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The importance of neutralization in the evaluation of triclosan-containing products

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A variety of bactericidal tests are available for evaluating the antimicrobial activity of products. An often overlooked variable in these types of studies is adequate neutralization. Triclosan is a widely used antimicrobial agent and has been shown to be difficult to neutralize. Incomplete neutralization may overestimate the efficacy of triclosan-containing products.

Keywords: neutralization; triclosan

Introduction

A variety of methods are available for evaluating the antimicrobial activity of active ingredients used in antiseptic and disinfectant products. An important but often overlooked variable in these types of studies is adequate neutralization [5]. Neutralization is essential to stop the antimicrobial activity of a test product at a particular exposure time. Incomplete neutralization allows continued antimicrobial activity and misleading interpretation of results.

Triclosan (2,4,4′trichloro-2′hydroxydiphenyl ether, Irgasan DP 300) is a halogenated bisphenol which exhibits broad-spectrum antimicrobial activity and is widely used in toiletries, soaps and cosmetics [3,6]. Neutralization methods for triclosan have been described in the literature, but in our experience, triclosan is difficult to chemically neutralize. ASTM guidelines (E 1054-91; [4]) were used to evaluate triclosan neutralization at contact times similar to actual product use and to compare time-kill results in the presence and absence of adequate neutralization.

Methods

Antiseptic products

Handwash products contained 1%, 0.3% and 0.2% triclosan.

Neutralization broths, agars and additives

C/G broth: tryptone water (1% tryptone, 0.5% NaCl; pH 7.2) with 10% Tween 80, 3% lecithin, 0.1% histidine, 0.5% sodium thiosulfate; pH 6.86. C/G agar: plate count agar with 3% Tween 80, 0.3% lecithin. B/S broth: 0.04% potassium phosphate, 1.01% sodium phosphate, 0.1% Triton X-100, 2% Tween 80, 1.2% lecithin, 0.5% sodium thiosulfate, 1% Tamol SN; pH 7.6. TTL broth: 0.1% tryptone, 0.1% proteose peptone, 5% Tween 80 and 2% lecithin. Letheen broth and agar, D/E broth and agar, brain heart infusion agar (BHA), standard methods agar with lecithin and Tween 80 (STMA). The following additives were also used at the indicated concentrations: 6% asolecithin/Tween 80 (A/T; 3.5% asolecithin ('Alcolec deoiled' American Lecithin Co, Oxford, CT, USA), 25% Tween 80), and 5% fetal bovine serum (FBS). Butterfield's buffer and plate count agar were used as non-neutralizer controls in time-kill studies.

Preparation of inocula

One-tenth milliliter of the stock cultures of *Staphylococcus aureus* ATCC 6538 or *E. coli* ATCC 8739 were inoculated onto 10 ml BHA slants and incubated overnight (18–24 h) at 30°C. Overnight growth was harvested with 10 ml of 0.85% sterile saline, to yield an inoculum of approximately 10^9 CFU per ml.

Neutralization studies

Neutralization studies [4] were performed in triplicate to identify an adequate neutralizer of the test product at 0.25 and 30 min. One milliliter of the test product or of 0.85% sterile saline control were added to 9 ml of test neutralizer broth and vortexed. Following either 15 s or 30 min contact time, 0.1 ml of the *Staphylococcus aureus* inoculum was added and vortexed. After 15 s, a 2-ml aliquot was removed; 1 ml was directly placed into a petri plate and 1 ml was diluted into 9 ml neutralizer broth. A series of four serial dilutions were performed, plating 1 ml and diluting 1 ml. Melted agar (held at ~45°C) was added to each petri plate, swirled to mix and allowed to solidify at room temperature. Plates were incubated at 30°C for 48 h. Serial dilutions were performed on the original inoculum in the presence and absence of neutralizers to confirm compatibility of the test neutralizer with test organisms [4,5]. Plates with 30–300 colonies were counted and the original counts per ml determined. All dilution plates were examined to determine if dilution of the active ingredient was important for neutralization.

Time-kill studies

Ten-milliliter samples of test product were placed into 1-ounce Nalgene© jars and inoculated with 100 μl of the test inoculum. At indicated contact times, a 1-ml sample was removed and immediately placed into 9-ml aliquots of appropriate neutralizing or dilution broth. Serial dilutions were performed in neutralizing or dilution broths and

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counts were determined by a standard pour plate method. Serial dilutions of the inoculum were similarly prepared in Butterfield's buffer to determine original inoculum (baseline) counts. Plates were incubated at 30°C for 48 h and counts determined as described above.

Results

Triclosan neutralization

The ASTM method for evaluating inactivators [4] was used to test the ability of a variety of combinations of neutralizer broths/agars (from the literature and from our own laboratories) to inactivate a 1% triclosan-containing product after 15 s and 30 min contact time. *Staphylococcus aureus* ATCC 6538 was chosen as an indicator organism, since it is particularly sensitive to triclosan. A simple calculation for the efficiency of test combinations was determined using the following formula [1]:

$$\text{Efficiency of neutralizer} = 1 - (\log \text{NC} - \log \text{ND})$$
where NC = CFUs per ml for the control (buffer)
ND = CFUs per ml for the test product

A neutralizer is considered completely effective if the efficiency is 1 and ineffective if the efficiency is lower than 0.8. Multiple combinations were tested and the most efficient are presented in Table 1. These combinations appeared to be efficient neutralizers of triclosan within 30 min, based on ASTM guidelines; however, only three were efficient at 15 s, which more accurately reflects exposure times with handwashing products. The controls indicated that these combinations did not appear to have any detrimental effect on the test organism; an obvious reduction in growth was observed in the first, or first several dilution plates. These results indicated that antimicrobial activity had been carried over but could be diluted out; complete neutralization could only be achieved by a combination of inactivators and dilution. Of the test combinations, Letheen broth and agar with 6% A/T was found to be the most reliable, with a detection limit of <100 CFU ml^{-1}.

Time-kill studies

Time-kill studies with *E. coli* ATCC 8739 were performed to show the effect of inadequate neutralization on observed bactericidal activity of a 1% triclosan-containing product.

Table 1 Efficiency of triclosan neutralizer combinations

Neutralization broth	Neutralization agar	Efficiency[a]	
		15 s	30 min
D/E	D/E	0.6	0.9
Letheen	Letheen	0.7	0.9
Letheen + 5% FBS	Letheen + 5% FBS	0.7	1.0
Letheen + 6% A/T	Letheen + 6% A/T	1.0	1.0
C/G	C/G	1.0	1.0
B/S	C/G	0.9	1.0
TTL	STMA	0.7	1.0

[a]As described in text [1].

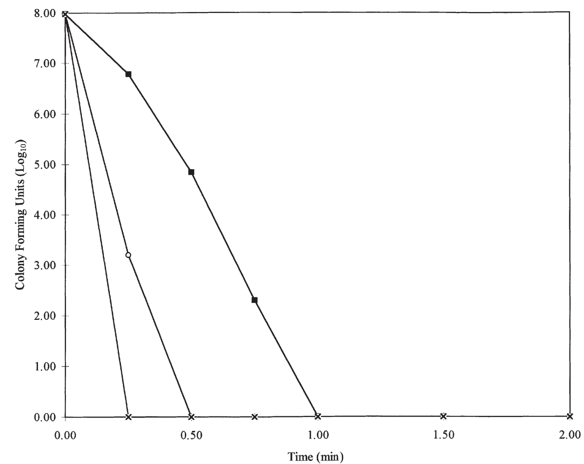

Figure 1 Effect of neutralization on the efficacy of a 1% triclosan-containing antiseptic product against *E. coli*. Adequate (-■-), inadequate (-○-) and no neutralizers (-×-) used are described in the text.

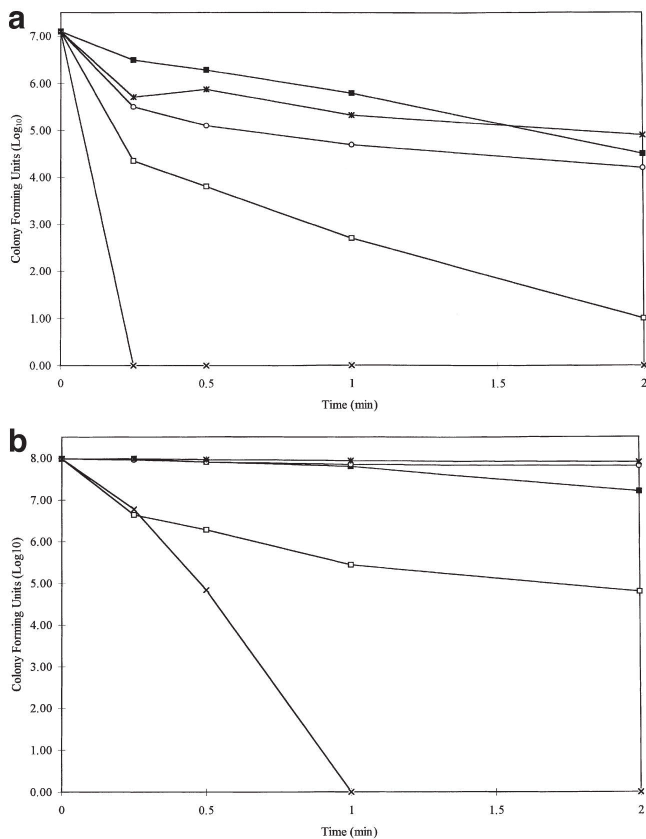

Figure 2 Time-kill studies comparing the efficacy of triclosan-containing products against *S. aureus* (a) and *E. coli* (b), with adequate neutralization. Triclosan concentrations in products were (-×-) 1%; (-■-) 1%; (-□-) 0.3%; (-○-) 0.2%; and (-✳-) 0.2%.

Log reductions were compared with no neutralization (Butterfield's buffer and plate count agar), inadequate neutralization (Letheen broth and agar) and adequate neutralization (Letheen broth and agar with 6% A/T; Figure 1). In the absence of neutralizers, complete log reduction was observed after 15 s and the efficacy of the test product was clearly overestimated with inadequate neutralization. With

adequate neutralization, an exposure time of 1 min was required to observe complete log reduction.

Further time-kill studies with adequate neutralization were performed on a number of triclosan-containing products using both *E. coli* and *S. aureus* (Figure 2). Test products had variable activity against both test organisms, independent of the concentration of triclosan. In the absence of neutralization, product efficacy could not be differentiated (results not shown).

Discussion

Formulation effects can both inactivate or potentiate the activity of triclosan and other biocides [2]. Products, even containing the same active concentration, can vary significantly in antimicrobial activity, but can be differentiated if efficiently neutralized. Triclosan is clearly a difficult biocide to neutralize chemically and many of the proposed inactivator combinations in the literature did not appear to be adequate. A number of combinations were efficient at 30 min, as required by the ASTM guidelines, but our studies indicate that neutralization should be adequate at the expected minimum exposure time to be used in subsequent efficacy studies, in this case 15 s. In this investigation, a combination of chemical and dilutional neutralization was optimal. However, examination of all plates in a dilution series clearly showed the carry-over of activity, which restricts the detection limit of the test to <100 CFU ml^{-1}. Alternative methods, in particular filtration, may also be useful [5], but adsorption of the active to membranes and the time required for sample manipulation, particularly for soap products, can also be limiting. In conclusion, closer inspection should be made to confirm adequate neutralization for reliable representation of antiseptic and disinfectant efficacy.

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