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The Effectiveness of Multi-purpose Disinfecting Solutions Against Clinically Isolated Micro-Organisms

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ABSTRACT

To quantitatively asses a contact lens multipurpose disinfecting solution's effectiveness, the ISO recommends a 3-log reduction against bacterial species and a 1-log reduction against fungal species. This standard, upheld by the FDA, is tested against pure strains of microorganisms from the ATCC. Recent concern has arisen regarding the use of these pure laboratory cultures as sufficient standards for evaluating the biocidal effectiveness of disinfecting solutions. In this study, the effectiveness of four disinfecting contact lens solutions was assessed by inoculation in three replicates with clinically isolated bacterial strains; one strain arose from an ocular infection, while two other strains were isolated from inanimate surfaces in a non-optometric clinic. The four solutions tested included Opti-Free Replenish®, Complete®, Re-Nu Sensitive®, and Equate®. All four solutions passed the 3-log reduction requirement when inoculated with the isolate from the ocular infection, later identified as *Staphylococcus warneri.* The four solutions failed to meet the 3-log reduction in one or more of the tests against the strains of bacteria which were non-ocular in origin.

Keywords: Contact lens, disinfecting solution, ocular infection, solution effectiveness, *Staphylococcus warneri.*

INTRODUCTION

Historically, contact lens wearers have been identified as a demographic group with increased susceptibility to ocular infections (Ritterband, 2007). For users of one extended wear silicone hydrogel, the rate of infectious incidence was 18 cases per 10,000 wearers (Schein et. al., 2005). Similarly, a study conducted in the United Kingdom regarding extended wear silicone hydrogels, found users of this type of contact to have an incidence rate of 19.8 cases per 10,000 wearers (Morgan, 2005). Therefore, while not every contact lens wearer should expect complications, doctors of optometry and ophthalmology, lens manufacturers, and manufacturers of lens care solutions share interest in ongoing research that can lead to the development of products and lens care guidelines to further improve contact lens wearers' health and safety.

Several factors contribute to the development of an infection. Specific wearing patterns have been linked to greater risk of bacterial colonization of lenses and associated care materials (Yung et. al., 2007). However, contamination alone does not guarantee that a user will develop an infection. Patient compliance and safe-handling of the lens are other contributing factors which Yung et. al. identified. Furthermore, the health of the cornea may be affected by dryness, trauma, or underlying conditions which may predispose a patient to a higher risk of infection (Stone, 2007). All other factors considered, the disinfecting solution's effectiveness is the one most easily manipulated and controlled by the eye care suppliers and healthcare providers.

A multi-purpose disinfecting solution's effectiveness depends upon the environment within which the solution is stored, the interaction of the solution with the corneal epithelium of the wearer, the binding properties of the solution with the case or contact, the solution's ability to attack biofilms, and the species of microorganism with which the solution interacts.

Solutions have been shown to lose bactericidal ability over a three month storage period, and when storage temperatures are altered (Leung et. al., 2004). Also, certain brands of silicone hydrogel lenses combined with solutions containing polyhexamethylene biguanide as the active ingredient are more prone to disturbing the protective corneal epithelium, thus leaving the tissue more vulnerable to potential infection (Stone, 2007). An ideal solution would not be absorbed into the lens surface or the surface of the case. Re-Nu with MoistureLoc® has previously shown to be less effective against *Fusarium* due in part to the disinfectant's absorbance into the case and lens materials, and also due to the inactive ingredients' ability to bind and mask the microorganism (Ritterband, 2007). Microbial communities can also become more resistant to solutions' active ingredients when the microorganisms form biofilms; thus testing a pathogenic strain in its "planktonic" form can yield different predictions of outcomes for a disinfecting solution's efficiency than that which is actually observed when the microorganism has formed biofilms on the lens surface (Flynn et. al., 2009). Finally, an effective solution must be able to perform adequately both against bacterial and fungal pathogens, which differ greatly in cellular structure (Boost et. al., 2010).

To quantitatively evaluate solution effectiveness, the ISO, International Organization for Standardization, requires a multi-purpose solution to demonstrate a 3-log reduction against bacterial pathogens and a

1-log reduction against fungal microorganisms in their planktonic form (Hume et. al., 2007). In accordance with the Food and Drug Administration, or FDA, guidelines, solutions are tested with pure laboratory strains which are cultured and recommended by the American Type Culture Collection, or ATCC, as a supplier (Hume et. al., 2007).

Recently, concern has arisen regarding the use of pure laboratory cultures as a sufficient standard for evaluating a solution's biocidal effectiveness. While proving to be effective against laboratory strains of microorganisms, certain multi-purpose solutions tested with environmentally and clinically isolated strains of fungi failed to meet the above criteria (Boost et. al., 2010). Also, a clinically isolated strain of *Serratia marcescens* was tested in five multipurpose solutions, two of which were found to be ineffective against the bacteria (Hume et. al., 2007). Thus, further study is needed to improve current testing procedures and to understand the interaction of solutions with clinically and environmentally encountered strains of microorganisms.

The objective of this study is to test multi-purpose solutions from the common classes of disinfectants including: polyhexamethylene biguanide, polyaminopropylbiguanide, myristamidopropyl dimethylamine, alexidine, and polyquaternium-1, against bacterial specimens of both ocular and non-ocular origin. These disinfecting agents function as bactericides by different means. However, each of the disinfectants affects the structural integrity and permeability of the bacterial cell membrane (McDonnell and Russell, 1999). The hypothesis is that all multi-purpose solutions will exhibit a 3-log reduction of the microorganisms from each of the three isolates after the manufacturers' recommended exposure time in solution.

MATERIALS AND METHODS

To collect clinical pathogens for isolation, contact lens wearers with an ocular infection agreed to contribute their disposable lenses. The lenses were kept in Blairex ® sterile saline solution until the samples could be transported to the laboratory for culture. While waiting for inoculating media to be prepared, the samples were stored in their cases in the refrigerator. All inoculating media were autoclaved at 121°C for a period of at least fifteen minutes; the total time in the autoclave averaged fifty-five minutes.

Once the inoculating media was prepared, samples were transported to the enclosed flow hood system. The hood work area and inoculating materials were first sterilized by ultraviolet radiation for three-five minutes. The contact lenses were transferred into tubes of nutrient broth (Oxoid) by tweezers which were sterilized with ultraviolet radiation and by flaming with isopropyl alcohol. If lenses adhered to the surface of the tube, a sterile micro-loop was used to push the lens into the media. After the inoculated tube exhibited growth, a sterile micro-loop was dipped into the media and used to create a streak plate on nutrient agar (Oxoid). Successions of streak plates were then generated in an attempt to produce an isolated colony. All inoculated materials were incubated at 37°C, except when transferred to the refrigerator for prolonged periods of storage.

The first infectious pathogen was collected 7-28-2010, and consisted of Gram negative rods as indicated by Gram staining. This strain eventually became unviable in laboratory culture, perhaps due to prolonged storage conditions in the refrigerator and incubator. A second sample consisting of cocci was collected 11-04-2010. Gram staining of this strain was ambiguous, as both Gram positive and Gram negative coloration was observed. This observation was attributed to the differences of smear thickness on the slide, which could create areas of denser staining. To further clarify the identity of the cocci, a test tube containing nutrient agar media (Oxoid) at a slant was prepared and inoculated from an isolated colony to be sent to Molecular Epidemiology, Inc., for genetic identification. The cocci isolated in the slanted tube of agar were used to inoculate a fresh tube of nutrient broth (Oxoid) and this tube was used to create all later tubes for testing this strain. Of notable interest, the wearer of the lens from which the cocci were isolated had suffered previous ocular trauma not related to use of the lens, and had since developed symptoms of infection after resuming use of the lens.

In order to diversify the collection of test organisms, a strain of Gram positive rods and a separate strain of Gram negative rods were contributed from the project conducted by Karissa Ferrell regarding the prevalence of oxacillin resistant microorganisms. These rods were therefore isolated from inanimate surfaces, and were not of ocular origin. Both strains had been plated on an oxacillin treated mannitol salt agar media originally. These two plated strains were used to inoculate tubes of nutrient broth (Oxoid), then transferred to streak plates. Isolated colonies were used to inoculate fresh tubes of the nutrient broth and were used to generate all tubes used for further testing of these strains. A Gram stain of the isolates confirmed the previous Gram staining observation conducted by Karissa Ferrell.

To determine the initial colony forming units, or CFU's, per tube of inoculate, a series of 1:10 dilutions was plated for each of the isolates. A single mL of inoculate was transferred into tubes containing 9 mL of nutrient broth to bring the total volume to 10 mL. Aliquots of 100 µL were plated and disbursed across the nutrient agar with the sterilized spreader tool. To ensure consistent concentration of cells in the inoculating media, all dilution tubes were vortexed for at least 30 seconds before the next aliquot was transferred to the next tube for dilution. The spectrophotometer was then used to measure the

absorbance of the undiluted inoculating media at 660 nm. According to previous studies, $1X10^8$ colony forming units in sterile saline has an absorbance of 0.1 at 660 nm (Hume et. al., 2007). By contrast, I measured absorbance values in the nutrient broth. The plates inoculated from the dilution tubes were then examined, and the plate yielding the clearest viable cell count was then utilized to calculate the undiluted concentration of colony forming units. The third 1:10 serial dilution tube was used to inoculate the multi-purpose solutions. The concentration of cells utilized to test the contact lens solutions varied for each of the three isolates due to the difference in growth rates and incubation times amongst the three microorganisms. Single mL aliquots of inoculate were placed in 9 mL of the multi-purpose solutions. The dilution tube was vortexed at least 30 seconds between each successive inoculation. Each of the three isolated strains was tested three times in each of the four multi-purpose solutions.

After the manufacturer recommended exposure period, the inoculated multipurpose solutions were vortexed for 30 seconds, and 100 µL of the inoculated disinfecting solution was plated onto nutrient agar. Colony counts were taken after incubation at 37°C for 35.5-42 hours.

Table 1. Disinfecting solutions specifications. Polyquad® is the trade name for polyquaternium-1 and Aldox® is the brand name for myristamidopropyl dimethylamine. PHMB is polyhexamethylene biguanide. Dymed® is merely a trade name for polyaminopropylbiguanide. The Exposure time is listed in hours.

Solution	Company	% Active Ingredient	Exposure Time (hrs)
Opti-Free Replenish®	Alcon	0.001% Polyquad® 0.0005% Aldox®	6
Complete®	Abott	PHMB 0.0001%	6
Re-Nu Sensitive®	Bausch& Lomb	Dymed® 0.00005%	4
Equate®	Wal-Mart	Polyaminopropyl biguanide 0.0001%	4

The one way ANOVA test for variance was utilized to identify any statistically significant difference amongst the plate counts from the four disinfecting solutions for each of the microorganisms tested. SigmaStat®. was utilized to perform the ANOVA test with $P<0.05$ used to determine significance. For the pairwise multiple comparisions test, the Holm-Sidak method was used to identify which solution interactions differed significantly.

RESULTS

The initial CFU/mL for the undiluted sample of cocci after 45 hours of incubation at 37°C was determined to be $1.19X10^8$ by serial dilution plate counts. The absorbance of the undiluted cocci was 0.308 at 660 nm. The plate count was relied upon more than the absorbance data because the absorbance varied based upon agar concentration and tube incubation time. More absorbance data would have been required for the construction of a calibration curve to directly associate concentrations of CFU/mL from the plate counts with the absorbances of the initial undilute cultures. Since $1.19X10^8$ CFU/mL was the original concentration by plate count, the disinfecting solutions would have been exposed to $1.19X10^5$ CFU/mL on the third dilution. The cocci was subsequently identified by Molecular Epidemiology, Inc., as being *Staphylococcus warneri.*

For the actual testing of the Gram negative rods, the fourth and fifth serial dilution plates provided an estimate of $1.7X10^7$ CFU/mL for the undiluted sample after 17 hours of incubation. I neglected to record an absorbance for the sample of Gram negative rods at the time of testing; however, a previous culture of the same Gram negative rods had yielded an absorbance of 0.119 at 660 nm after 24 hours of incubation and contained $3.7X10^7$ CFU/mL in the undiluted tube as determined by serial plate counts. Thus there is close agreement between the pilot trial and the actual test. The disinfecting solutions were therefore exposed to $1.7X10^4$ CFU/mL, which was the third dilution of the $1.7X10^7$ CFU/mL undiluted culture.

The Gram positive rods had an absorbance of 0.107 at 660 nm and 21 hours of incubation. The original concentration from the serial dilutions plate count was determined to be $3.6\ X10^7$ CFU/mL. Thus, the disinfecting solutions were exposed to $3.6X10^4$ CFU/mL.

The one way ANOVA failed to detect any significant differences amongst the four disinfecting solutions' performances against the cocci and the Gram positive rods. The ANOVA did detect a significant difference amongst the four solutions' performances with the Gram negative rods.

The Holm-Sidak test revealed a significant difference between Re-Nu Sensitive® and Complete® as well as for Re-Nu Sensitive® and Opti-Free Replenish®. The level used to determine significance was 0.05 for each statistical test.

As previously stated, each strain of microorganism was tested in each of the four multi-purpose disinfect-

ing solutions three times. The direct colony counts after being plated and incubated are displayed below.

Table 2. Raw data. In each column with a bacterial test strain label, the exp. denotes the expected CFU/mL after exposure to the solution. The actual CFU/mL as determined from the plate counts is displayed below. The "p" or "f" designation notates whether the solution passed or failed to meet the 3-log reduction.

Incubation Period 35.5-42 hours for all plates	Ocular Cocci Exp. 119	Gram (-) Rods Exp. 17	Gram(+) Rods Exp. 36
Opti-Free Replenish ®			
1	10 P	50 F	60 F
2	0 P	0 P	90 F
3	10 P	70 F	0 P
Complete® 1	0 P	20 F	10 P
2	20 P	10 P	30 P
3	0 P	10 P	100F
Re-Nu Sensitive®			
1	10 P	120F	80 F
2	10 P	90 F	110F
3	0 P	210F	120F
Equate®			
1	20P	60 F	40 F
2	0 P	60 F	30 P
3	0 P	90 F	40 F

DISCUSSION

Of considerable notability, the solutions all met the 3-log reduction when exposed to the ocular strain of cocci; however, no solution met the 3-log reduction for all three repetitions of the Gram positive and Gram negative rods which were not ocular in origin. The results cannot be interpreted as absolute regarding the comparisons between solutions for each bacterial strain tested. The power of the ANOVA tests was considerably lower for those comparisons which failed to detect any significant difference. The power was only 0.145 whereas the desired power was 0.800 for the evaluation of the differences between solutions when exposed the Gram positive rods. Power of the ANOVA used to evaluate the difference in interactions amongst solutions exposed to cocci was only 0.05 rather than 0.800. However, the power of the ANOVA which detected the significant difference amongst the solutions exposed to the Gram negative rods was 0.915. The Holm-Sidak test revealed a significantly greater CFU/mL remaining after exposure to Re-Nu Sensitive® in contrast to CFU/mL remaining after exposure to either Complete® or Opti-Free Replenish®. To further increase the power of the tests and reduce the variability of the results, one should use more repetitions of each solution-strain combination. Accuracy of the initial inoculating concentrations used to test solutions could be further ensured by making replicate serial dilution plates to verify the reproducibility of colony counts.

One additional possible source of error in this experiment is the prolonged storage periods in both the incubator and refrigerator. Multiple re-cultures in the laboratory and varying storage conditions can cause the bacterial population to deviate from the original sample; previous studies recommended that no more than five re-cultures should be taken if the subcultures are to remain representative of the original bacterial population. The samples used in solution testing were re-cultured more than five times, and therefore there may be genetic discrepancy between the test culture and the original culture (ATCC, 2010).

When considering the results of this experiment, one should note that the methodology of this study differed from the previous experiment conducted by Hume et. al. (2007), because their team used 10 µL of cell culture in 1 mL of disinfecting solution. Hume et. al. previously noted that 10 mL of disinfecting solution is the volume recommended by the ISO rather than 1 mL; therefore the larger test volume was implemented in this study. Also note that this study used longer incubation periods in contrast to previous studies. The extended incubation period was implemented to allow for sufficient time to detect the growth of the slower growing ocular strain.

While these results suggest that certain disinfecting solutions may be less effective against the non-ocular isolates of bacteria, further testing of clinically isolated bacterial strains of ocular origin is necessary to draw conclusions sufficient to warrant any change in the current disinfection systems. In addition, future experiments could test microorganisms representative of the fungal or protozoal pathogens. One could also evaluate the effects of biofilms and solution interactions since the observations of this study were restricted to the evaluation of microbial behavior in the planktonic form.

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