RESEARCH ARTICLE

Validation of Microbial Recovery From Disinfectants

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ABSTRACT: We evaluated the efficacy of different neutralizing solutions for use in disinfectant efficacy assays. Our approach used comparisons between the recoveries of low inocula in different treatment populations. The challenge organisms employed were those described by the AOAC for use in determining germicidal, tuberculocidal, sporicidal, and fungicidal activity of disinfectants. Neutralizer efficacy (NE) ratios were determined by comparing the recovery of identical inocula from the neutralizing solution in the presence, or the absence, of a 1:10 dilution of the biocide. Neutralizer toxicity (NT) ratios were determined between recovery of viable microorganisms incubated for a short period in peptone, and in the neutralizing medium without the biocide. An effective and non-toxic neutralizer was initially identified by NE and NT ratios of ≥ 0.75. Statistical evaluation of the data was performed by ANOVA, with Dunnett's test for multiple comparisons used to confirm failures. By this analysis, 239/244 identified failures were confirmed by ANOVA of 588 NT and NE comparisons (5 presumptive failures were not confirmed by statistical analysis). We therefore conclude that recovery of 75% is a suitable criterion (2% false negative rate) for neutralizer evaluations. There was a wide degree of variability seen among the responses of different test organisms to the different recovery broths. *Pseudomonas aeruginosa* and *Salmonella choleraesuis* were particularly sensitive to commercial neutralizer broths. Interestingly, no commercially available neutralizer proved adequate for all index organisms against all biocides tested. This finding underscores the need to evaluate potential neutralizers against all microorganisms and biocides employed.

Introduction

A recently proposed *United States Pharmacopoeia* (USP) guidance chapter (1) describes the use, evaluation and control of disinfectants and antiseptics. This *Pharmacopoeial Preview* recommends demonstration of the efficacy of the disinfectants used in a manufacturing facility and notes the need to adequately neutralize the disinfectant under test. Similar concerns are expressed for the evaluation of personal antiseptics used by personnel. The USP offers guidance on evaluation of neutralizers in chapter <1227> (2). Complete neutralization of disinfectants is important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survivors with time, and inhibition of microbial growth by low levels of residual biocide would lead to exaggerated measures of microbicidal activity (2, 3, 5).

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Common methods for inhibition of residual biocide include dilution or chemical neutralization of the biocide. Dilution is useful for those biocides with a large concentration exponent and little propensity for binding to the cell (6, 7, 8). A variation on dilution is filtration of the suspension to remove the biocide. This technique must be approached with caution, however, as the biocide may bind either to the membrane filter or to the cells, inhibiting recovery (9-14). Finally, one can inhibit residual biocide by chemical neutralization (see 4, 15, and 16 for reviews). Several classes of biocides have well-established chemical neutralizers. Examples of these are noted in Table 1. A potential difficulty of chemical neutralization of biocides is the toxicity displayed by several types of neutralizers. Examples of these are also provided in Table 1. Evaluation of a chemical neutralizer or a physical neutralization scheme must examine the potential toxicity of the neutralizer as well as its efficacy.

An excellent review by Russell (15) describes three criteria for an effective neutralizer. First, the neutralizer must effectively inhibit the action of the biocidal solution. Second, the neutralizer must not itself be unduly toxic to the challenge organisms. Finally, the neutralizer and active agent must not combine to form a toxic compound.

Table 1. Previously reported efficacy and toxicity of chemical neutralizers. ***Overview of literature references to biocide neutralizers. In addition to chemically neutralizing biocides, many of these compounds are toxic also to microbial cells.***

CHEMICAL NEUTRALIZER	BIOCIDE CLASS	POTENTIAL TOXICITY	REFERENCES
Bisulphate	Glutaraldehyde, Mercurials	Non-sporing Bacteria	17, 18, 19
Dilution	Phenolics, Alcohol, Glutaraldehyde	- - -	18, 20, 21
Glycine	Glutaraldehyde	Growing Cells	22, 23
Lecithin	Quaternary Ammonium Compounds (QACs), Parabens, Bis-biguanides	Bacteria	4, 5, 24, 25
Mg^{+2} or Ca^{+2} ions	EDTA	- - -	26
Polysorbate	QACs, Iodine, Parabens	- - -	27, 28, 29, 30
Thioglycollate	Mercurials	Staphylococci and Spores	31, 32, 33, 34
Thiosulphate	Mercurials, Halogens, Glutaraldehyde	Staphylococci	17, 33, 35, 36

Table 2. Neutralizing broths evaluated in this study. ***All neutralizers were prepared as cited, with the final concentrations as listed (g/L). Neutralizing broths evaluated included AOAC Diluting Broth (AOAC), Dey-Engley Neutralizing Broth (DEB), Letheen (LET), NIH Thioglycollate Broth (NIH), Trypticase with Tween (TAT), and Trypticase Soy Broth with Polysorbate 80 and Lecithin (TPL).***

Ingredient	AOAC	DEB	LET	NIH	TAT	TPL
Beef extract	5.0		5.0			
Casitone				15.0		
Cystine				0.5		
Dextrose		10.0		5.5		2.5
Lecithin		7.0	0.7		5.0	0.7
Peptamin	10.0		10.0			
Polysorbate 20					43.2	
Polysorbate 80		5.0	5.0			15.0
Sodium bisulfite		2.5				
Sodium chloride	5.0		5.0	2.5		
Sodium thioglycollate		1.0		0.5		
Sodium thiosulfate		6.0				
Soytone						3.0
Tryptone		5.0			20.0	17.0
Yeast extract		2.5		5.0		

Three methods have been published describing methods of neutralizer evaluation. Dey and Engley (37) describe a procedure utilizing *Staphylococcus aureus* as the index organism that measures survival with time. The challenge organism is inoculated directly into the biocide and sampled with time. The relative efficacy of the neutralizer is measured by comparing the relative recovery of the challenge organism among differ-

Table 3. Biocides evaluated in this study. ***The labeled concentrations of active ingredients for all biocides tested are presented. All biocides were diluted as appropriate with tap water to use concentration.***

Biocidal Solution	Active Ingredients
Chlorox	5.25% sodium hypochlorite
Alcide	2.73% sodium chlorite
Rocall II (Quat-alcohol)	10% alkyl dimethyl benzyl ammonium chloride 1.25% ethanol
Lysol Deodorizing Cleaner	2.7% alkyl (50%C_{14}, 40% C_{12}, 10%C_{16}) dimethyl benzyl ammonium chloride
Real Pine	25% pine oil
Lysol Disinfectant Spray (Phenol-alcohol)	0.1% o-phenyl phenol; 79% ethanol
Amphyll	10.5% o-phenylphenol; 5.0% o-benzyl-p-chlorophenol
Amerse	p-chlorophenol/phenylphenol
Cidex Plus	3.2% glutaraldehyde
Cidex 7	2% glutaraldehyde
Lysol Pine Action	15% pine oil; 11.7% isopropyl alcohol; 0.78% o-phenylphenol
Staphene	0.077% o-benzyl-p-chlorophenol; 0.041% o-phenylphenol; 0.074% p-tertiary amylphenol; 0.041% n-alkyl dimethylbenzyl ammonium chloride; 0.041% n-alkyl dimethyl ethylbenzyl ammonium chloride; 4.548% propylene glycol, 53.096% ethanol
Sporocidin	7.05% phenol; 2.0% glutaraldehyde 1.2% sodium phenate

ent treatments. This protocol is useful in identifying neutralizers. However, it has several limitations. First, it does not distinguish between neutralization of the biocide versus recovery of organisms injured by sublethal exposure to the biocide. An apparent increase in recovery could result either from a decrease in the activity of the biocide upon treatment, or from improved recovery of crippled organisms. A second concern is that this method utilizes only one organism, *S. aureus*. The assumption is that a neutralizer acts upon the biocide, independent of the challenge organism.

Terleckyj and Axler (38) describe a control procedure to demonstrate neutralization for a fungicidal experiment. The basic design for this experiment was first described in 1972 by Bergan and Lystad (39). First, the biocide is exposed to the neutralizer for a specified time. The challenge organism (*Candida albicans*) is then added to a final concentration of approximately 106 CFU/mL. Survival is determined after an additional 15 minute incubation. While this design separates recovery from inactivation of the biocide, several concerns remain. This method assumes that a neutralizer proven effective for

one organism will be equally effective for all. In addition, this method uses extensive dilution of the organism prior to plating. Dilution of the sample is required due to the high number of organisms in the challenge, but serves to dilute the biocide, which compromises the stringency of the procedure. Finally, this method does not allow the investigator to separate any toxic effect of the neutralization treatment from the effects of the biocide.

The final method was described for use in testing contact lens disinfectants (40). This method is similar in overall design to that described above (39). However, it addresses some of the concerns suggested by earlier protocols. This method employs a smaller inoculum concentration to avoid dilution of the sample. It also takes advantage of statistical analysis of the data to ensure that apparent differences among populations are supported. Finally, and perhaps most importantly, this method evaluates the potential neutralizer with all index organisms. This strategy is employed by the American Society for Testing and Materials (ASTM) in the evaluation of neutralizers (41). It is also the method recommended by USP for validation of microbial recovery (2).

Regardless of the method used to evaluate a neutralizer, there must be a population of organisms included that serve as a growth control. This control population is exposed to neither the potential neutralizer nor the biocide. We suggest two comparisons among three populations, providing evidence for fulfillment of the three criteria outlined by Russell. The first comparison is Neutralizer Efficacy (NE) which can be determined by evaluating survivors in the neutralizing broth in the presence and the absence of the biocide. The ability of the neutralizing broth alone to allow survival is a second important consideration in this analysis. The second comparison is Neutralizer Toxicity (NT). This aspect of neutralization is determined by comparing survivors in the neutralizing medium without the biocide with the viability (growth) control.

We report the results of studies conducted to evaluate six neutralizing broths (Table 2). These neutralizing media were tested against 13 commercially available surface disinfectants chosen to represent a wide range of biocide types (Table 3). We utilized seven challenge organisms recommended by the Association of Analytical Chemists (AOAC) as bioindicators of the chemical inactivation that occurred between the biocide and neutralizer. Our results show that each biocide-neutralizer-organism combination is unique. Neutralizer evaluation must be performed for each combination to be tested in any assay involving biocides or preservatives. In addition, the current study provides a good guideline for levels of recovery to be expected from neutralizer efficacy studies.

Methods

The same basic procedure was followed for all populations examined and is a modification of the method previously described (40). We assayed the recovery of index microorganisms under several different conditions. First, a determination of a standard inoculum was made to confirm the presence of 10 – 100 CFU per plate (102 – 103 CFU/mL). This low number of cells was preferred to enhance the sensitivity of the assay. The second treatment population was exposed to the neutralizing broth without the biocide. Table 2 provides a listing of the neutralizing broths studied. The population exposed to neutralizer served as the control population for all statistical analysis. The final treatment population consisted of the neutralizing broth in the presence of the particular disinfectant and referred to as the neutralizer and biocide population. All biocides evaluated are described in Table 3 and were prepared according to label instructions and used within the stated shelf-life.

Test organisms

Test organisms used include *Trichophyton mentagrophytes* (ATCC 9533), *Pseudomonas aeruginosa* (ATCC 15442), *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* (ATCC 6538), *Clostridium sporogenes* (ATCC 3584), *Salmonella choleraesuis* (ATCC 10708), and *Escherichia coli* (ATCC 11229). These organisms were grown to confluence at 30-35°C on nutrient agar slants of Trypticase Soy Agar (TSA - Difco Laboratories, Detroit, MI) and harvested into Phosphate Buffered Saline (PBS), pH 7.2. Samples were washed twice in PBS and then standardized to approximately 108 CFU/mL by turbidity.

Mycobacterium bovis Type BCG was also used to assay the effective neutralization of the surface disinfectants. This organism was grown on Middlebrook 7H10 Agar (Difco) and required three weeks incubation at 30-35°C.

Test solutions

All neutralizers were prepared as cited, with the final concentrations as listed in Table 2. All biocidal solutions were prepared as directed on the label instructions for general disinfection at the time of testing. Concentrated solutions were diluted to use concentrations in tap water.

The concentration of active ingredients for each of the biocides is provided in Table 3.

Neutralizer evaluation procedure

This procedure provides three treatment populations for comparison. One mL of biocide or PBS was added to a tube containing 9 mL of neutralizing broth. These suspensions were then incubated for 10 minutes on the benchtop at room temperature. These tubes represented the "neutralizer and biocide" and "neutralizer exposed" populations. A third tube, containing 10 mL PBS, was prepared and served as the "viability" control. Each of these solutions was inoculated with 103 to 104 CFU of the challenge organisms (final concentration of 103 to 104 CFU/mL). Each inoculated suspension was incubated for an additional 10 minutes on the benchtop at ambient temperature.

Recovery of all organisms was performed by plating 10 samples of 0.1 mL each on TSA (Difco) supplemented with 0.5% glucose (TSAG) and incubating at 30-35°C for three days. The only exception to this recovery scheme was *M. bovis* which was plated on Middlebrook 7H10 agar as described above. All plates were examined for recovery of CFUs, and the populations analyzed as described below.

Analysis of data

NT was estimated by comparing the recovery of the specific challenge organism in the neutralizer exposed population and the viability population. NE was estimated by comparing the recovery of the challenge organisms in the neutralizer exposed population and the neutralizer with biocide population.

NT and NE ratios were derived utilizing the geometric mean of the recovery in the different populations. Acceptable NT and NE ratios are defined as ≥ 0.75.

Failures on toxicity or efficacy comparisons were confirmed by statistical analysis. The first statistical analysis was done by ANOVA on the log10 transform of the surviving CFU for all populations. Populations that were obviously different (i.e., the Cidex 7 and Cidex Plus for most organisms) were excluded from this analysis. If significant differences were indicated by ANOVA (p=0.05 or less), then the mean of the two populations (log10 transform) were compared by Dunnett's test (α = 0.05) (42). If q' (the critical value in the Dunnett's test) did not exceed the critical value for α = 0.05, then the comparison passed the statistical test. It should be noted that these were employed as one-tailed tests of significance. If the NT or NE ratio was greater than 1.0, then no statistical tests were performed and the comparison was defined to be acceptable.

Results

Neutralizer toxicity

Evaluation of NT was performed by comparison between the viability population and the neutralizer exposed population. NT ratios were determined for all neutral-

Table 4. Toxicity of neutralizing broths. ***Neutralizer toxicity was determined for the various neutralizing broths as the percent ratio of recoverable microorganisms from the "minus biocide" population and the "viability" population. Failures were initially identified as a recovery ratio of less than 0.75 and confirmed by ANOVA analysis. See text for details.***

	AOAC	DEB	LET	NIH	TAT	TPL
B. subtilis	1.02	1.25	0.64	1.36	1.01	0.79
E. coli	0.92	0.80	0.98	0.99	0.59	0.59
M. bovis	0.67	0.98	1.01	0.79	0.68	0.83
P. aeruginosa	0.87	0.76	0.84	0.79	0.93	0.78
S. choleraesuis	0.81	0.84	0.74	0.85	0.81	0.79
S. aureus	0.97	0.89	0.99	1.03	0.90	0.93
T. mentagrophytes	1.04	0.97	1.02	0.93	0.97	1.02

izer-organism combinations. These results are shown in Table 4. Most neutralizers examined showed toxicity against at least one index organism by these analyses, with the exception of Dey-Engley Broth and NIH Thioglycollate Media. AOAC broth was toxic to *M. bovis*, and LET was toxic to *B. subtilis* and *S. choleraesuis*. TAT demonstrated toxicity toward both *E. coli* and *M. bovis*, while TPL was toxic to *E. coli.*

Several organisms were robust to the different neutralizers. Recovery of *P. aeruginosa, S. aureus*, and *T. mentagrophytes* was not affected significantly by incubation in the different neutralizers. Other organisms, notably *B. subtilis, E. coli,* and *M. bovis*, show marked toxic responses to different neutralizing broths. It is important, therefore, to determine NT separately for each index organism and neutralizer combination. Data showing lack of toxicity against one microorganism is not sufficient evidence for the benign nature of a neutralizing solution for all organisms.

Neutralizer efficacy

Determination of NE requires evaluation of the neutralizing broth's ability to neutralize the biocide at a specified dilution. We evaluated NE by the comparison between the neutralizer exposed population to the neutralizer plus biocide population. Tables 5a-5f provide the results of this comparison. Acceptable neutralization is defined as whenever a NE ratio of ≥ 0.75 is obtained, or when the populations are indistinguishable by Dunnett's test. An acceptance criterion of 0.75 (or 75% recovery) in the NT and NE ratios proved to be a useful presumptive test. A total of 588 comparisons were performed in this study. Of these, 244 failed to meet the presumptive test. Only 5 of the 244 failures were not supported by the ANOVA and Dunnett's analysis (2% false negative rate).

AOAC and NIH thioglycollate broths performed dismally as neutralizing broths at a 1:10 dilution for all challenge organisms. DEB, LET, TAT, and TPL provided broad-range neutralization by this analysis. TAT may not be the best choice for a neutralizer, however, due to its high inherent toxicity.

No neutralizing broth was suitable for all organisms and all biocides. For example, TAT was the only effective neutralizer for *B. subtilis* with Staphene, but it was not effective as a neutralizing broth for *B. subtilis* with the Lysol products or with the Cidex products.

Different organisms behaved differently in the NE evaluation. *T. mentagrophytes* provided evidence for effective biocide neutralization with virtually all broths and biocides tested. It proved to be much more difficult to adequately neutralize the biocides when tested by the bacteria. In ad-

Table 5. Efficacy of neutralizing broths. ***Neutralizer efficacy was determined for the various neutralizing broths as the percent ratio of recoverable microorganisms from the "plus biocide" population and the "minus biocide" population. Failures were initially identified as a recovery ratio of less than 0.75 and confirmed by ANOVA analysis. See text for details. †Mean was not statistically different from control value at α=0.05 by Dunnett testing.***

Table 5a. Neutralizer efficacy (AOAC).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	0.09	0.87	0.27	0.07	0.01	0.86	0.83
Amerse	0.04	0.01	0.01	0.02	0.01	0.02	1.00
Amphyll	0.06	0.02	0.28	0.02	0.01	0.02	0.82
Cidex Plus	0.04	0.01	0.01	0.02	0.01	0.02	0.98
Cidex 7	0.04	0.01	0.67†	0.02	0.01	0.02	0.68†
Clorox	1.61	0.82	0.00	0.97	0.83	0.92	0.00
Lysol Deodorant Cleaner	0.00	0.00	0.00	1.35	0.00	0.00	0.80
Lysol Pine Action	0.00	0.00	1.41	0.84	0.99	0.39	0.91
Lysol Disinfectant Spray	0.00	0.00	0.62†	0.79	0.00	0.00	0.93
Real Pine	0.00	0.00	1.52	0.86	0.89	0.00	0.63
Rocall II	0.03	0.02	0.52	0.21	0.01	0.02	0.60
Sporocidin	0.04	0.42	0.69	0.82	0.90	0.03	0.61
Staphene	0.03	0.02	0.28	0.04	0.01	0.02	0.58

Table 5b. Neutralizer efficacy (DEB).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	1.39	1.04	1.05	1.20	0.81	0.97	1.50
Amerse	0.71	1.05	0.82	1.22	0.89	0.98	1.41
Amphyll	0.86	0.97	0.84	1.02	1.01	1.07	1.02
Cidex Plus	0.04	0.02	0.69	0.02	0.01	0.02	0.95
Cidex 7	0.06	0.02	0.33	0.02	0.01	0.02	2.05
Clorox	1.32	0.83	0.00	0.90	0.62	1.15	1.00
Lysol Deodorant Cleaner	0.95	1.09	0.00	1.25	0.65	0.98	0.64
Lysol Pine Action	0.49	0.91	0.77	1.01	0.72	1.12	0.97
Lysol Disinfectant Spray	1.29	1.09	0.00	1.38	0.92	1.10	0.81
Real Pine	1.59	1.05	0.00	1.19	0.85	0.88	0.53
Rocall II	1.38	0.86	0.75	1.25	1.06	0.83	1.04
Sporocidin	0.23	1.06	1.07	1.25	0.77	0.89	1.12
Staphene	0.04	1.19	0.83	1.20	0.91	0.94	1.06

Table 5c. Neutralizer efficacy (LET).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	0.13	0.55	1.03	0.91	0.02	0.92	0.82
Amerse	1.69	0.88	0.83	1.20	1.11	0.94	0.81
Amphyll	1.84	0.83	0.69	1.07	1.02	0.98	1.08
Cidex Plus	0.05	0.01	0.40	0.03	0.02	0.02	0.66
Cidex 7	0.07	0.01	0.92	0.02	0.02	0.02	1.14
Clorox	1.12	0.98	0.00	0.86	1.16	1.22	1.23
Lysol Deodorant Cleaner	1.07	0.99	0.00	0.87	1.02	0.86	1.19
Lysol Pine Action	1.06	0.95	0.46	0.89	0.94	1.22	0.00
Lysol Disinfectant Spray	1.09	0.92	0.00	0.89	0.91	1.35	0.96
Real Pine	1.05	0.92	0.00	0.92	0.95	0.71	1.05
Rocall II	1.79	0.83	0.90	1.26	1.12	0.28	0.71
Sporocidin	1.66	0.63	1.03	1.02	1.02	0.71	0.78
Staphene	0.08	0.82	0.49	1.14	1.07	0.38	0.77

Table 5d. Neutralizer efficacy (NIH).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	0.36	1.09	1.14	0.88	0.75	0.76	0.98
Amerse	0.04	0.01	0.04	0.03	0.02	0.02	0.75
Amphyll	0.04	0.01	0.01	0.02	0.01	0.03	0.92
Cidex Plus	0.03	0.01	0.01	0.02	0.01	0.02	0.81
Cidex 7	0.03	0.01	1.07	0.02	0.01	0.02	0.86
Clorox	1.50	0.75	1.50	1.24	0.88	1.13	0.90
Lysol Deodorant Cleaner	0.00	0.00	1.69	0.52	0.00	0.00	1.06
Lysol Pine Action	0.00	0.00	0.72	0.98	0.69[†]	0.88	1.02
Lysol Disinfectant Spray	0.00	0.00	0.00	0.84	0.00	0.00	1.04
Real Pine	0.00	0.00	1.21	0.88	0.63	0.05	0.98
Rocall II	0.03	0.01	0.74	0.02	0.01	0.02	0.59
Sporocidin	0.02	0.01	0.12	0.03	0.01	0.02	0.29
Staphene	0.03	0.86	1.11	0.91	0.80	0.02	0.73

Table 5e. Neutralizer efficacy (TAT).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	0.92	1.39	0.74	0.87	0.97	1.00	1.08
Amerse	1.10	1.40	0.28	1.15	1.40	0.82	1.16
Amphyll	1.23	1.67	0.19	1.07	1.17	0.99	1.13
Cidex Plus	0.02	0.02	0.01	0.02	0.02	0.02	1.07
Cidex 7	0.05	0.02	1.23	0.02	0.02	0.02	1.09
Clorox	1.30	0.80	0.00	0.77	0.99	1.43	1.23
Lysol Deodorant Cleaner	1.12	1.05	0.00	0.69	0.97	1.38	1.02
Lysol Pine Action	1.18	0.97	0.00	0.76	0.98	1.20	1.12
Lysol Disinfectant Spray	1.23	1.10	0.00	0.50	0.97	1.41	1.14
Real Pine	1.20	0.77	0.00	0.55	0.64	1.09	1.00
Rocall II	1.65	1.47	0.44	1.12	1.09	0.87	1.02
Sporocidin	1.66	1.64	1.03	1.02	1.10	1.00	1.18
Staphene	0.84	1.38	0.22	1.21	1.03	0.95	0.86

Table 5f. Neutralizer efficacy (TPL).

	B. subtilis	*E. coli*	*M. bovis*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*	*T. mentagrophytes*
Alcide	1.08	1.46	1.27	0.89	0.92	0.36[†]	0.88
Amerse	1.07	1.60	1.03	1.32	1.03	0.79	0.65
Amphyll	1.06	1.49	0.74	1.27	0.99	0.90	0.84
Cidex Plus	0.01	0.02	0.79	0.02	0.02	0.02	1.02
Cidex 7	0.01	0.03	1.16	0.03	0.02	0.02	0.53
Clorox	1.25	0.93	0.00	0.89	0.84	1.22	0.52
Lysol Deodorant Cleaner	0.99	0.89	0.00	1.29	0.61	1.03	0.85
Lysol Pine Action	1.31	1.02	0.00	0.68	0.95	1.18	0.95
Lysol Disinfectant Spray	1.10	0.94	0.00	1.08	0.89	1.31	0.65
Real Pine	0.54	0.94	0.57	0.92	0.90	0.97	0.64
Rocall II	0.95	1.48	1.20	1.23	1.05	0.25	0.71
Sporocidin	1.06	1.14	1.28	0.99	0.81	0.83	0.71
Staphene	0.63	1.40	1.02	1.23	1.00	0.30	0.71

dition, the bacteria showed differential responses among the different biocides and neutralizing broths.

Discussion

Effective neutralization of a chemical biocide is critically important to the quality of the data derived from any assay of biocidal efficacy (43). Care must be taken to avoid carry-over of active biocide to the recovery media, which may result in biostasis of the organism. This biostasis would lead to an overestimation of the biocide's efficacy. Therefore, the experimental design used to establish the efficacy of biocide neutralization has a major impact on the estimation of antimicrobial efficacy.

We studied several recommended neutralizing broths cited in the literature. DEB (37, 44) was formulated to neutralize a wide range of biocidal agents (compare ingredients as listed in Table 2 with Table 1). The neutralizer efficacy of the formulation was originally demonstrated with *S. aureus* (37), then later with a variety of microorganisms (38, 40). Letheen broth is effective in recovering bacteria exposed to quaternary ammonium compounds and biguanides (5, 45). The thioglycollate medium has been shown effective against mercurials (5). Other recommended neutralization (or dilution) broths include TAT (46), AOAC Disinfectant Neutralization Solution (47), Fluid Casein Digest-Soy with Lecithin and Polysorbate (TPL - 49), and NIH Thioglycollate Medium (49).

Two separate characteristics must be assessed in an evaluation of a potential neutralizer or neutralization procedure. The first is the toxicity of the neutralizer. The

second characteristic is the efficacy of that chemical or treatment in neutralizing the target biocide for the challenge organism of interest. The neutralizer toxicity is an important consideration as growth inhibition introduced by the intended neutralizer will affect the recovery of microorganisms. Similarly, if only the efficacy of the neutralizer is measured, little is learned by a failure in the study. This failure could be due either to inadequate neutralization, or due to toxicity of the neutralizer confounding the results of the study.

The determination of NT and of NE should be a comparison between a test and a control population. We determined NT as the ratio of recovery between a viability population, and a population exposed to the neutralizer. This comparison directly examined the toxicity of the individual neutralizing media for the different microorganisms. The efficacy of a particular neutralizer was defined as the ratio of recovery between the neutralizer and the biocide, and the neutralizer exposed populations. Therefore, only the affect of the biocide in the system was measured. These ratios allowed for a threshold value (0.75) as the first test. The second test was a statistical one to confirm failures. This two-tiered acceptance criterion ensured against the inadvertent rejection of an effective, non-toxic neutralizer.

The acceptance criterion of 0.75 (75% recovery) was used in the presumptive test for both the NT and the NE comparisons. It was chosen based on preliminary work, and confirmed in this study. An alternate acceptance criterion of 0.5 (50% recovery) was considered as this has been suggested as a suitable criteria (50), but this was rejected as too permissive. Expansion of the acceptance range from 0.74 to 0.50 would have included an additional 57 comparisons. While this would have captured 4 of the 5 false negatives seen, it would have also included 53 comparisons whose differences were statistically significant (true failures). We therefore believe that a ratio of 0.75 (75% recovery) is an appropriate criterion for neutralizer evaluations.

Determining the efficacy of a neutralizing solution must be done operationally. The same neutralizer may effectively inhibit a particular biocide against one index organism while not effectively protecting a second. An example is provided in the analysis of the biocide neutralization afforded by AOAC broth. Although relatively non-toxic as a suspension broth, it fails to neutralize most biocides. However, if the fungal organism *T. mentagrophytes* had been the only index organism utilized, then a false estimation of the efficacy of AOAC would have been unavoidable. The neutralizer efficacy of the entire test should be determined using all organisms of the test.

Finally, a neutralizer evaluation must be conducted under conditions that mimic the most stringent conditions of the biocidal experiment. The neutralization procedure must be examined, not just the solution. A critical concern to this point is the dilution ratio of biocide:neutralizer. We employed a 1:10 dilution, as in most kinetic studies, the number of survivors are quantified with time by plating serial 10-fold dilutions. Therefore, the most concentrated biocide would be present in the initial 10^{-1} dilution tube. No neutralizing broth adequately inhibited the biocidal activity of Cidex 7 or Cidex Plus at this dilution. Greater dilution, or filtration coupled with neutralization, should be employed to perform a biocidal study in this instance. Other types of biocidal tests may require greater dilution ratios or recovery conditions. For example, the Use-dilution test may require a different dilution ratio, as biocidal retention on the carrier is low. It may also require recovery in the neutralization broth, not performed in the current study.

We found the Dey-Engley neutralizing broth to be the best overall neutralizing broth. It offered no evidence of toxicity in our hands, and displayed effective neutralization against most biocides for most of the organisms. However, even the Dey-Engley neutralizing broth was ineffective against several biocide-organism combinations, and completely ineffective against Cidex Plus and Cidex 7 in a 1:10 dilution. Higher dilutions of the biocides into Dey-Engley broth may, however, be adequately neutralized.

It is important to realize that this design for neutralizer evaluations provides no information on the recovery of organisms sub-lethally injured by exposure to the biocidal agent. These crippled organisms may have specific nutritional requirements for survival and repair, or may be more sensitive to toxic component of the neutralizer. The procedure described by Dey and Engley provides a useful technique to compare the ability to recover injured organisms among several effective neutralizers.

Acknowledgements

The authors thank Dr. Joseph Ascenzi for supplying the *M. bovis* used in this study. The expert technical assistance of Robert Manchester and John Barber is appreciated. This study was supported by a cooperative agreement (#CR81753-01-0) with the Environmental Protection Agency.

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