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In vitro and *in vivo* evaluation of efficacy of citrate/methylene blue/parabens/IPA solution as a skin disinfectant

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Summary *Objective:* The objective of this study was to evaluate the bactericidal activity of a new antiseptic in an in vitro model using reference bacterial strains and on abdominal and inguinal skin of healthy human subjects. ZuraPrep™ (C/MB/P/IPA) contains citrate, methylene blue, parabens and isopropyl alcohol 70%.
Methods: In vitro and *in vivo* studies evaluate immediate and persistent activity of the tested solution using bovine rawhides inoculated with single strains of microorganisms and natural bacterial flora on skin of adult human subjects. Depending upon the anatomical site (abdominal or inguinal), several different configurations of test times were executed. Post-prep surface cultures were performed at 10 min and 6 h after application of the test solution.
Results: Tested solution showed high efficiency in log reduction of viable microbes both *in vitro* and *in vivo*. Post-prep activity 10 min after application ranged from 2.5 to 3 $\log_{10}$ reduction from baseline on abdominal sites and 3.5—4.5 on inguinal sites. Similar levels of reduction persisted 6 h after application.
Conclusion: The level of antisepsis provided by the tested solution is similar or greater than that obtained with other antiseptics currently in use, and further clinical testing of the new antiseptic is warranted.

Introduction

Antiseptics are antimicrobial substances that are applied to living tissue/skin to destroy or inhibit the growth of microorganisms and in consequence reduce the possibility of infection and sepsis.[1] Therefore antiseptics are an important component in avoiding healthcare-associated infections (HAI) which are connected to invasive

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procedures, such as surgery or intravascular devices insertion. Commonly the infective agents are the microorganisms found on the patient's own skin flora.[2–4]

An estimated 18 million surgical procedures are performed in United States each year. Of these more than 500,000 are complicated by nosocomial infections.[5] Surgical site infections (SSI) are responsible for 77% of the deaths in nosocomially infected surgical patients.[6] Bacterial colonization originating from microflora of patients skin is also a common cause of bloodstream infection (BSI). Patients who acquire an SSI have a 2-fold increase in the length of hospital stay and the risk of death[5] and annual cost to the US healthcare system is in excess of $1.8 billion.[7–9]

The purpose of topical antiseptics is to decrease quickly a broad spectrum of resident and transient microbes to subpathogenic levels and to prevent the rebound of growth for up to 6 h after use. Currently most common skin antiseptic agents offered in the US include isopropyl alcohol, parachlorometaxylenol (PCMX), povidone-iodine (PVI), chlorhexidine gluconate alone (CHG), chlorhexidine/70% isopropyl alcohol (CHG/IPA), iodophor/isopropyl alcohol, or zinc pyrithione/73%ethanol (ZPT). The selection of the appropriate antimicrobial agent is a very crucial step before application. Together with efficacy, immediate action and persistence, the problem of direct and indirect tissue injury should be taken under account. The most common injuries include ototoxicity, skin irritation, ophthalmic damage and anaphylactic reactions.[10] Also, depending upon the application, the effectiveness in the presence of blood, necrotic tissue, or purulence may be an issue.

The Food and Drug Administration (FDA) approved the 2% formulation of CHG/IPA for use as a topical antiseptic for preoperative skin preparation. This product was extensively analyzed and its efficacy was compared to the above most cited topical antiseptics. *In vivo* and *in vitro* studies showed high activity of CHG/IPA in eradication of microbes from patients' skin,[11–13] and substantial reduction of planktonic and biofilm *Staphylococcus epidermidis* bacteria in a short time.[14] However because of rare but possible side effects a new highly efficient antiseptic solution would be a beneficial addition to the existing line of products.

The aim of this study was to evaluate antimicrobial efficacy of the antiseptic solution newly developed in our lab. *In vitro* protocols compared antimicrobial properties on bovine rawhide contaminated by a group of selected bacteria strains. *In vivo* protocols tested the effect of C/MB/P/IPA solution on natural bacterial flora on intact skin of human subjects.

Material and methods

Composition of the two tested antiseptic solutions

The newly tested antiseptic C/MB/P/IPA is comprised of 70% IPA, 4.6% (w/v) citrate (citric acid/sodium citrate, pH ~3.5), 0.2% methyl paraben (MP), 0.1% propyl paraben (PP) and 0.05% methylene blue (MB). This solution in an *in vitro* study was compared to commercially available CHG/IPA (1.5 mL applicators containing 2% of CHG in 70% IPA) from Enturia.

Neutralizer system and validation study

Sterile Stripping Suspending Fluid (SSF+) with appropriate product neutralizers contained in 1 L of solution the following compounds: 10.1 g Na_2HPO_4, 0.4 g KH_2PO_4, 1 mL Triton X-100, 11.67 g Lecithin, 100 mL Polysorbate 80, 5 g $Na_2S_2O_3.5H_20$, and 10 g of Tamol SN (Sodium Naphthalate).

A neutralization study was performed to assure the neutralizers used in the recovery medium quenched the antimicrobial activity of the test products. The neutralization followed guidelines set forth in ASTM E 1054-02,[15] except that the microorganism was added to the neutralizer prior to the addition of the test or comparison products. Common skin contaminant strains of Staphylococcus epidermidis (ATCC #12228 and 51625) were used as the challenge species in the neutralizer validation study. The neutralization assay included four phases.[16] I — Establishment of baseline population of challenge microorganisms grown in non-inhibitory medium. It is recommended that colony counts are in the range of 30—300 CFU per plate. II. — Exposure of challenge microorganisms to antimicrobial product at use-strength to show its antimicrobial efficacy. III — Exposure of challenge microorganisms to neutralizer system to demonstrate non-toxicity of neutralizer. IV — Test solution and neutralizer are mixed together, followed by exposure to challenge microorganisms to confirm efficacy of the neutralizing system.

In vitro test on bovine rawhide

A few gram negative and gram positive organisms from the list recommended by FDA for testing of healthcare antiseptic drug products[17] were used for contamination of bovine rawhide surface. This set included ATCC strains of: *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, *Staphylococcus aureus* 29213, *Enterococcus faecalis* 29212 and also MRSA 33591(not on FDA list). Single colonies from fresh Trypticase Soy Agar (TSA) plates with sheep blood were used for preparations of overnight inocula. The next day bacteria slurries were diluted to match the #3 McFarland standard ($\sim 9 \times 10^8$ CFU/mL) and 1 mL of the dilutions were spread separately under the laminar flow hood onto sterile bovine rawhide squares of dimension 7 cm × 7 cm. Before inoculation rawhide sections were sterilized with alcohol and next presoaked in 0.9% sterile saline for 48 h. The saline solution was exchanged every 6 h to remove all impurities. Sterility was check by swabbing with cotton and plating on blood sheep agar. The bacteria solutions were kept on the square segments for 10 min and then withdrawn carefully with sterile Pasteur pipette. All segments were allowed to dry for 30 min. The sections of rawhide were prepared for each bacteria strain in multiples for controls and two different post-application times (10 min, 6 h) during the experiment.

Both challenge solutions were tested in the following way. One and a half mL of C/MB/P/IPA or CHG/IPA (applied to sterile gauze) was scrubbed against rawhide for 30 s. Following the designated time of the contact of antiseptics with microorganisms (10 min and 6 h), a sterile metal cylinder with an inside area of 3.46 cm^2 was held firmly onto the central position of the rawhide segments for sampling. The sampling was performed by dispensing into the cylinder 1.0 mL of stripping fluid with neutralizers and massaging

the surface in a circular manner for 1 min with a sterile rubber policeman. The solution was removed with a pipette and transferred to a sterile tube. A second 1.0 mL aliquot was applied into the cylinder and the rawhide area again was massaged for 1 min with a rubber policemen. The solutions were combined and serial dilutions of samples were plated on TSA for colony enumeration after 24 h incubation at 37 °C. The experiments were carried out at room temperature under aerobic conditions and repeated three times. Results were calculated as described for *in vivo* tests, below.

In vivo test on the skin of human volunteers

Healthy subjects of either sex, at least 18 years of age and of any race, free from dermatoses, injuries, lesions, inflammation, tattoos or other skin disorders on or around test sites (abdominal and inguinal areas) were eligible to participate in the study. The study was approved by the Institutional Review Board (IRB) of Bioscience Laboratories, Inc. Subjects had to have not received topical or systemic antimicrobials, antibiotics or steroids for seven days pretest conditioning period and until the completion of the study. During this time, subjects used no medicated soaps, lotions, shampoos, deodorants and antibacterial hygiene products, or other agents known to affect normal skin flora. Subjects also avoided UV tanning beds, or bathing in antimicrobial-treated (e.g. chlorinated) pools and/or hot tubs. Instead, subjects were given nonbactericidal personal hygiene kits to use throughout the duration of the study. The shaving or waxing of the anatomical sites to be treated was prohibited within 5 days prior to the screening period. If needed, the hair on sampling sites was clipped at least 72 h prior to the test period. No bathing was allowed within 72 h before the microbial testing day. This regimen allowed for the stabilization of the normal microbial flora on the skin. All subjects gave written informed consent before entering the test.

Subjects were included in the study if microbial counts on sampling sites were $>2.5\ \log_{10}$ colony forming units (CFU) per square centimeter (cm^2) of abdominal skin and $>4.5\ \log_{10}$ CFU/cm^2 of inguinal skin.

On the testing day, skin irritation was rated just before study began. Antiseptic application skin areas were first sampled for baseline microbial counts. The four abdominal test configurations (one pass of C/MB/P/IPA, swabbing with C/MB/P/IPA for 15,30 and 120 s) and four inguinal test configurations (30, 60, 90 and 120 s swabbing with C/MB/P/IPA respectively) were assigned randomly and bilaterally to the subjects per a computer generated randomization schedule. The tested solution was administrated using sterile Type VII gauze saturated with C/MB/P/IPA. Antiseptic was applied using a vigorous back-and-forth motion. The antiseptic was allowed to dry for 30 s after application. Sample times were 30—60 s post-prep, 10 min post-prep and 6 h post-prep. The 6 h sampling sites on the inguinal and abdominal skin were covered with sterile gauze and semi-occlusive bandages after antiseptic was applied.

At the designated time, a sterile cylinder with an inside area of 3.46 cm^2 was held firmly onto the anatomical test site to be sampled. Two and half mL of sterile stripping suspending fluid (SSF+) with appropriate antiseptic neutralizers was installed into the cylinder and the skin area inside the cylinder was massaged with a sterile rubber policeman in a circular manner for 1 min.[17] The product neutralizer's effectiveness in inactivating the antimicrobial activity of the C/MB/P/IPA was verified prior to evaluation.[15] After removal of SSF+ to a sterile test tube a second 2.5 mL aliquot of SSF+ was pipetted into the cylinder and skin area was treated as before. The solution was collected and pooled in the test tube with the first aliquot. The number of CFUs/cm^2 of skin was determined using 10-fold dilution methods utilizing Butterfield's phosphate buffer with neutralizer. Duplicate spread plates and/or spiral plates were prepared for each dilution on Tryptic Soy Agar with product neutralizers (TSA+). Plates were incubated at 30 ± 2 °C for approximately 72 h, or until sufficient growth was observed. Plates containing 25—250 CFUs were used to determine the number of CFU/cm^2 on the skin. To convert the volumetric measure of the sample into the number of CFU/cm^2 the following formula was employed:

$$R = \mathrm{Log}_{10}\frac{F(\sum c_i/n)10^{-D}}{A}$$

where: R = the average CFU count in $\log_{10}$ scale per cm^2 of sampling surface; F = total number of mL of stripping fluid added to the sampling cylinder; in this study, $F = 5$ mL; $\sum c_i/n$ = average of the duplicate colony counts used for each sample collected; D = dilution factor of the plate counted; A = inside area of the cylinder in cm^2 (3.46 cm^2 in this study).

Suspension test model

The suspension test model was performed as follows. Ten μL of overnight growth of microbes (*S. aureus*/*P. aeruginosa*) in LB medium was diluted with sterile saline to attain a suspension of approximately 10^9 CFU/mL of microorganism. Fifty μL of this concentration was mixed with 5 mL testing solutions and after 15 and 60 s of contact time at room temperature 100 μL aliquots were removed and added to 900 μL neutralizing fluid. This solution was used for preparation of serial dilutions spread onto TSA agar. After 24—48 h of incubation at 37 °C plates were used for colony enumeration. The evaluations were carried out in triplicate.

Statistical analysis

All *in vitro* experiments were performed in triplicate. *P* values were calculated using Student's *t*-test (two-tailed, paired). For the human subjects study, confidence intervals were determined for baseline and post-application microbial recovery for each test configuration.

Results

Validation of neutralizer system used in this study

Many antimicrobial efficacy evaluations of antimicrobial substance(s) involve measurements of microbial population reductions at a specific time point after exposure to the tested product. Therefore antimicrobial action of the

product has to be stopped at the specified time. For this purpose the neutralizer system is utilized. In the presented study the validity of this system was established prior to performing all antimicrobial efficacy experiments. The results are summarized in Table 1. They indicate that the neutralizer system developed for this study is quenching antimicrobial properties of the tested product and is not toxic to test microorganism(s).

Antimicrobial effectiveness of the new antiseptic solution. *In vitro* comparison study with CHG/IPA

Antibacterial properties of C/MB/P/IPA and CHG/IPA were tested against gram positive and gram negative strains of bacteria obtained from ATCC and from hospital isolates. The baseline levels for all tested microorganisms appeared to be similar ($\sim 10^6$ CFU/cm^2 of rawhide) and did not change significantly during the time course of experiment (0–6 h). C/MB/P/IPA revealed antimicrobial properties, substantially decreasing the number of viable bacteria (Fig. 1). The decline to the level 5×10^1–5×10^2 CFU/cm^2 was achieved for most tested strains after 10 min of application of C/MB/P/IPA onto contaminated bovine rawhide. This efficacy remained unchanged to the end of the experiments (6 h). Moreover, for some bacteria further reduction in living cells was noted at 6 h after application. Comparison of both products demonstrates that the $\log_{10}$ reductions in bacterial counts with C/MB/P/IPA solution were similar to or exceeded those observed with CHG/IPA. Presented results are statistically significant for each tested antiseptic (baseline versus sampling times, $P < 0.05$). Within the two groups (C/MB/P/IPA versus CHG/IPA) statistically significant differences were noticed after 6 h for *E. coli* ($P = 0.009$), *P. aeruginosa* ($P = 0.025$) and *E. faecalis* ($P = 0.037$). Treatment of other microorganisms with two antiseptics resulted in very similar $\log_{10}$ reductions.

Microbial reductions from baseline of natural skin flora of volunteers by C/MB/P/IPA application using four different test configurations

For product evaluation the screening samples were collected from 42 White/Caucasian volunteers who met inclusion/exclusion criteria. Median age was 37 years (18–74) and in this group were 27 males and 15 females. The main reason for exclusion from the study was lower than allowed bacteria count on abdominal and inguinal site (see Material and Methods). No adverse effects were noticed in inclusion/exclusion groups after application of test solution onto human skin.

The mean microbial declines from baseline of all different test configuration treatments for the abdominal and the inguinal sites are presented in Table 2. Mean baseline microbial counts from the abdominal sites were in similar range for each configuration: ($\log_{10}$cfu/cm^2 between 3.44 and 3.08). Significant $\log_{10}$ reduction in microbial counts compared with baseline was noticed for each post-prep time in all 4 test configurations ($P < 0.05$). As can be seen from Table 2, the test product is effective even after a very short application time (single pass, 15 or 30 s). This high efficiency remains even after 6 h of product contact with the skin. Furthermore, expanding application time of C/MB/P/IPA to 120 s reduced bacteria viability counts close to zero.

Table 2 reports also the results from inguinal sites following 4 different times of C/MB/P/IPA application; 30, 60 and 90 and 120 s. Mean microbial recovery at baseline for all 4 test configurations were similar ($\log_{10}$ cfu/cm^2 between 5.59 and 5.40). As in the case of abdominal sites, broad $\log_{10}$ reduction in microbial counts was observed for each post-application time in all test configurations ($P < 0.05$). For example immediate post-prep $\log_{10}$ reduction was 2.93, at 10 min post-prep 3.40 and at 6 h 4.08 after skin was exposed for 30 s to antiseptic solution. The highest $\log_{10}$ reduction from the baseline in the inguinal study was attained after 10 min (4.66) and 6 h (4.02) sampling following a 90 s application of C/MB/P/IPA.

All results from the C/MB/P/IPA treated abdominal site exceeded the FDA criteria for preoperative skin preparation of a 2.0 $\log_{10}$ reduction/cm^2 (compared to baseline) within 10 min after application of the study antiseptic agents.[17] Similarly FDA requirement of 3.0 $\log_{10}$ reduction/cm^2 on inguinal site after 10 min of antiseptic application and not exceeding the baselines on both anatomic treated sites after 6 h were easily accomplished. In one part of the inguinal study (Table 2; 30 s post-prep) $\log_{10}$ reduction was a little below 3, but statistically significant ($P < 0.05$). However

Table 1 Neutralization evaluation results for *Staphylococcus epidermidis ATTC #51625*.

Phase	Test description[a]	Mean $\log_{10}$	95% confidence interval	Mean $\log_{10}$ change
I	Baseline population 1 min exposure	1.98	1.95–2.01	N/A
	Baseline population 30 min exposure	2.05	1.95–2.15	−0.07
II	Test solution efficacy test solution 1 min exposure	1.19	0.82–1.56	0.79[b]
III	Neutralizer fluid toxicity evaluation 1 min exposure	2.03	2.00–2.06	−0.05
	Neutralizer fluid toxicity evaluation 30 min exposure	2.00	1.97–2.03	−0.02
IV	Neutralizer efficacy evaluation versus test solution 1 min exposure	1.98	1.92–2.04	0
	Neutralizer efficacy evaluation versus test solution 30 min exposure	1.99	1.95–2.03	−0.01

[a] Sample size 4.
[b] Significantly different from inoculum population ($p \leq 0.05$ and greater than 0.25 $\log_{10}$ difference).

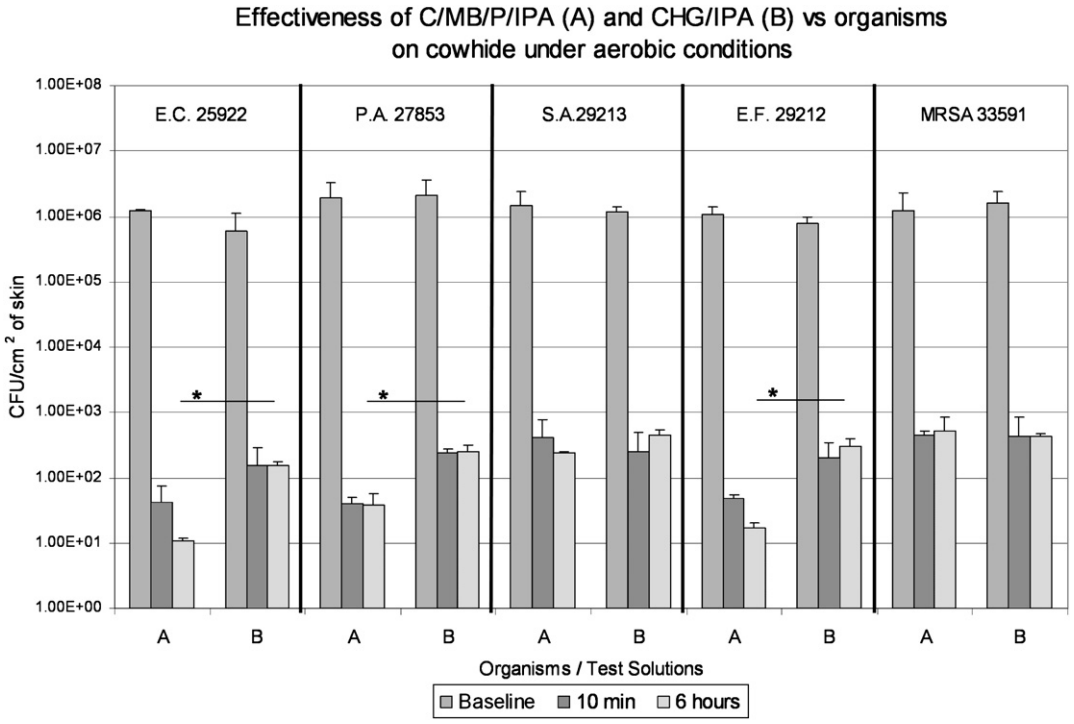

Figure 1 Changes in colony counts of tested organisms after application of C/MB/P/IPA or CHG/IPA onto rawhide infected with single bacterial strains. Changes were statistically significant for each time versus baseline ($P < 0.05$). Changes within groups were statistically significant for *E. coli* ($P = 0.009$), *E. faecalis* ($P = 0.037$) and *P. aeruginosa* ($P = 0.025$) at 6 h after product application (*). Error bars indicate 1 SD.

the FDA monograph does not necessitate checking microbial recovery at this point in time.

Assessing the efficacy of C/MB/P/IPA components in suspension model

A bacterial suspension model was applied to evaluate effectiveness of individual components of C/MB/P/IPA in lowering viable cells count. Two bacteria strains were used: *S. aureus* and *P. aeruginosa*. In a preliminary experiment (not shown) the concentration of IPA in which tested bacteria may survive for a minimum of 10 min was evaluated. It was found that *S. aureus* may remain alive in 30% IPA, but *P. aeruginosa* is more sensitive and the concentration of IPA was lowered to 20%. A range of concentrations of each individual component was tested to assess the effect on cells viability (citrate: 4.6%—1%, MP/PP: 0.2%/0.1%—0.03%/0.015% and MB: 0.05%—0.01%). The lowest concentration of single components having distinctive impact on *S. aureus* cell death is shown on Fig. 2. The results disclosed that the greatest antimicrobial efficacy in 15 s can be achieved by a combination of 30% IPA with citrate or MP/PP. As low as 1% citrate or 0.06%/0.03% MP/PP can reduce living cells practically to zero. The combination of 30% IPA with 0.02% MB performed more slowly and 60 s' contact time is needed to achieve this same goal. Similar results (not shown) were obtained testing components of C/MB/P/IPA on *P. aeruginosa*; however sensitivity of this organism to a lower pH may be an additional factor.

Table 2 Mean microbial counts (log_{10}) following application of tested solution on the skin of human volunteers.

Test site	Test application format	# Subjects	Mean log_{10} baseline count	Mean log_{10} count reduction from baseline post-prep		
			(95% CI)	30 s (95% CI)	10 min (95% CI)	6 h (95% CI)
Abdomen	Single pass	6	3.44 (2.77—4.11)	2.73 (1.52—3.93)	2.52 (1.08—3.96)	2.30 (1.01—3.60)
Abdomen	15 s	8	3.45 (3.04—3.78)	2.87 (1.97—3.77)	3.00 (2.41—3.58)	2.97 (2.37—3.57)
Abdomen	30 s	9	3.11 (2.58—3.64)	2.48 (1.94—3.02)	2.58 (2.03—3.12)	2.44 (1.82—3.06)
Abdomen	120 s	12	3.08 (2.88—3.28)	N/A	3.01 (2.81—3.21)	2.80 (2.32—3.29)
Inguinal	30 s	13	5.49 (5.13—5.86)	2.93 (2.17—3.70)	3.40 (2.62—4.18)	4.08 (3.14—5.01)
Inguinal	60 s	15	5.58 (5.28—5.88)	3.92 (3.06—4.79)	4.37 (3.56—5.19)	3.85 (3.13—4.57)
Inguinal	90 s	13	5.59 (5.14—6.03)	3.48 (2.42—4.54)	4.66 (3.80—5.53)	4.02 (3.22—4.81)
Inguinal	120 s	14	5.40 (5.04—5.76)	N/A	4.23 (3.36—5.11)	4.17 (3.56—4.79)

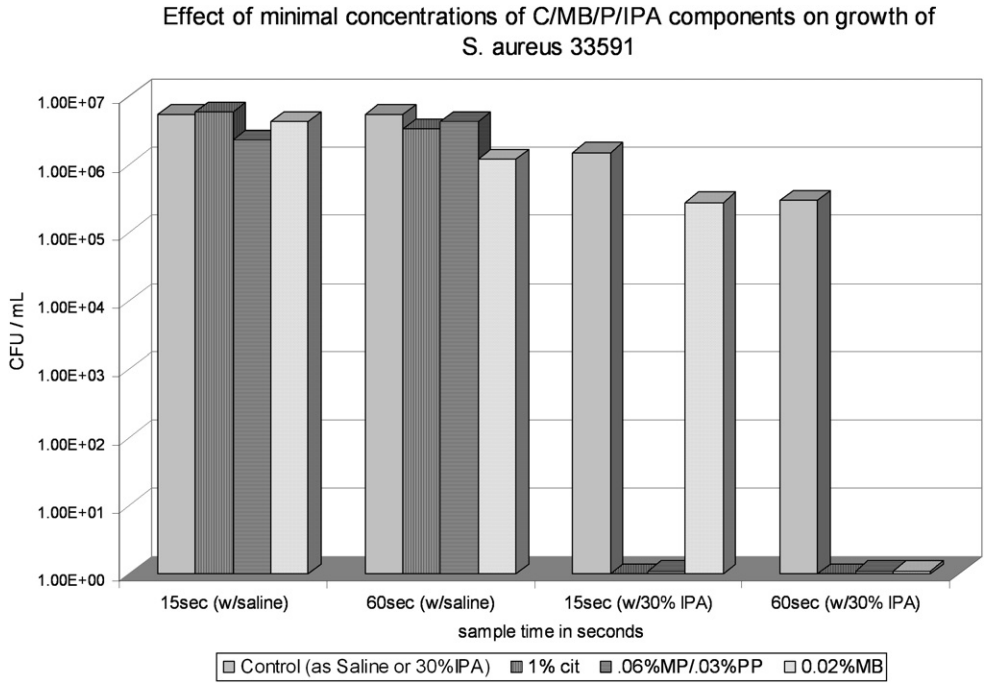

Figure 2 Reduction in *S. aureus* colony counts in suspension test after contact of microorganisms with three individual components of C/MB/P/IPA in the presence of saline or 30% IPA.

Discussion

Effective skin antisepsis is essential in preventing increased incidence of infections associated with invasive procedures, such as surgery or intravascular catheter insertion and use. Over the past 20 years, many studies have been published evaluating efficacy of skin antiseptic agents such as iodophor, povidine-iodine and chlorhexidine compounds at various concentrations in aqueous or alcoholic solutions. According to the evidence-based practice in infection control (EPIC)[18] and CDC guidelines,[19] 2% (w/v) chlorhexidine solution is the recommended agent to be used prior to invasive procedures. Although 2% CHG is able to significantly reduce intravascular catheter-related infections,[20] the CHG/IPA combination displays activity higher than that of aqueous CHG solution in a preoperative skin preparation[12] and *in vitro* tests.[14] The optimal disinfection regimen for avoiding postoperative infections has not yet been defined. Many other antiseptics or their combinations[21,22] are still being used and investigated and research efforts to identify improved antisepsis approaches continue. Studies comparing the efficacy of antiseptic skin cloths with other bottled preoperative antiseptic formulations or with the addition of a preoperative shower (4%CHG soap) are also in progress.[23,24]

The research presented in this publication describes the antibacterial properties of a new antimicrobial solution, containing C/MB/P/IPA. This solution was developed utilizing Zuragen™ platform technology. Zuragen™ is a new catheter lock solution consisting of citrate/methylene blue/parabens. Prior studies have shown that the components of this lock solution act synergistically in eradication of both planktonic and sessile bacteria and fungi in a short time.[25] Zuragen™ has rapid bactericidal effect on the preformed mature biofilm and its architecture.[26] C/MB/P/IPA solution has a different balance of chemical components than Zuragen™ (lower level of citrate, higher level of parabens), lower pH and IPA at a concentration of 70%. These changes in composition create a very high level of immediate activity (conventionally measured after 10 min) and persistent activity when applied *in vitro* and *in vivo* to the skin as a preoperative skin antiseptic.

In *in vitro* laboratory studies on bovine rawhide, a group of gram negative and gram positive bacteria was tested. Applying each bacteria strain separately on rawhide allowed a comparative observation of antimicrobial properties of tested antiseptics. Ten minutes' application of C/MB/P/IPA or CHG/IPA caused an approximately 3.5 $\log_{10}$ reduction CFU/cm^2 of skin for all evaluated microorganisms (Fig. 1). Persistent activity remained at this same high level as immediate activity for both tested antiseptics. However, immediate activity in the case of *E. coli* and *E. faecalis* was higher after application of C/MB/P/IPA than CHG/IPA ($\log_{10}$ reduction 4.45 versus 3.58 for CHG/IPA and 4.34 versus 3.60 respectively). Moreover this trend was even greater after 6 h of treatment (5.04 against 3.58 and 4.79 against 3.43 respectively). *P. aeruginosa* also demonstrates somewhat greater log reduction with C/MB/P/IPA. The remaining two bacteria show equal reduction of viable cells by either tested antiseptic.

Comparative clinical studies with different antiseptics and their combinations have a long history. It was found that CHG is more effective in preventing catheter-relating bloodstream infections than povidine-iodine.[27] The combination of IPA and iodophor results in a product with immediate efficacy that requires less time for application in comparison with typical iodophor agents.[28] Similarly ZPT

with alcohol demonstrates greater antimicrobial efficacy than 10% PVI.[8] In a study conducted by Guthery et al. the combination of ZPT/alcohol exceeded the efficacy of both CHG and iodine.[29] Hibbard[12,13] in his clinical trials found that the mixture of CHG/IPA was superior in all tested criteria (immediate, persistent and cumulative action) in comparison with four other antiseptics: 70% IPA, 2% CHG and 4% CHG or PVI. The $\log_{10}$ reduction observed in our *in vivo* study with new antiseptic solution are similar to those reported by Hibbard et al. For abdominal sites 30 s of product application caused a 2.58 $\log_{10}$ reduction CFU/cm^2 of skin in 10 min and 2.44 in 6 h. The increased initial scrubbing time of abdominal sites for 2 min resulted in reduction of bacterial count to almost zero. The reductions on inguinal sites are also very high. The immediate efficacy (after 10 min) expressed as $\log_{10}$ reduction from baseline is 4.66, and persistent activity (after 6 h) 4.02 when tested solution was applied for 90 s. The product may be applied in even shorter time (60 s) with almost this same efficacy.

C/MB/P/IPA, similar to Zuragen™, is composed of several compounds which if used together disclose a synergistic effect.[25] One important modification in the composition of a new preoperative solution is the presence of IPA in C/MB/P/IPA antiseptic. Therefore we investigated the influence of alcohol on the effectiveness of three other components of C/MB/P/IPA: citrate, parabens and methylene blue separately. This was done in a lower concentration of IPA than in C/MB/P/IPA to ensure survival of bacteria cells for 10 min or more in a suspension test. As can be seen on Fig. 2, *S. aureus* cells grow well when exposed to low concentration of citrate, parabens and methylene blue solutions containing no IPA. In the presence of 30% IPA, concentrations as low as 1% citrate or 0.06%MP/0.03% PP reduced viability of the cells practically to zero after 15 s of contact. In the case of methylene blue 60 s is required to accomplish this same total reduction of living cells. High concentration of alcohol may satisfactorily kill bacteria by itself. However, because of its rapid vaporization on skin surface it is mainly used in combination with other antiseptics as preoperative skin solution. It has been pointed out that addition of other antiseptic agents is necessary to extend alcohol persistance.[10] Our results suggest rather that IPA may act synergistically with some other antiseptics. The effectiveness of 30% ethanol combined with 4% trisodium citrate as an antithrombotic/antibacterial lock solution in eradicating bacteria and fungi and preventing biofilm formation[30,31] may also have its origin in synergism between ethanol and citrate in the mixture. The results from our suspension test cannot be directly equated to a preoperative skin setting, but they are valuable and informative. A low concentration of alcohol (generally desire for safety reason) should be able to stimulate other used antiseptics when utilized moderately in an application where efficacy is important but a high level of some component may be unpleasant or harmful to patients. These areas of application include, for example, topical wound treatments, wound/ulcer irrigants or healing creams or gels.

Our study has some limitations, mainly in data for human subjects. While the enrollment was limited and comparisons were not performed head-to head, the data presented in this paper nonetheless show the high efficacy of a newly tested disinfectant solution for preoperative skin antisepsis. The level of immediate and persistent effectiveness of this solution appears to be as high as that of CHG/IPA and in some circumstances may be even better. Strikingly, a very high level of $\log_{10}$ reduction was noticed in as short a time as 30 s after C/MB/P/IPA application for abdominal and groin areas. Another advantage could be the good penetration of the skin by the C/MB/P/IPA components, as predicted in several studies.[32,33] Microorganisms colonizing the skin are also found to inhabit hair follicles, sebaceous glands and lower skin depth.[34] Chlorhexidine, especially in aqueous solutions, demonstrates poor penetration into the deeper layers of the skin, which restricts the efficacy of skin antisepsis with this agent.[35] The eradication of bacterial biofilm may also be an important factor. Chlorhexidine may inhibit biofilm formation of different bacterial species but when used at sub-MIC concentration it actually can induce biofilm development.[36] On the basis of our prior studies[25,26] C/MB/P/IPA should not only strongly prevent growth of sessile microorganisms but also eradicate existing biofilm from insertion sites of indwelling devices. Immediate hypersensitivity and eczema have occurred with chlorhexidine, sometimes taking the form of anaphylactic shock (more than fifty cases reported worldwide from 1994 to 2004).[37,38] The components of C/MB/P/IPA are safe and use frequently in clinical applications. One exception is that some patients have an allergic reaction to parabens[39,40] but if used in higher concentration than in tested solution. However expanded clinical studies are needed to determine the incidence of sensitivity, irritation and injuries of patients' skin or mucosa.

Conflict of interest statement

There has been no research support received from granting agencies or industry sources used to generate the information disclosed in this publication. J.S. and L.B. are fulltime employees of Ash Access Technology, Inc. S.R.A. (Chairman & Dir. R&D) and A.G. are part-time paid consultants with the Company. All own shares and hold stock options in Ash Access Technologies, Inc.

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