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Development and Validation of a Neutralizer System for In Vitro Evaluation of Some Antiseptics

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A neutralizer system was developed and validated for use in the in vitro bactericidal evaluation of three commonly used antiseptics, namely, Hibiclens (4% [wt/vol] chlorhexidine gluconate), Betadine (7.5% [wt/vol] povidone-iodine), and pHisoHex (3% [wt/vol] hexachlorophene). The neutralizer finally selected after a screening of 12 potential candidates consisted of 3% Asolectin, 10% Tween 80, and 0.3% sodium thiosulfate in diluent, and 0.3% Asolectin, 1% Tween 80, and 0.3% sodium thiosulfate in the recovery agar. This neutralizer system was tested and validated for its neutralizing capacity for the three antiseptics, as well as for its lack of inherent bactericidal action against *Staphylococcus aureus* and a number of gram-negative bacteria of clinical significance. With no more than a 10-fold dilution of the antiseptic, the selected neutralizer system was 100% effective in neutralizing all the bacteriostatic carry-over of the three antiseptics and was also completely without any inherent bactericidal action against all the test organisms used. Sodium sulfite (considered to be a potential inactivator for iodophores such as Betadine), even in concentrations as low as 0.1%, was found to be ineffective or inherently bactericidal, whereas 0.3% sodium thiosulfate, in combination with Asolectin and Tween 80, was adequate (effective as well as non-bactericidal) and was considered to be essential for the neutralization of the three test antiseptics, namely, Hibiclens, Betadine, and pHisoHex.

Chemical agents commonly known as inactivators or neutralizers are often used for (i) the bactericidal evaluation of antimicrobial agents, antiseptics, and disinfectants; (ii) the evaluation of preservative efficacy in many pharmaceuticals, toiletries, and cosmetic products; and (iii) the microbial limit testing of products containing antimicrobial agents. The need for an adequate neutralizer in some of these applications is well documented and rightfully stressed (2-4, 11-14). The selected neutralizer should not only be able to completely inactivate all of the bacteriostatic activity of the residual antimicrobial agent likely to be carried over into recovery media, but also be inherently non-bactericidal to the test organisms (4).

As part of an extensive study recently completed in our laboratory on the comparison of three commercially available antiseptic detergent preparations, Hibiclens Surgical Scrub, Betadine Surgical Scrub, and pHisoHex, a neutralizer system was developed, and its adequacy was established. The main objective of the present study was to develop one neutralizer system that could be used effectively and uniformly for the in vitro bactericidal evaluation of the three test antiseptics.

MATERIALS AND METHODS

Test organisms. Recent clinical isolates of the following bacteria, obtained from Wilmington Medical Center, Wilmington, Del., were used: *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus rettgeri, Proteus mirabilis, Escherichia coli,* and *Serratia marcescens.* In addition, one water isolate, a *Pseudomonas* species, was also included. FDA agar (BBL Microbiology Systems, Cockeysville, Md.) slopes in Roux culture bottles were inoculated with 24- to 48-h FDA broth (BBL) cultures of the test organisms. After a 24- to 48-h incubation at 35°C, the growth from the slopes was carefully washed off with sterile 0.85% saline and centrifuged at 30,000 rpm for 10 min. The supernatant was removed, and the sediment was suspended in 5 ml of sterile 0.85% saline. This represented the stock suspension. Viable counts were performed on these stock bacterial suspensions by standard surface plate-counting technique employing FDA agar. The plates were incubated at 35°C up to 72 h before counting. Stock suspensions were kept at 4°C up to the 4-week duration of the present study.

Test antiseptics. The following antiseptics were used: Betadine, lot 7517077 from the Perdue Frederick Co., Norwalk, Conn., povidone-iodine surgical scrub solution containing 7.5% (wt/vol) povidone-iodine (0.75% [wt/vol] titratable iodine as determined by the method described in the *United States Pharmacopeia* XX for povidone-iodine [14]); pHisoHex, lot no. 0560-B from Winthrop Laboratories, Div. Sterling Drug, Inc., New York, N.Y., surgical scrub emulsion containing 3% (wt/vol) hexachlorophene; Hibiclens, lot no. PD27-21-11H from Stuart Pharmaceuticals, Division of ICI Americas, Wilmington, Del., surgical scrub solution containing 4% (wt/vol) chlorhexidine digluconate.

Procedure for evaluating the adequacy of neutralizer. Previously, we developed a neutralizer system for the in vitro bactericidal evaluation of Hibiclens (to be published soon). This system consisted of 3% (wt/vol) Asolectin (95% purified soy phosphatides—Associated Concentrates Inc., Long Island, N.Y.) and 10% (wt/vol) Tween 80 (ICI Americas Inc., Wilmington, Del.) in diluent (0.1% [wt/vol] aqueous Thiotone peptone [BBL Microbiology Systems], pH 7.2 ± 0.1) and one-tenth levels of the same ingredients in a recovery agar medium. Based on this previously developed neutralizer system, which was found adequate for Hibiclens and pHisoHex but not for Betadine, 12 potential neutralizer systems containing Asolectin and Tween 80 combined with various levels of sodium sulfite (SS) or sodium thiosulfate (STS) were investigated in the preliminary evaluation (Table 1). Experiments were conducted to investigate and compare the recovery of *S. aureus* in the presence, as well as in the absence, of Hibiclens, pHisoHex, and Betadine to select the most promising neutralizer system. This was further evaluated for its satisfactory neutralizing capacity, as well as for its lack of inherent antibacterial action against *P. aeruginosa* and other test organisms.

The procedure used for determining neutralizing capacity, briefly outlined in Fig. 1, consisted of: (i) equilibrating at 23°C duplicate 9-ml tubes of diluent containing appropriate test neutralizer, pH 7.2 ± 0.1, (ii) transferring into it and mixing 1 ml of the test antiseptic and equilibrating for 5 min, (iii) inoculating with 0.1 ml (containing 1×10^4 to 3×10^4 viable cells) of a suitable dilution of the stock suspension of the test organism so as to give 1×10^3 to 3×10^3 viable cells per ml in the reaction mixture, (iv) mixing and withdrawing from the reaction mixture 0.1-ml samples at 10- and 60-min intervals to prepare duplicate surface plates with FDA agar containing appropriate test neutralizer. The plates were incubated at 35 ± 1°C up to 72 h, and mean plate counts were determined. Appropriate neutralizer controls (no neutralizer), antiseptic controls (no antiseptic), or neutralizer-antiseptic controls (no neutralizer, no antiseptic) were included in each series of experiments. Antiseptic controls were included only in the final experiments with the most promising neutralizer system, i.e., no. 12 (see Tables 3 and 4).

The neutralizer present in diluent used in all 12 test systems consisted of 3% Asolectin, 10% Tween 80, and 0.1 to 0.3% SS or STS. In half of these systems, neutralizer was also incorporated in the recovery agar and consisted of 0.3% Asolectin, 1% Tween 80, and 0.1 to 0.3% SS or STS. In any given system, however, the concentration of SS or STS, both in diluent and recovery agar, was kept the same. For example, if 0.2% SS was used in diluent, the same level of SS was maintained in the recovery agar (Table 1).

The procedure for determining the inherent bactericidal action, if any, of the test neutralizer system to the test organism was similar to that used for determining the neutralizing capacity, except that no antiseptic was used (Fig. 2). Ten milliliters of diluent containing the test neutralizer and equilibrated at 23°C was inoculated with 0.1 ml of the test suspension

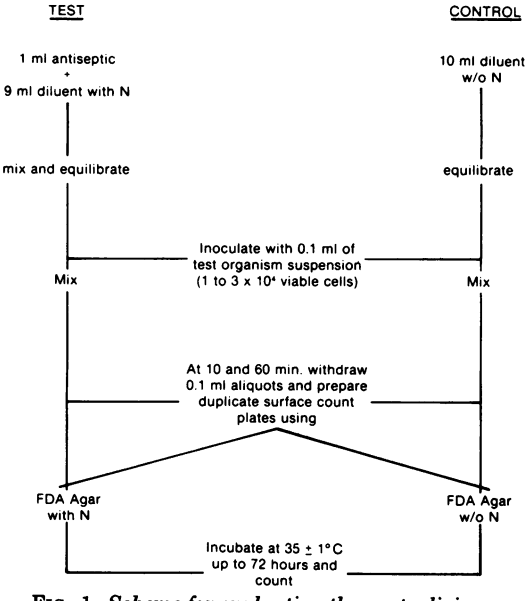

FIG. 1. *Scheme for evaluating the neutralizing capacity of a test neutralizer. N, Test neutralizer; w/o N, without neutralizer.*

TABLE 1. *Neutralizer systems investigated*[a]

Neutralizer system no.	Diluent (0.1% aqueous Thiotone peptone [BBL]) containing:	Plate count agar (FDA agar [BBL]) containing:
1.	3% Asolectin + 10% Tween 80 + 0.1% SS	Nil neutralizer
2.	3% Asolectin + 10% Tween 80 + 0.2% SS	Nil neutralizer
3.	3% Asolectin + 10% Tween 80 + 0.3% SS	Nil neutralizer
4.	3% Asolectin + 10% Tween 80 + 0.1% STS	Nil neutralizer
5.	3% Asolectin + 10% Tween 80 + 0.2% STS	Nil neutralizer
6.	3% Asolectin + 10% Tween 80 + 0.3% STS	Nil neutralizer
7.	3% Asolectin + 10% Tween 80 + 0.1% SS	0.3 Asolectin + 1% Tween 80 + 0.1% SS
8.	3% Asolectin + 10% Tween 80 + 0.2% SS	0.3 Asolectin + 1% Tween 80 + 0.2% SS
9.	3% Asolectin + 10% Tween 80 + 0.3% SS	0.3 Asolectin + 1% Tween 80 + 0.3% SS
10.	3% Asolectin + 10% Tween 80 + 0.1% STS	0.3 Asolectin + 1% Tween 80 + 0.1% STS
11.	3% Asolectin + 10% Tween 80 + 0.2% STS	0.3 Asolectin + 1% Tween 80 + 0.2% STS
12.	3% Asolectin + 10% Tween 80 + 0.3% STS	0.3 Asolectin + 1% Tween 80 + 0.3% STS

[a] Neutralizer control consisted of diluent and FDA agar without neutralizer.

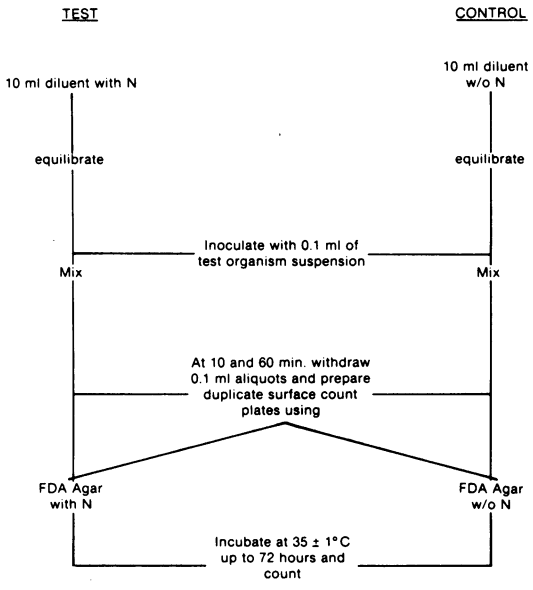

FIG. 2. *Scheme for evaluating the potential toxicity to test organisms of a neutralizer. N, Test neutralizer; w/o N, without neutralizer.*

so as to give 0.5×10^4 to 3×10^4 viable cells per ml in reaction mixture. After 10 and 60 min, 0.1-ml samples were withdrawn and surface plated, in duplicate, with FDA agar containing appropriate test neutralizer. The plates were incubated, and mean colony counts were computed. The controls in these experiments consisted of recovering the test organisms by the above procedure but without any neutralizer in the diluent or in the agar. The same procedure was used with all the test organisms employed in this study.

RESULTS

Attempts to recover viable *S. aureus* from plain diluent (no neutralizer) in the presence of one-tenth dilution of Hibiclens, Betadine, or pHisoHex, employing plain recovery agar (no neutralizer) failed. This indicated bacteriostatic carry-over of the antiseptics and hence the need to use a suitable neutralizer (Table 1). The results of the preliminary experiments on the 12 test neutralizer systems revealed that the recovery of *S. aureus* was significantly improved whenever STS (and not SS), in combination with Asolectin and Tween 80, was used both in diluent and in the recovery agar (Table 2). Based on these results, three neutralizer systems, no. 10, 11, and 12 containing 0.1, 0.2, and 0.3% STS, respectively (along with Asolectin and Tween 80), were further evaluated using *P. aeruginosa*. The experiments with *S. aureus* using fresh suspension were also repeated to confirm the previous results. The neutralizer system no. 12, consisting of 0.3% STS in combination with Asolectin and Tween 80, was found to be the best, allowing full recovery of the test organisms in the presence of the three antiseptics (Table 3).

The validity of selecting this neutralizer system for use in the comparative bactericidal evaluation of Hibiclens, Betadine, and pHisoHex was established by determining its neutralizing capacity, as well as its inherent bactericidal activity, employing a panel of gram-negative test bacteria which included *K. pneumoniae, P. rettgeri, E. coli, S. marcescens*, and *Pseudomonas* species. Indeed, the neutralizer system no. 12 was able to neutralize all the bacteriostatic carry-over of the three antiseptics, allowing full recovery of all the test organisms (Table 4). Further, when the test organisms were allowed to be in contact with the diluent containing the above neutralizer (no. 12) without the presence of an antiseptic, full recovery of the test organisms was again obtained, indicating a lack of inherent bactericidal action to the test organisms of the neutralizer system (Table 5).

An increase in contact time from 10 to 60 min between the test organisms and neutralizer no. 12 in the diluent, with or without the presence of an antiseptic, had no effect on the recovery of the organisms (Tables 2–5). This ensures the adequacy of results when samples from diluent must be delayed for as long as 30 and sometimes even 60 min before transfer into a recovery medium, an unavoidable occurrence during some bactericidal evaluation procedures.

When neutralizer systems no. 1 to 3 and 7 to 9, in which SS (0.1 to 0.3%) combined with Asolectin and Tween 80 were used in diluent, or in both diluent and recovery agar, little or no recovery of *S. aureus* was obtained in the presence of Betadine. This indicates the lack of neutralizing capacity of these systems for Betadine (Table 2). Further, as the concentration of SS was increased from 0.1 to 0.3%, a reduction in the recovery of *S. aureus* occurred, indicating inherent bactericidal action of SS toward the test organism. The bactericidal effect of SS seemed to increase with the increase in contact time from 10 to 60 min.

DISCUSSION

It was desirable to develop a neutralizer system that could be used with all three test antiseptics, Hibiclens, Betadine, and pHisoHex. There is no known single inactivator that could be used for the neutralization of all of the three test antiseptics. Recently, a universal neutralizing medium containing a battery of different inactivators was recommended for use in the bactericidal evaluation of various types of dis-

TABLE 2. *Screening of various neutralizer systems: evaluation of neutralizing capacity for Hibiclens, Betadine, and pHisoHex using S. aureus*[a]

Test neutralizer system no.	Mean colony counts after contact times					
	Hibiclens		Betadine		pHisoHex	
	10 min	60 min	10 min	60 min	10 min	60 min
1.	NG	NG	NG	NG	232	183
2.	NG	NG	NG	NG	205	121
3.	NG	NG	NG	NG	159	33
4.	NG	NG	1	NG	222	206
5.	NG	NG	206	200	237	240
6.	NG	NG	214	223	236	225
7.	226	221	NG	NG	232	148
8.	205	194	NG	NG	258	170
9.	170	128	NG	NG	175	201
10.	234	263	72	66	244	230
11.	237	220	228	215	241	253
12.	258	239	240	252	261	255
Neutralizer control[b]	NG	NG	NG	NG	NG	NG
Neutralizer-antiseptic control[c]	—	—	—	—	—	—

[a] See Table 1, footnote *a*, for the composition of the neutralizer systems. NG, No growth.
[b] No neutralizer.
[c] No neutralizer and no antiseptic. Mean for 10 min, 258; for 60 min, 242.

infectants and antimicrobial agents (3). This growth medium was inadequate for our study, however, because the levels of Tween 80 (0.5%) and lecithin (0.7%) advocated in this medium are insufficient to completely neutralize all the bacteriostatic carry-over of Hibiclens (and possibly of pHisoHex) unless samples from the reaction mixture are diluted several 100-fold before transfer into the growth medium. Also the level of STS (0.6%) recommended in the medium for the neutralization of iodophores such as Betadine is above the level (0.5%) considered to be inherently toxic to bacteria, especially staphylococci (6–8). Therefore, the present study was undertaken to develop an adequate (effective and inherently non-bactericidal) neutralizer system for use in the bactericidal evaluation of the three test antiseptics. These two criteria were fully evaluated in the present study to establish the adequacy of the selected neutralizer system.

S. aureus was selected for use in all the preliminary experiments and also for the final evaluation of the selected neutralizer because (i) it is known to be very sensitive to the bacteriostatic action of antiseptics (1, 9) and hence is a good indicator of the adequacy of the neutralizing capacity, (ii) it is claimed to be sensitive to the action of SS or STS (6–8) and hence is a good indicator of inherent bactericidal action of these inactivators, and (iii) it is an important clinical organism, often associated with human skin and widely used in antiseptic evaluation studies. Further, the use of *S. aureus* would detect even small deficiencies in the neutralizing capacity or inherent bactericidal activity of the neutralizer. The panel of gram-negative bacteria selected for use in the study mostly represents clinical isolates from human skin and wound infection. These organisms also represented a part of the panel of test organisms which were eventually used for the comparative bactericidal evaluation of the three antiseptics (manuscript in preparation).

The results from the present study have established the satisfactory development of a neutralizer system for use in the evaluation of Hibiclens, Betadine, and pHisoHex, with minimum sacrifice in the sensitivity of the method to detect antiseptic-treated viable organisms. The selected neutralizer system was able to meet fully the two most important criteria (neutralizing capacity and the inherent lack of bactericidal action) discussed above. The neutralizer system, consisting of relatively high levels of Tween 80 (10%) and Asolectin (3%) in diluent and one-tenth of these levels in the recovery agar plus 0.3% STS used both in the diluent and in the agar, had no detectable bactericidal effect on any of the test organisms, including *S. aureus*. The fact that the recovery of *S. aureus* in the presence of 0.1 or 0.2% STS was not as good as that in the presence of 0.3% STS indirectly indicates the inadequacy of Tween 80 as the sole neutralizer for povidone-iodine (such as Betadine) recently recommended by MacKinnon (11).

There is a lack of agreement in the literature on the use of STS as a neutralizer for iodine and

TABLE 3. *Evaluation of the neutralizing capacity of some of the selected neutralizer systems for Hibiclens, Betadine, and pHisoHex employing S. aureus and P. aeruginosa*[a]

Test organism	Test neutralizer system no.	Mean colony counts ± SEM after contact times							
		Hibiclens		Betadine		pHisoHex		No antiseptic	
		10 min	60 min	10 min	60 min	10 min	60 min	10 min	60 min
S. aureus	10	110 ± 8.5	127 ± 3.9	13 ± 1.6	9 ± 1.3	128 ± 5.5	138 ± 4.2	ND	ND
	11	119 ± 5.9	122 ± 4.6	76 ± 3.5	81 ± 4.8	141 ± 5.5	127 ± 4.9	ND	ND
	12	139 ± 3.5	135 ± 5.9	130 ± 5.2	136 ± 5.6	140 ± 5.9	137 ± 6.9	128 ± 4.0[b]	121 ± 6.5[b]
	Neutralizer[c] control	NG	NG	NG	NG	NG	NG	148 ± 5.9	131 ± 4.5
P. aeruginosa	10	ND	ND	ND	ND	ND	ND	ND	ND
	11	114 ± 4.7	79 ± 3.1	92 ± 5.1	97 ± 4.1	108 ± 6.7	102 ± 5.3	ND	ND
	12	104 ± 4.7	99 ± 6.9	113 ± 6.5	102 ± 6.8	111 ± 10.5	113 ± 6.3	111 ± 8.2[c]	107 ± 5.8[b]
	Neutralizer[c] control	NG	NG	NG	NG	NG	NG	102 ± 7.1	84 ± 6.8

[a] See Table 1, footnote *a*, for the composition of the neutralizer systems. SEM, Standard error of the mean; ND, not done; NG, no growth.
[b] These data represent antiseptic controls, i.e., neutralizer present, antiseptic absent.
[c] No neutralizer.

iodophores (3, 6, 7). Concentrations of STS ranging from 0.1 to 1.0% have been recommended or used in diluents or in growth media for the neutralization of iodophores (3, 5, 8, 10). STS in concentrations of 0.25 to 0.5% or above has been shown to be inherently bactericidal against some gram-positive cocci, especially staphylococci (6–8). However, the researchers (8) showed that the inherent antibacterial action of STS was significantly reduced in the presence of Tween 80. Our results are in agreement with this finding. It is conceivable that Tween 80, being present in relatively high concentrations (above its critical micelle concentration), would tend to solubilize STS, rendering it nontoxic to the bacterial cells, but would still not affect its ability to inactivate iodine. Our results, however, are in apparent variance with others who recommend 0.1% SS as a better neutralizer than 0.5% STS for povidone-iodine (6). We attribute this apparent discrepancy to the excessive dilution of the primary diluent containing 0.1% SS, used by these authors. Such dilution would reduce the concentration of SS, as well as that of iodine, too low to be toxic or inhibitory to the test organism. Based on our results, 0.1 to 0.3% SS, even in the presence of nonionics Tween 80 and Asolectin, was unable to neutralize Betadine's bacteriostatic activity. Furthermore, an increase in contact time from 10 to 60 min between *S. aureus* and SS seemed to reduce the recovery of the organism, indicating the inherent bactericidal activity of SS for *S. aureus*.

In conclusion, the present study demonstrates the approaches and the methodology required to develop and validate a neutralizer system that can be used in the bactericidal evaluation of several antiseptics containing high levels of antimicrobial agents.

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TABLE 4. *Evaluation of the finally selected neutralizer system:[a] recovery of a panel of test organisms in the presence of Hibiclens, Betadine, and pHisoHex*

Test organism	Mean plate colony counts after contact times							
	Hibiclens		Betadine		pHisoHex		Control (no antiseptic, no neutralizer)	
	10 min	60 min	10 min	60 min	10 min	60 min	10 min	60 min
S. aureus	139	135	130	136	140	137	148	131
P. aeruginosa	104	99	113	102	111	113	102	84
K. pneumoniae	120	138	155	148	135	162	142	136
P. rettgeri	159	200	169	188	175	202	181	180
E. coli	101	92	112	113	113	108	109	103
S. marcescens	123	121	125	122	120	116	114	85
Pseudomonas species	162	169	157	175	175	185	177	195

[a] Neutralizer system no. 12 (see Table 1, footnote *a*) consisted of 3% Asolectin, 10% Tween 80, and 0.3% STS in diluent; 0.3% Asolectin, 1.0% Tween 80, and 0.3% STS in the recovery agar.

TABLE 5. *Final evaluation of the selected neutralizer system: recovery of a panel of test organisms in the absence of antiseptics*

Test organism	Mean plate colony counts after contact times			
	Neutralizer system no. 12[a]		No neutralizer used	
	10 min	60 min	10 min	60 min
S. aureus	128	148	130	146
P. aeruginosa	182	196	190	186
K. pneumoniae	265	225	232	222
P. rettgeri	220	189	212	216
E. coli	183	218	233	217
S. marcescens	219	218	217	233
Pseudomonas species	142	140	144	144

[a] See Table 1, footnote *a*, for composition.

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