

Neutralization efficacy of Dey-Engley medium in testing of contact lens disinfecting solutions

S.V.W. Sutton, T. Wrzosek and D.W. Proud

Bausch & Lomb Inc., Rochester, NY, USA

Accepted 3 September 1990

Paper number: 3365/04/90

S.V.W. SUTTON, T. WRZOSEK AND D.W. PROUD. 1991. A quantitative assay for the demonstration of neutralizer efficacy was developed to monitor contact lens disinfecting solutions. Adequate neutralization of disinfecting agents is essential to the accurate determination of disinfecting activity with time. This method employed the recovery of small numbers of micro-organisms from neutralizing medium containing a disinfectant. A statistical estimation of significance between treatments demonstrated that Dey-Engley medium (DE; Difco) was generally effective when tested as an agar growth medium with several bacterial test organisms. DE medium from another vendor was less effective, underscoring the need for laboratory quality control and monitoring. DE agar (Difco) adequately neutralized all solutions tested at a 1:20 dilution. The solutions included those containing Dymed™ (polyaminopropyl biguanide, 0.00005%), chlorhexidine (0.005%), Polyquad® (0.001%), chlorhexidine (0.005%) and thimerosal (BP, 0.001%), thimerosal (BP, 0.002%) and Tris(2-hydroxyethyl) tallow ammonium chloride (0.013%), and a solution preserved with 115 ppm benzalkonium chloride (BAK). A modification of this medium was developed which retained virtually all of the neutralizing efficacy for the solutions tested while allowing the use of automated testing procedures.

INTRODUCTION

The validation of biocidal assay conditions before commencing microbiological tests is critical in accurate determination of disinfecting efficacy. A medium that adequately neutralizes the disinfecting formulation under investigation is necessary to avoid an over-estimation of efficacy. Dey-Engley medium was formulated to inhibit the activity of a wide range of disinfectants while allowing bacterial growth (Engley & Dey 1970). Disinfectants are neutralized either by dilution or by inclusion of specific reagents that inhibit their activity. A number of reagents are known to inhibit specific disinfectants (reviewed by Russell 1981; Furr & Rogers 1987). These compounds may be toxic to the test organisms (Reybrouck 1978) and so it is imperative to test the growth promotion properties of the neutralizing medium. These two activities, neutralizer efficacy and growth promotion (or neutralizer toxicity), are equally important for the accurate determination of the efficacy of a disinfecting, or a preserved solution.

It is important to know if the presence of the disinfectant in the neutralizing medium affects the viability of micro-organisms. We therefore attempted to recover small numbers of micro-organisms from a neutralizing agar (DE agar) in the presence, or the absence, of the disinfectant. We are primarily concerned with the study of ophthalmic disinfectants and so examined solutions formulated for contact lens and eye care.

MATERIALS AND METHODS

Test organisms

Test organisms included *Staphylococcus hominis* (ATCC 17917), *Pseudomonas aeruginosa* (ATCC 15442), *Serratia marcescens* (ATCC 14041), *Candida albicans* (ATCC 10231), and *Aspergillus fumigatus* (ATCC 10894). The bacteria were grown to confluence on slopes of Trypticase Soy Agar (TSA; Difco) and harvested into phosphate buffered saline (PBS; pH 7.2). Samples were washed twice in PBS and then standardized to 108 cfu/ml by turbidimetric measurement (Spectrometer 20, Bausch & Lomb,

Rochester, NY). *Candida albicans* was grown to confluence on slopes of Sabouraud Dextrose Agar (SDA; Difco). Samples were washed twice in PBS and then standardized to 108 cfu/ml by turbidity. *Aspergillus fumigatus* was grown, and allowed to sporulate, on SDA slopes. These slants were stored for up to 1 month at 3°C. The spores were harvested in TPBS (PBS with 0.1% Tween 80) and washed in PBS.

Procedure

The disinfecting solutions studied are listed in Table 1. One ml of solution was mixed with neutralizing agar (DE Agar; Difco) maintained at 45°C until use in a 1:20 dilution. This solution was poured into a petri dish inoculated with 10–100 cfu of the challenge micro-organism. The plate was swirled several times to distribute the cells evenly, the agar allowed to solidify, then incubated at 33°C for 2 to 4 d. The total numbers of colonies growing in the media were counted, and analysed as described below.

Statistical analysis

All statistical analysis was performed on colony counts transformed to \log_{10} values. Neutralizer toxicity was determined by comparing the population consisting of mean cfu (\log_{10}) recoverable from rich medium with those from the neutralizing medium in the absence of the disinfectant. Neutralizer efficacy comparisons were made between the populations consisting of the neutralizing media in the presence and the absence of the disinfectant. Student's *t* test was performed on the transformed data in each case to evaluate the statistical difference between the two popu-

lations ($P = 0.01$ in a one-tailed test). Equivalence of variance between the two populations was tested by the *F* test at $P = 0.01$ (Dowdy & Weardon 1983). As all comparisons were made between two populations (neutralizing medium without disinfectant vs another) Student's *t* test is appropriate for evaluating the relationship. Use of this test, however, precludes any comparisons among the populations.

Finally, the estimations of the resolution of the assays were made by determining the ratio of the means having the minimum difference detectable with the data set at a beta risk of 0.05 and an alpha risk of 0.01 (Freund & Walpole 1987). The resolution of the assay was calculated using the formula:

$$(\mu_1 - \mu_2)^2 = \frac{(S_1^2 + S_2^2) \times (Z_\alpha + Z_\beta)^2}{n}$$

As the population variances are not known, the pooled sample variance is used as an estimate, after the equivalence of variation is established. The respective *t* values are substituted for the *Z* values as the variance is estimated from the data, and the sample size is small, giving the result:

$$(\mu_1 - \mu_2)^2 = \frac{2 \times (\text{Pooled Sample Variance}) \times (t_\alpha + t_\beta)^2}{n} = \delta^2$$

This equation provides an estimation of the smallest discernible difference between the two means, μ_1 and μ_2 . These are the means of the transformed (\log_{10}) cfu recoverable, therefore the antilog of δ is equal to the ratio of the two most similar sample means discernible by the data. The resolution of the assay is defined as 10^δ .

Table 1 Soft contact lens disinfecting solutions tested

Solution	Active agent(s)
Bausch & Lomb® Sterile Preserved Disinfecting Solution	Chlorhexidine (0.005%) Thimerosal (0.001%) Edetate disodium (0.1%)
Allergan® Hydrocare® Cleaning and Disinfecting Solution	Tris (2-hydroxyethyl) tallow ammonium chloride (0.013%) Thimerosal (0.002%)
Durasoft™ Colors Disinfecting Solution	Chlorhexidine Gluconate (0.005%) Edetate Disodium (0.1%)
Bausch & Lomb® ReNu® Multi-Purpose Solution	Dymed™ (0.00005%) (polyaminopropyl biguanide)
Alcon Opti-free® Rinsing, Disinfecting & Storage Solution	Polyquad® (0.001%) (polyquaternium-1) Edetate disodium (0.1%)
Bausch & Lomb® Moisture Drops® Validation Lot (excess BAK)	Benzalkonium chloride (0.00115%) Edetate Disodium (0.1%)

Table 2 Neutralizer efficacy of Dey-Engley agar

	<i>Staphylococcus hominis</i>	<i>Serratia marcescens</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>	<i>Aspergillus fumigatus</i>
Neutralizer toxicity	+	+	+	+	+
	(1·286)	(3·050)	(1·509)	(1·333)	(1·542)
B&L Sterile Pres. Dis. Sol.	+	+	+	+	+
	(1·359)	(2·857)	(1·626)	(1·484)	(1·423)
Hydrocare	+	+	+	+	+
	(1·374)	(2·673)	(1·498)	(1·341)	(1·416)
Durasoft	+	+	+	+	+
	(1·822)	(2·479)	(1·521)	(1·524)	(1·413)
ReNu MPS	+	+	+	+	+
	(1·337)	(2·601)	(1·506)	(1·516)	(1·514)
Opti-free	+	+	+	+	+
	(1·446)	(2·750)	(1·531)	(1·671)	(1·472)
Moisture drops	+	+	+	+	+
	(1·462)	(2·532)	(1·527)	(1·344)	(1·497)

The medium (DEA) was tested for neutralizer efficacy of disinfecting solutions in a 1:20 dilution. +, Indicates an inability to distinguish between the means of 10 replicates in the presence or in the absence of the disinfecting solution. —, Indicates a difference distinguishable by Student's *t* test (checked for equivalence of variance with the F test) at $P = 0.01$. The number in parentheses below the response is the resolution of the means of the data assuming a beta risk of 0.0 and an alpha risk of 0.01.

RESULTS AND DISCUSSION

The purpose of a neutralizing efficacy validation study is to demonstrate the ability of the system adequately to neutralize the disinfecting agent, allowing unrestrained microbial growth. This is important for the accuracy of a biocidal assay. As microbicidal activity is reflected by survivors with time, care needs to be taken to avoid inhibition of microbial growth by low levels of residual disinfectant which would

lead to exaggerated measures of microbicidal activity. The neutralizing efficacy of the medium was determined by comparing growth supported by the neutralizing media in the presence and the absence of the specified amounts of the disinfectant solutions. The ability of the neutralizing medium to promote growth is a second important consideration in this analysis. Neutralizer toxicity is determined by comparing growth in the neutralizing medium without the

Table 3 Efficacy of different potential neutralizing media with staphylococcus

	TSA	DEA	Minus dye	Minus lecithin	1/7 lecithin
Neutralizer toxicity	NA	+	+	+	+
		(1·286)	(1·242)	(1·994)	(1·722)
B&L Sterile Pres. Dis. Sol.	—	+	+	—	+
	ND	(1·359)	(1·245)	(1·970)	(1·793)
Hydrocare	—	+	+	+	+
	ND	(1·374)	(1·240)	(1·864)	(1·736)
Durasoft	—	+	+	+	+
	ND	(1·822)	(1·274)	(1·981)	(1·686)
ReNu MPS	+	+	+	+	+
	(1·295)	(1·337)	(1·439)	(2·263)	(1·853)
Opti-free	+	+	+	+	+
	(1·378)	(1·446)	(1·308)	(2·005)	(1·791)
Moisture drops	—	+	+	—	+
	ND	(1·462)	(1·336)	(1·759)	(1·292)

The media tested included Trypticase Soy Agar (TSA: Difco), Dey-Engley Agar (DEA), DEA minus the bromocresol purple (minus Dye), DEA lacking dye and lecithin (minus lecithin), and DEA lacking dye but containing 1 g lecithin per litre. See footnote to Table 2 for explanation of symbols. All comparisons satisfied the F test for equivalence of variance. The number in parentheses below the response is an estimation of the resolution of the assay (see the footnote to Table 2). NA refers to the identity of TSA: TSA counts; ND is not derivable—the organisms would not grow in the presence of the test solution and so the resolution of the assay could not be determined.

disinfectant with growth in a rich medium (TSA or SDA). All tests were initially made at a 1:20 dilution in agar and a pour plate method.

Dey-Engley Agar (Difco) was found to be a suitable neutralizing medium for all contact lens disinfecting solutions tested using the five test micro-organisms (Table 2). (Note that solutions containing hydrogen peroxide were not included in this study. Hydrogen peroxide solutions are neutralized with the enzyme catalase before plating.) Dey-Engley Medium from an alternate vendor exhibited significant toxicity to *Staph. hominis* and thus did not meet our quality control standards. This was brought to the attention of the vendors who corrected the problem and now incorporates this bacterium in their monitoring programme.

One limitation of this procedure is that it does not measure recovery of crippled organisms. Although it is a sensitive assay for the neutralizing efficacy of a medium, it uses healthy cells which have not been exposed to disinfectants. The recovery of damaged micro-organisms is extremely difficult to quantify (reviewed by Gilbert 1984). Consequently, this method provides no estimation of bacterial recovery from a crippled state in the presence of the disinfectant. While some estimation of recovery would be of interest (Mossel & van Netten 1984), it lies outside the scope of neutralizer efficacy determination.

We were interested in decreasing the turbidity of Dey-Engley broth for use in suspension tests. This turbidity is caused by high levels of lecithin and the presence of bromocresol purple. While the bromocresol purple indicator is useful for some organisms, several test systems do not acidify the medium sufficiently for the colour change. In addition, the presence of the indicator itself precludes the use of Dey-Engley medium in many automated tests that rely upon changes in optical density to indicate growth. While removal of the dye resolves one problem, the turbidity due to high levels of lecithin hampers the use of this medium. A limited study was undertaken to reduce the amount of lecithin present in the medium without forfeiting neutralizer efficacy. The test organism *Staph. hominis* was the most sensitive indicator of neutralizing efficacy and toxicity of the five test organisms studied. Results from comparative tests with *Staph. hominis* are shown in Table 3. These results were confirmed with the remaining test organisms (results not shown). The level of lecithin could be reduced to one-eighth the concentration found in Dey-Engley without sacrificing the neutralizing efficacy of the medium for the solutions tested, resulting in a solution sufficiently clear for use. The only exceptions to the 1:20 ratio were Durasoft™, which was found to require a 1:40 ratio for neutralization with *Candida albicans*, and Hydrocare®, which required a similar dilution with *Aspergillus fumigatus*.

Dey-Engley medium is an effective medium for the neutralization of currently marketed contact lens disinfecting solutions. Removal of the pH indicator dye bromocresol purple and reduction of the levels of lecithin did not significantly reduce this efficacy for the disinfectant solutions tested and improved the clarity of the medium for suspension disinfection assays. In conclusion, Dey-Engley medium is a good neutralizer of currently marketed contact lens disinfecting solutions (with the exception of 3% hydrogen peroxide solutions), although demonstration of neutralizer efficacy should be performed prior to testing of an new solution.

ACKNOWLEDGEMENTS

The authors wish to thank Nydia Montag, Denise Kolb, Lynne Brunner, and Marlene Ciulla for their expert technical assistance.

REFERENCES

- DOWDY, S. & WEARDEN, S. (1983) *Statistics for Research* pp. 193–194. New York: John Wiley.
- ENGLEY, F.B. & DEY, B.P. (1970) A universal neutralizing medium for antimicrobial chemicals. In *Proceedings of the 56th Mid-year Meeting of the Chemical Specialties Manufacturers Association* pp. 100–106. New York.
- FREUND, J.E. & WALPOLE, R.E. (1987) *Mathematical Statistics* p. 422. Englewood Cliffs, NJ: Prentice-Hall.
- FURR, J.R. & ROGERS, D.T. (1987) Preservation of sterile pharmaceutical products. In *Preservatives in the Food, Pharmaceutical and Environmental Industries* pp. 211–230. Oxford: Blackwell Scientific.
- GILBERT, P. (1984) The revival of micro-organisms sublethally injured by chemical inhibitors. In *The Revival of Injured Microbes*. Andrew, M.H.E. & Russell, A.D. pp. 175–197. Society for Applied Bacteriology Symposium Series No. 12. London: Academic Press.
- MOSSEL, D.A.A. & VAN NETTEN, P. (1984) Harmful effects of selective media on stressed micro-organisms: nature and remedies. In *The Revival of Injured Microbes*. ed. Andrew, M.H.E. & Russell, A.D. pp. 329–369. Society for Applied Bacteriology Symposium Series No. 12. London: Academic Press.
- REYBROUCK, G. (1978) Bactericidal activity of 40 potential disinfectant inactivators. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene Series B* 167, 528–534.
- RUSSELL, A.D. (1981) Neutralization procedures in the evaluation of bactericidal activity. In *Disinfectants: Their Use and Evaluation of Effectiveness*. ed. Collins, C.H., Allwood, M.C., Bloomfield, S.F. & Fox, A. pp. 5–59. Society for Applied Bacteriology Technical Series No. 16. London: Academic Press.