant organisms (MDROs), have been recently reviewed [4]. Due to this increased usage, concerns about the possible development of resistance to CHG have emerged, and several studies have attempted to answer this question [5–8]. However, there is currently no consensus as to how to test the susceptibility of organisms to CHG. As a result, numerous methods have been used and it is difficult to compare data from different studies [9].

This study compared the antimicrobial efficacy of a new film-forming CHG skin prep with a commercially available 2% CHG/isopropyl alcohol (IPA)-based solution, in the presence and absence of an organic soil load. It also wanted to assess the

* Corresponding author.
E-mail address: sfbernatchez@mmm.com (S.F. Bernatchez).

https://doi.org/10.1016/j.jgar.2018.12.008

potential for developing resistance to the new skin prep compared with CHG alone (2% aqueous CHG), and evaluate the potential development of cross-resistance to antibiotics.

2. Materials and methods

This work was performed by an independent laboratory (Microbac Laboratories, Inc., Sterling, VA). The tested formulations were a new skin prep under development (2% CHG/70% IPA with a tint and a film-forming acrylate copolymer; 3M, St. Paul, MN) and either a commercially available skin prep without colpolymer (ChloraPrep® Patient Preoperative Skin Preparation, tinted; active ingredients 2% w/v CHG and 70% v/v IPA; CareFusion Inc., San Diego, CA) or a 2% CHG solution in water as a control (prepared by dilution of 20% CHG from Medichem, Barcelona, Spain). These were named Product A, Product B, and Product C, respectively. Other materials that were used were: Butterfield's Phosphate-Buffered Dilution Water (PBDW); trypticase soy agar (TSA); trypticase soybroth containing 5% defibrinated sheep's blood (TSB + S); Müeller-Hinton agar (MHA); Müeller-Hinton broth (MHB); Gram stain reagents, Etest® strips for clindamycin, oxacillin, vancomycin, ampicillin, ceftazidime, imipenem, piperacillin, and tobramycin (BioMérieux Clinical Diagnostics, 69280 Marcy l'Etoile, France); and laboratory grade penicillin-G potassium salt (Fisher Scientific, Fair Lawn, NJ).

An in vitro time-kill method was used to evaluate the antimicrobial activity of the products at different concentrations. Forty-eight repository isolates (12 species, four isolates per species) and 144 clinical isolates (12 species, 12 isolates per species) were evaluated. The twelve lab reference strains included six resistant and six nonresistant species. All strains and their sources are listed in Supplementary Table 1.

Another in vitro method was used to determine the potential for development (emergence) of resistance to CHG by sequential passage of several clinically relevant microorganisms [10] through increasing concentrations of the antimicrobial included in the culture medium. Clinically, for antibiotic resistance to be determined, it is necessary to have blood levels of the antibiotic in question [11]. By comparison, measurable blood levels of antiseptics are typically considered adverse events in susceptible populations [12]. Resistance to antiseptics must be operationally defined, since there is no direct correspondence to antibiotic resistance. Functional resistance to antiseptics, including chlorhexidine, implies a higher than typical minimal inhibitory concentration (MIC) [13]. Serial passage of organisms is an accepted method to determine the evolution of resistance patterns [14]. Ten repository isolates from eight species were evaluated. Eight clinical isolate strains equal to the repository isolates were also evaluated (four isolates, two resistant and two non-resistant, per species). All challenge microorganisms for this assay (total of 42 isolates) are described in Supplementary Table 2. The criterion for establishment of resistance was defined as the ability of the microorganisms to acclimate to at least a four-fold increase (a generally accepted threshold [8,15–17]) in the concentration of the test or control product and maintain that increase after three serial passages on media that does not contain the antimicrobial. Due to differences in the reported MICs for the organisms being evaluated, the initial round of testing was conducted against high, medium, and low concentration ranges of the products (see Supplementary Table 3). Dilutions of the CHG-containing products were prepared at 10 times the actual test concentrations due to the subsequent addition of 9 parts of agar. The full-strength products were aseptically diluted (one-part product plus 0.95-part culture medium). This resulted in a CHG concentration of 10 250 mg/L (before adding agar). Two-fold serial dilutions were carried out aseptically in culture medium until the lowest concentration needed for testing was achieved.

In addition, an evaluation of the potential for antibiotic cross-resistance was performed by comparing the MICs of several antibiotics, before and after extended exposure to sub-lethal levels of CHG. The antimicrobial resistance of each microorganism to clindamycin, oxacillin, vancomycin, ampicillin, ceftazidime, imipenen, piperacillin or tobramycin, as appropriate, was determined by Etest®. The MIC of penicillin was determined by the well-established broth dilution method (CLSI M07-A10) [17]. The same organisms as for the emergence of resistance were used (Supplementary Table 2).

2.1. In vitro time-kill assay

2.1.1. Preparation of microorganism suspension

Bacteria and yeast suspensions were prepared using 18–24-h cultures grown at 36 ± 1 °C in TSB or TSB + S. Each culture was adjusted by dilution using PBW to yield a concentration of approximately 1×10^8 CFU/mL, where attainable.

2.1.2. Preparation of CHG-based skin preps

The concentration of actives tested for Products A and B were full strength (2% CHG/70% IPA), a two-fold dilution considered a secondary active concentration (1% CHG/35% IPA), and an inactive concentration (0.0002% CHG/0.007% IPA).

2.1.3. Determination of antimicrobial activity

Antimicrobial testing was performed based on the ASTM E2315-03 method and the *Manual of Clinical Microbiology* [18,19]. For each challenge microorganism, 9.9 mL of the test or control product was used. The test (Product A) or control (Product B) was dispensed into sterile test tubes. Tubes were sealed and equilibrated to 30 °C for at least 10 min. A 0.1-mL aliquot of inoculum was added to each tube and mixed in. To minimize both buffer interference and dilution of the antimicrobial concentration, the volume of inoculum was kept at ≤1% of the total volume. After 3 min, a 1-mL sample was removed and neutralized. Serial dilutions were performed and plated in triplicate. The plates were incubated and recovery from each sample was expressed as $\log_{10}$ CFU/mL (colony forming units) recovered per mL. Microbial $\log_{10}$ reductions were calculated by subtracting the mean $\log_{10}$ recovery of the surviving microbial population from that of the initial microbial population. In addition, the activity of Product A was evaluated against a subset of three species (two strains each) of drug-resistant organisms in the presence of a 5% organic soil load (heat inactivated fetal bovine serum).

2.1.4. Neutralization of antimicrobial

Samples were neutralized using Butterfield's PBDW containing 0.3% lecithin, 1.0% Tween® 80, and 1.0% Tamol. The effectiveness of the neutralizer was determined based on the procedures outlined in ASTM E1054 [20] using one Gram-negative (*Escherichia coli*, ATCC 11229) and one Gram-positive (MRSA, ATCC 33591) challenge microorganism. At the end of the contact time, a 1-mL sample was transferred to a tube containing 9 mL of neutralizer. Serial ten-fold dilutions were made in Butterfield's PBDW, and triplicate aliquots from selected dilutions were transferred to TSA pour plates (1-mL aliquots) or TSB + S spread plates (0.1-mL aliquots). All plates were inverted and incubated for 48 ± 2 h at 36 °C. Colonies were counted and surviving microorganisms were determined (CFU/mL). The neutralizer was considered effective if the $\log_{10}$ CFU/mL of the test product sample was ≤0.3 $\log_{10}$ less than the test microorganism viability control sample. The neutralizer was considered nontoxic if the neutralizer toxicity control sample was ≤0.3 $\log_{10}$ less than the test microorganism viability control.

2.1.5. Sterility controls

Triplicate plates of each agar type were incubated at 36 °C for 48 h to check for sterility. In addition, triplicate 1-mL aliquots of PBDW and neutralizer were plated in at least one type of agar used.

2.2. Emergence of resistance (EoR) procedures

2.2.1. EoR inocula preparation

Bacteria were sub-cultured from stock cultures onto agar and incubated overnight at 36 ± 1 °C in ambient air. At least five colonies from the overnight cultures were inoculated into 4 mL broth. One-tenth mL of this suspension was transferred into 10 mL broth and incubated at 36 ± 1 °C for 2–6 h. The suspension of the challenge organism was adjusted with PBDW to contain approximately 1–2 × 10^6 CFU/mL using spectrophotometry. The inoculum was utilized within 30 min.

2.2.2. EoR test and control product preparation

Products A and B were diluted as described above. Two-fold serial dilutions of the products were prepared in culture medium to yield a total of nine separate dilutions. The dilutions were prepared and stored for less than 24 h before use.

2.2.3. EoR media preparation

For each test (Product A) and control (Product C), ten 450-mL portions of agar were prepared in flasks and steam sterilized. Then, 50 mL of the test or control product at appropriate dilutions was added to a flask and gently mixed (one volume of each dilution to nine volumes of agar). Next, 50 mL of the culture medium used to dilute the products was added to the tenth agar flask as a control. The contents of the flask were mixed thoroughly and poured quickly into the plates (before cooling and partial solidification).

2.2.4. EoR test

For each microorganism, per product, the agar surface of 10 plates containing the dilutions of the test and control products and the control plates containing no antimicrobial agent were spot inoculated with 0.01 mL. Approximately 10^4 CFU were delivered to an area 5–8 mm in diameter. Inoculated agar plates were allowed to stand undisturbed until the inoculum spots were completely absorbed and were incubated at 36 ± 1 °C for 18–20 h.

2.2.5. EoR recovery

A total of ≥2 CFU present in an inoculated area were considered positive. Surviving organisms from the maximum non-inhibitory concentration (MNC) were passaged twice in medium containing that same concentration of product. Two to five colonies were transferred from the appropriate plate to broth. The suspension was adjusted to approximately 1–2 × 10^6 CFU/mL using spectrophotometry. The inoculum was utilized within 30 min. Approximately 1.0 × 10^4 CFU (0.01 mL) was applied to 5–8 mm diameter areas (as described in EoR test above). A subsequent two-fold dilution series of the product was prepared, with the lowest concentration being equivalent to the MNC observed in the previous step; testing was repeated using the new dilution series. Since the MIC from the new dilution series did not increase compared with the initial MIC, testing was terminated and the product was not considered to have the potential for developing resistance.

2.2.6. EoR negative/positive controls

Negative: Duplicate plates prepared from the control flask (50 mL culture medium + 450 mL agar) were incubated with the test plates.

Positive: For each microorganism, duplicate plates prepared from the control flask as above were inoculated as described for the test plates and incubated with the test plates.

2.2.7. EoR inoculum counts control

A 1-mL aliquot of the challenge microorganism inoculum was serially diluted tenfold in PBDW. Selected dilutions were plated in appropriate agar in triplicate. All plates were allowed to solidify, then inverted and incubated with the test.

2.2.8. EoR confirmation of the challenge microorganism

Confirmation of each challenge organism was done through a comparison of colonies from the inoculum control and test plates. Gram stains were performed on an isolated colony from the positive control, and any suspicious colonies noted in the test plates. This procedure ensured the purity of each challenge microorganism.

Test acceptance criteria: The test was considered acceptable if the positive controls exhibited growth of the challenge microorganisms and if the negative controls did not exhibit any growth.

2.3. Cross-resistance procedures

2.3.1. Cross-resistance inocula preparation

This was performed as described in the Emergence of Resistance procedure above.

2.3.2. Cross-resistance Etest®

The Etest® strips consist of a predefined gradient of antibiotic concentrations on a plastic strip and are used to determine the MICs of antibiotics. A single MHA plate for each organism was inoculated in a cross-hatch pattern. The appropriate Etest® strip was added to each plate following the manufacturer's directions. The plates were incubated at 36 ± 1 °C for 20–24 h and observed for growth. The zones of inhibition were measured and reported as equivalent MICs.

2.3.3. Cross-resistance MIC broth dilution

For each applicable organism, 12 two-fold dilutions of penicillin were prepared in MHB. A total of 2 mL from each dilution were placed into sterile tubes and inoculated with 0.05 mL of a 1:10 dilution of one of the challenge organisms. The tubes were incubated at 36 ± 1 °C for 20–24 h. Tubes exhibiting growth at the most concentrated level of the antibiotic were streaked onto MHA and incubated at 36 ± 1 °C for 20–24 h along with the corresponding viability control tube.

2.3.4. Cross-resistance controls

Sterility control: A single Etest® strip representing each antibiotic was added to a sterile MHA plate and incubated with the test. In addition, duplicate 1-mL aliquots of PBDW and MHB were plated on MHA.

Viability control: A single tube containing 2 mL of MHB was inoculated with 0.05 mL of the organism and incubated with the test.

Negative control: A total of 2 mL of each prepared dilution of penicillin was dispensed into sterile tubes and incubated as the test. This determined the sterility of the antibiotic and served as a comparison for growth determinations (e.g. sediment or turbidity can occasionally be caused by the test substance or the antibiotic itself).

Organism confirmation: For each organism, Gram stains were performed from the Etest® strip plate and the viability control streak.

Due to the nature of the data, no statistical analysis of the results was performed. A four times MIC increase would have been considered significant and this was not observed.

3. Results

3.1. In vitro time-kill study

Products A and B demonstrated >5 $\log_{10}$ reduction against all Gram-positive and Gram-negative bacteria and fungi tested

Table 1
Viability of challenge microorganisms after 3-min exposure to various concentrations of CHG/IPA preparations, expressed in bacterial log reduction.

	1X 2% CHG/70% IPA Product A or B[a] (results were the same for both)		0.5X 1% CHG/35% IPA Product A		0.0001X 0.0002% CHG/0.007% IPA Product A	
Challenge microorganism	Susceptible strain	Drug-resistant strain	Susceptible strain	Drug-resistant strain	Susceptible strain	Drug-resistant strain
Burkholderia cepacia	5.87	5.67	5.87	5.67	0.45	0.28
Candida albicans	5.75	5.27	5.75	5.27	0.44	0.17
Enterococcus faecalis	5.70	5.70	5.70	5.70	0.41	0.40
Enterococcus faecium	5.83	5.68	5.83	5.68	0.45	0.37
Escherichia coli	5.93	5.64	5.93	5.93	0.51	0.31
Klebsiella pneumoniae	5.84	5.78	5.84	5.78	0.51	0.40
Pseudomonas aeruginosa	5.81	5.92	5.81	5.92	0.46	0.52
Serratia marcescens	5.69	5.73	5.69	5.73	0.39	0.36
Staphylococcus aureus	5.59	5.59	5.59	5.59	0.23	0.18
Staphylococcus epidermidis	5.97	5.73	5.97	5.73	0.59	0.41
Streptococcus pneumoniae	5.30	5.57	5.30	5.57	0.23	0.35
Streptococcus pyogenes	5.81	5.39	5.81	5.39	0.43	0.27

Abbreviations: CHG, chlorhexidine gluconate; IPA, isopropyl alcohol.
[a] At this concentration, both test solutions gave the same log reductions on all microorganisms tested. For this reason, only Product A was tested at the subsequent dilutions.

(>99.999% reduction). Neutralization effectiveness and toxicity of the microbicide were confirmed using one Gram-negative and one Gram-positive organism (antibiotic resistant) following the standard ASTM E1054 [20]. A >5 $\log_{10}$ reduction was also observed when the challenge organisms (susceptible and drug-resistant) were exposed to a diluted form of Product A at 0.5× concentration (1% CHG/35% IPA). Dilution of both active agents (CHG and IPA) to 0.0001X concentration (0.0002% CHG/0.007% IPA) resulted in decreased efficacy on all microbial strains tested. Chlorhexidine is bacteriostatic at low concentrations ranging from 0.02–0.06%, and bactericidal at concentrations >0.12%. Less than a one $\log_{10}$ reduction (0.70 $\log_{10}$ reduction) was observed when the inactive concentration of chlorhexidine was used. The average $\log_{10}$ reductions for the different concentrations are presented in Table 1.

The addition of a 5% organic load to Product A was tested to determine if the antimicrobial efficacy of the solution would be compromised. A >5 $\log_{10}$ reduction (>99.999% reduction) was observed in the presence of serum for all microorganisms tested, which was comparable with the efficacy observed in the absence of the serum, as shown in Fig. 1. For the in vitro kill study, each challenge organism was confirmed by Gram stain and colony morphology, and all sterility controls exhibited no growth.

3.2. Emergence of resistance test

The MIC did not increase for any of the strains evaluated; therefore, Products A and C were not considered to have the potential for the development of resistance. Table 2 displays this data and shows that MICs are the same for both products, pre-exposure and post-exposure, for every strain of every organism tested. This also demonstrates that CHG alone and in combination with a film-forming copolymer and a tint shows the same MIC on all bacteria tested, showing that these components do not affect the antimicrobial activity of CHG.

3.3. Cross-resistance test

Antibiotic cross-resistance results are presented in Tables 3a, 3b, and 3c. There was no indication of a significant change in MIC (four-fold or more) related to cross-resistance observed for any of

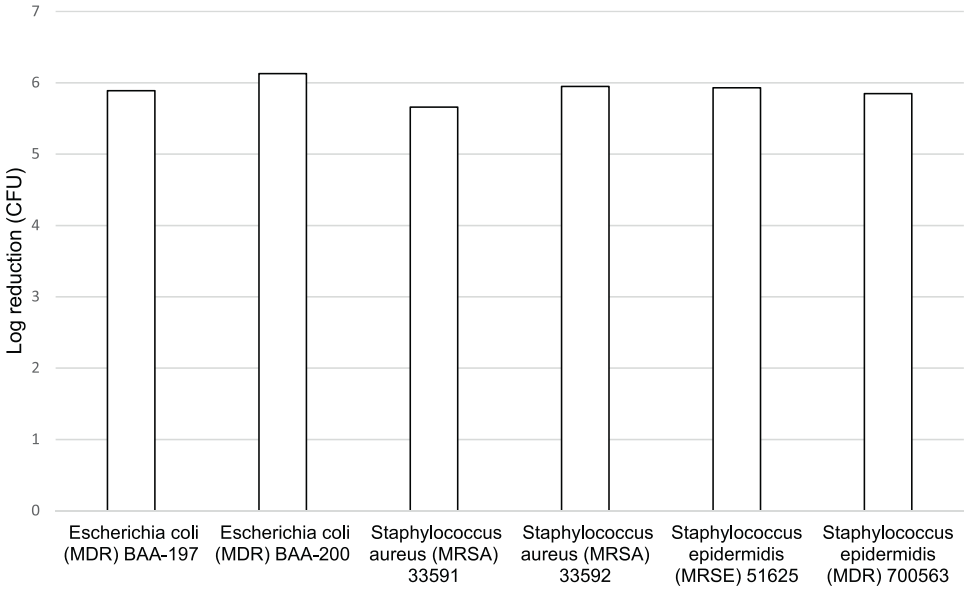

Fig. 1. Average $\log_{10}$ reduction (CFU) for drug-resistant microorganisms exposed for 3 minutes to the new investigational skin prep in the presence of serum (all showed > 5 $\log_{10}$ reduction).

Table 2
Emergence of resistance results.

Organism	ID	Minimum inhibitory concentration (mg/L)			
		Product A		Product C	
		Initial	Post	Initial	Post
Acinetobacter baumannii	ATCC 17904	64	64	64	64
	CI 14002[a]	64	64	64	64
	CI 10057[a]	64	64	64	64
	CI 10058[a]	64	64	64	64
	CI 10059[a]	32	32	64	64
Burkholderia cepacia	ATCC 25608	513	513	513	513
	CI 13052[a]	256	256	256	256
	CI 13053[a]	32	32	32	32
	CI 13054[a]	128	128	128	128
	CI 13055[a]	32	32	32	32
Enterococcus faecalis	ATCC 51299[b]	32	32	32	32
	CI 99824	32	32	16	32
	CI 99825	8	8	8	8
	CI 13046[b]	8	8	16	16
	CI 13047[b]	8	8	8	8
Escherichia coli	ATCC 11229	2	2	2	2
	CI 99903	2	2	2	2
	CI 99904	2	2	2	2
	CI 10100[a]	2	2	2	2
	CI 10101[a]	2	2	2	2
Pseudomonas aeruginosa	ATCC 15442	128	128	256	256
	CI 99791	64	64	64	64
	CI 99792	32	32	32	32
	CI 13015[a]	128	128	256	256
	CI 13016[a]	128	128	128	128
Serratia marcescens	ATCC 14756	128	128	128	128
	ATCC 43297[a]	128	128	128	128
	CI 99413	32	32	32	32
	CI 99452	64	64	64	64
	CI 13026[a]	64	64	128	128
	CI 13027[a]	513	513	513	513
Staphylococcus aureus	ATCC 33591[c]	2	2	2	2
	ATCC 25923[d]	2	2	2	2
	CI 99510	2	2	2	2
	CI 99511	1	1	2	2
	CI 10113[c]	1	1	1	1
	CI 10114[c]	2	2	1	1
Staphylococcus epidermidis	ATCC 51625[c]	16	16	16	16
	CI 99530	2	2	2	2
	CI 99532	2	2	2	2
	CI 13031[a]	2	2	2	2
	CI 13032[a]	2	2	2	2

[a] Multidrug-resistant.
[b] Vancomycin-resistant.
[c] Methicillin-resistant.
[d] Methicillin-sensitive.

the organism/antibiotic combinations tested. Some organisms showed MICs $\geq$ 256 ug/mL (indicating resistance to the highest gradient of antibiotic in the test strip) but this was also the case before their exposure to the CHG products. Therefore, CHG exposure was not responsible for this higher resistance. The Clinical and Laboratory Standards Institute (CLSI) states that the acceptable reproducibility for this type of testing is within one two-fold dilution of the actual end point [21]. All of the control strains met the criteria established for a valid test: all sterility and negative controls exhibited no growth, and all positive controls exhibited growth. Each challenge organism was confirmed by Gram stain and colony morphology.

4. Discussion

These results show that the film-forming copolymer and the tint present in the test product did not compromise the antimicrobial efficacy of the CHG, and suggest that the products tested do not have the potential for development (emergence) of resistance. In addition, there was no indication of cross-resistance for any of the organism/antibiotic combinations tested.

The mechanisms of bacterial resistance to biocides can be intrinsic (e.g. impermeability of the membrane) or acquired (by acquisition of genetic material such as a plasmid, or through mutation) [22,23]. In terms of intrinsic resistance, Gram-positive bacteria are generally more susceptible, followed by Gram-negative organisms, then mycobacteria, with bacterial spores being the most resistant [24]. This is consistent with the current results, showing for the most part lower MICs for the Gram-positive organisms (See Table 2: *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* as Gram-positives) than the Gram-negative organisms. There is debate and conflicting results in the literature about the emergence of resistance to antimicrobials and biocides in microorganisms, and the potential of this resistance to lead to cross-resistance to antibiotics. Various studies have been published on specific microorganisms. Bhardwaj et al. found that chlorhexidine induced VanA-type vancomycin resistance genes in Enterococci species in vitro, suggesting a

Table 3a
Development of cross-resistance results.

Organism	ID	Minimum inhibitory concentration (mg/L)							
		Ceftazidime		Imipenem		Piperacillin		Tobramycin	
		Initial	Post	Initial	Post	Initial	Post	Initial	Post
Acinetobacter baumannii	ATCC 17904	8	8	0.38	0.38	24	24	0.75	0.75
	CI 14002[a]	≥256	≥256	1.5	1.5	≥256	≥256	≥256	≥256
	CI 10057[a]	≥256	≥256	≥256	≥256	≥256	≥256	256	256
	CI 10058[a]	≥256	≥256	≥256	≥256	≥256	≥256	1.5	1.5
	CI 10059[a]	≥256	≥256	≥256	≥256	≥256	≥256	192	192
Burkholderia cepacia	ATCC 25608	4	2	8	8	3	3	128	192
	CI 13052[a]	24	16	≥256	≥256	≥256	≥256	≥256	≥256
	CI 13053[a]	2	1	0.38	0.25	0.75	0.75	6	6
	CI 13054[a]	3	3	12	24	8	8	0.38	0.38
	CI 13055[a]	2	2	8	8	12	8	0.5	0.5
Escherichia coli	ATCC 11229	0.38	0.38	0.25	0.25	3	3	0.5	0.5
	CI 99903	0.19	0.19	0.25	0.25	1.5	1.5	1	1
	CI 99904	0.047	0.047	0.19	0.19	0.38	0.38	0.25	0.25
	CI 10100[a]	0.25	0.25	0.19	0.19	≥256	≥256	≥256	≥256
	CI 10101[a]	1	1	0.25	0.25	≥256	≥256	64	64
Pseudomonas aeruginosa	ATCC 15442	3	1.5	1	1	12	3	1.5	1
	CI 99791	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256
	CI 99792	3	3	1.5	1.5	12	12	0.38	0.38
	CI 13015[a]	2	1	≥256	≥256	24	2	0.38	0.125
	CI 13016[a]	3	3	≥256	≥256	24	12	0.38	0.38
Serratia marcescens	ATCC 14756	0.094	0.19	0.5	0.5	1	2	3	3
	ATCC 43297[a]	1.5	1	1	0.38	64	6	8	6
	CI 99413	0.19	0.125	1	0.75	1.5	1	3	1.5
	CI 99452	0.5	0.38	3	0.016	4	2	0.25	0.25
	CI 13026[a]	0.25	0.125	1.5	0.75	3	3	2	1.5
	CI 13027[a]	1	0.25	3	0.25	8	2	4	3

[a] Multidrug-resistant.

Table 3b
Development of cross-resistance results.

Organism	ID	Minimum inhibitory concentration (mg/L)							
		Clindamycin		Oxacillin		Vancomycin		Penicillin	
		Initial	Post	Initial	Post	Initial	Post	Initial	Post
Staphylococcus aureus	ATCC 33591[a]	≥256	≥256	≥256	≥256	1	1	250	250
	ATCC 25923[b]	0.047	0.047	0.38	0.38	≥256	≥256	1	1
	CI 99510	≥256	≥256	16	16	1.5	1.5	2	2
	CI 99511	0.032	0.032	0.5	0.5	1	1	7.8	7.8
	CI 10113[a]	0.016	0.016	48	48	0.75	0.75	250	250
	CI 10114[a]	0.047	0.047	32	32	0.75	0.75	125	125
Staphylococcus epidermidis	ATCC 51625[a]	96	96	≥256	≥256	≥256	≥256	<0.5	<0.5
	CI 99530	48	48	≥256	≥256	≥256	≥256	500	500
	CI 99532	≥256	≥256	≥256	≥256	3	3	7.8	7.8
	CI 13031[c]	0.125	0.125	≥256	≥256	2	2	2	2
	CI 13032[c]	≥256	≥256	≥256	≥256	3	3	1	1

[a]Methicillin-resistant.
[b] Methicillin-sensitive.
[c] Multidrug-resistant.

Table 3c
Development of cross-resistance results.

Organism	ID	Minimum inhibitory concentration (mg/L)			
		Ampicillin		Vancomycin	
		Initial	Post	Initial	Post
Enterococcus faecalis	ATCC 51299[a]	≥256	≥256	≥256	≥256
	CI 99824	≥256	≥256	≥256	≥256
	CI 99825	4	2	1.5	1.5
	CI 13046[a]	1.5	1	≥256	≥256
	CI 13047[a]	1	1	≥256	≥256

[a] Vancomycin-resistant.

possible long-term impact of chlorhexidine bathing on vancomycin-resistant Enterococci (VRE) [5]. A study from the food sciences, also in vitro, found that gradual exposure of bacteria from organic foods to chlorhexidine resulted in decreased susceptibility to other antibiotics and biocides and other phenotypic alterations [6]. Wand et al. have identified, through in vitro studies, a novel mechanism of resistance to chlorhexidine (involving mutations in a regulator gene and a repressor gene adjacent to the superfamily efflux pump gene smvA) that may potentially operate in a number of different species [8]. On the other hand, a clinical study looking at bacterial isolates from patients subjected to a chlorhexidine bath intervention and including pre- and postintervention periods showed no reduced susceptibility to chlorhexidine over time, and no correlation between antibiotic resistance and the MIC for chlorhexidine for the nine different antibiotics used during the

study [7]. These authors observed a significant reduction in the frequency of MRSA isolates, confirming the clinical benefit of the intervention.

If reduced susceptibility to chlorhexidine became prevalent, the healthcare implications could be potentially serious, and research in this field is very active. A factor increasing the risk of this emergence is the wide range of chlorhexidine concentrations used (0.02% in catheter maintenance solutions, 0.2% in mouthwash, 0.5% in wound dressings, and 2% and 4% in solutions for skin antisepsis). The potential to develop resistance is increased due to the variable selection pressure [8]. In a point/counterpoint review, Harbarth et al. summarized their findings by saying that there are clinical examples of reduced susceptibility to antiseptics, but no strong evidence at this point that this is a major clinical problem [25]. They concluded that changes in the way antiseptics are clinically used should be matched with surveillance studies to understand the consequences of the new practices, including the possible emergence of microorganisms that can survive exposure to these substances. The same conclusion was also reached by Septimus and Schweizer, in a review on decolonization to prevent hospital-acquired infections, who stated that although the incidence of CHG resistance is currently low and of uncertain clinical significance, resistance to CHG should be monitored with more widespread use [4].

The current study had some limitations. It was performed in vitro and did not reflect the complex biological systems of patients. The methods reflect those customarily used to establish MIC for antibiotics in biological fluids, and this may not be adequate for antiseptics on the skin surface. It also studied a limited number of antibiotics. However, no other methods specific to this application have yet been established. A strength of this study is that it tested multiple strains of each organism, including repository and clinical isolates.

5. Conclusions

With the methods used in this study, the microbicidal activity of the new skin prep containing a tint and a film-forming copolymer was equivalent to that of a currently available skin prep without a film forming copolymer. The MIC for chlorhexidine did not increase for any of the evaluated bacterial strains, suggesting no potential for development (emergence) of resistance. In addition, there was no indication of a change in MIC related to cross-resistance observed for any of the organism/antibiotic combinations tested. However, there are currently no standardized methods to test the susceptibility of organisms to chlorhexidine, and there is a need to establish surveillance to identify and monitor potential increases in resistance to antimicrobials commonly used in healthcare settings. In the assays looking at time-kill and development of resistance and cross-resistance, the antimicrobial efficacy of the new formulation was equivalent (MIC) to that of the control (e.g. not compromised by the film-forming copolymer or the tint), other manuscripts just published and in preparation present data from additional studies showing the advantages of the film-forming copolymer (better resistance to wash-off [26] and improved incise drape adhesion compared to a commercially available skin prep with similar CHG/IPA concentrations but no film-forming copolymer) and the tint (improved visibility).

Conflict of interest statement

K. Dormstetter is an employee of Microbac Laboratories, Inc. The study was performed by this laboratory under a contract with 3 M. LKM Olson, A Bennaars-Eiden, and SF Bernatchez are employees of 3 M. The investigative product described received FDA approval, since this work was performed and will be available under the brand name 3M™ SoluPrep™ Film-Forming Patient Prep.

Funding source

This study was paid by 3M. The study was designed in collaboration with 3M and Microbac Laboratories. Microbac Laboratories performed the studies and issued the final study reports. 3M decided to submit the manuscript for publication and prepared the manuscript. All authors contributed to data interpretation and critical review of the manuscript for intellectual content. Ethical approval was not required for this study (in vitro work).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.12.008.

References

[1] Hemani ML, Lepor H. Skin preparation for the prevention of surgical site infection: which agent is best? Rev Urol 2009;11:190–5.
[2] Maiwald M, Chan ES. The forgotten role of alcohol: a systematic review and meta-analysis of the clinical efficacy and perceived role of chlorhexidine in skin antisepsis. PloS one 2012;7:e44277.
[3] Zhang D, Wang XC, Yang ZX, Gan JX, Pan JB, Yin LN. Preoperative chlorhexidine versus povidone-iodine antisepsis for preventing surgical site infection: a meta-analysis and trial sequential analysis of randomized controlled trials. Int J Surg 2017;44:176–84.
[4] Septimus EJ, Schweizer ML. Decolonization in prevention of health care-associated infections. Clin Microbiol Rev 2016;29:201–22.
[5] Bhardwaj P, Ziegler E, Adams H, Palmer KL. Chlorhexidine induces VanA-type vancomycin resistance genes in enterococci. Antimicrob Agents Chemother 2016;60(4):2209–21.
[6] Gadea R, Glibota N, Perez Pulido R, Galvez A, Ortega E. Adaptation to biocides cetrimide and chlorhexidine in bacteria from organic foods: association with tolerance to other antimicrobials and physical stresses. J Agric Food Chem 2017;65:1758–70.
[7] Velazquez-Meza ME, Mendoza-Olazaran S, Echaniz-Aviles G, Camacho-Ortiz A, Martinez-Resendez MF, Valero-Moreno V, et al. Chlorhexidine whole-body washing of patients reduces methicillin-resistant Staphylococcus aureus and has a direct effect on the distribution of the ST5-MRSA-II (New York/Japan) clone. J Med Microbiol 2017;66:721–8.
[8] Wand ME, Bock LJ, Bonney LC, Sutton JM. Mechanisms of increased resistance to chlorhexidine and cross-resistance to colistin following exposure of klebsiella pneumoniae clinical isolates to chlorhexidine. Antimicrob Agents Chemother 2017;61.
[9] Williamson DA, Carter GP, Howden BP. Current and emerging topical antibacterials and antiseptics: Agents, action, and resistance patterns. Clin Microbiol Rev 2017;30:827–60.
[10] Mangram AJ, Horan TC, Pearson ML, Silver LC, Jarvis WR. Guideline for prevention of surgical site infection, 1999. Committee HICPA 1999;97–134.
[11] Mouton JW, Brown DF, Apfalter P, Canton R, Giske CG, Ivanova M, et al. The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach. Clin Microbiol Infect 2012;18:E37–45.
[12] Chapman AK, Aucott SW, Milstone AM. Safety of chlorhexidine gluconate used for skin antisepsis in the preterm infant. J Perinatol 2012;32:4–9.
[13] Sheng WH, Wang JT, Lauderdale TL, Weng CM, Chen D, Chang SC. Epidemiology and susceptibilities of methicillin-resistant *Staphylococcus aureus* in Taiwan: emphasis on chlorhexidine susceptibility. Diagn Microbiol Infect Dis 2009;63:309–13.
[14] Lahiri SD, Alm RA. Identification of non-PBP2a resistance mechanisms in *Staphylococcus aureus* after serial passage with ceftaroline: involvement of other PBPs. J Antimicrob Chemother 2016;71:3050–7.
[15] Sole M, Fabrega A, Cobos-Trigueros N, Zamorano L, Ferrer-Navarro M, Balleste-Delpierre C, et al. In vivo evolution of resistance of Pseudomonas aeruginosa strains isolated from patients admitted to an intensive care unit: mechanisms of resistance and antimicrobial exposure. J Antimicrob Chemother 2015;70:3004–13.
[16] Ismail N, Omar SV, Ismail NA, Peters RPH. In vitro approaches for generation of *Mycobacterium tuberculosis* mutants resistant to bedaquiline, clofazimine or linezolid and identification of associated genetic variants. J Microbiol Methods 2018;153:1–9.
[17] CLSI. M07-A10 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard. 10th ed Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
[18] ASTM. ASTM E2315-03. Standard guide for assessment of antimicrobial activity using a time-kill procedure. 2008.

[19] Balows A, Hausler Jr. WJ, Herrmann KL, Isenberg HD, Shadomi HJ. Manual of clinical microbiology. Washington: American Society for Microbiology (ASM Press); 1991.
[20] ASTM. ASTM E1054-08 (Reapproved 2013): Standard test methods for evaluation of inactivators of antimicrobial agents. 2013.
[21] CLSI M07-A8. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved Standard. 8th Ed. 2009.
[22] Russell AD, Day MJ. Antibacterial activity of chlorhexidine. J Hosp Infect 1993;25:229–38.
[23] Russell AD. Bacterial resistance to disinfectants: present knowledge and future problems. J Hospital Infect 1998;43:S57–68.
[24] Tumah HN. Bacterial biocide resistance. J Chemother 2009;21:5–15.
[25] Harbarth S, Tuan Soh S, Horner C, Wilcox MH. Is reduced susceptibility to disinfectants and antiseptics a risk in healthcare settings? A point/counter-point review. J Hosp Infect 2014;87:194–202.
[26] Hamid Bashir M, Hollingsworth A, Schwab D, Prinsen KS, Paulson JE, Morse DJ, et al. Ex vivo and in vivo evaluation of residual chlorhexidine gluconate on skin following repetitive exposure to saline and wiping of 2% chlorhexidine gluconate/70% isopropyl alcohol preoperative skin preparations. J Hospital Infect 2018, doi:http://dx.doi.org/10.1016/j.jhin.2018.10.004 (in press) (on line at https://www.journalofhospitalinfection.com/article/S0195-6701(18)30539-5/fulltext)..