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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