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ORIGINAL PAPER

Recovery of Gram-Positive Cocci and *Candida albicans* from Peroxygen/Silver-Based Disinfectants

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Abstract

Neutralization method evaluation is an important first step in a disinfectant validation study program. It is also crucial in assessment of the efficiency of microbial recovery media in the presence of a residual biocidal agent. In the present study, four commercially available peroxygen/silver-based disinfectant formulae – intended to be used in a pharmaceutical facility sanitization program – were tested at two dilutions against three Gram-positive cocci and one yeast; *Staphylococcus aureus* (ATCC 6538), *Kucoria rhizophila* (ATCC 9341) and *Candida albicans* (ATCC 10231) and *Staphylococcus capitis* as an environmental monitoring (EM) isolate sample (identified by miniaturized biochemical identification system). Disinfectants preparation, dilutions and the test procedure were carried on in laboratory under conditions simulating the working environment of 20–25°C and RH% 40–60. In-house made neutralizing broth was mixed with biocidal agents to make two dilutions of each disinfectant forming Peroxygen: neutralizing broth ratios of 1:10 and 1:100 (v/v). Three populations were established and two comparison ratio groups were examined, namely neutralizer efficacy and neutralizer toxicity. Two acceptance criteria were tested. One criterion showed higher rate of neutralization success than the other. *S. aureus* showed the highest rate of successful microbial recovery from neutralization process. The Disinfectant (Bixco) was exceptionally neutralized at all dilutions with all microorganisms. In conclusion, in-house made neutralizing broth effectively neutralized all disinfectants with all the tested microorganisms at 1:100 (v/v); thus, it can be used in sanitizer validation studies and EM media.

Key words: disinfectant validation, neutralization method, peroxygen/silver, recovery media, sanitization program

Introduction

The harmonized methods in the compendia (United States Pharmacopoeia [USP], European Pharmacopoeia [EP], and Japanese Pharmacopeia [JP]) provide detailed tests for the detection of specified organisms, including *Staphylococcus aureus* and *Candida albicans*. These organisms are considered objectionable if present in certain types of products, taking into consideration their known pathogenicity as well as contamination potential during pharmaceutical manufacturing. However, the presence of other organisms that might be considered objectionable to a product or manufacturing process must not be overlooked (Clontz, 2008).

Commercial disinfectants are usually composed of a mixture of biocidal agents to provide synergistic activity against a wide of microorganisms. In one form or another, silver and its compounds have long been used as antimicrobial agents (Brown and Anderson, 1968; Russell and Hugo, 1994). The most important silver compound currently in use is silver sulfadiazine (AgSD), other examples of silver-based compounds possessing antimicrobial activities include silver metal, silver acetate, silver nitrate, and silver protein, which have been listed in Martindale, The Extra Pharmacopoeia (McDonnell and Russell, 1999).

The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl, SH) groups (Fuhrmann and Rothstein, 1968; Bragg and Rainnie, 1974; Belly and Kydd, 1982; Furr *et al.*, 1994), although other target sites remain a possibility (Richards, 1981; Thurman and Gerba, 1988). The study of Liau *et al.* (1997) demonstrated that amino acids such as cysteine and sodium thioglycollate containing thiol groups possess the ability to neutralize the activity of silver nitrate against *Pseudomonas* aeruginosa. In contrast, amino acids containing disulfide (-S-S-) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, l-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that the

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interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved.

Hydrogen peroxide (H_2O_2) is a common biocide which is widely used for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H_2O_2 is considered environmentally friendly, because it can rapidly degrade into the innocuous products; water and oxygen. H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores. In general, greater activity is seen against Gram-positive than Gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance at lower concentrations. On the other hand, higher concentrations of H_2O_2 (10 to 30%) and longer contact times are required for sporicidal activity (Russell, 1991), although this activity is significantly increased in the gaseous phase. Mechanistically, H_2O_2 acts as an oxidant by producing hydroxyl free radicals (·OH) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that the accessible sulfhydryl groups and double bonds are particularly targeted (Block, 1991).

Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than H_2O_2, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (< 0.3%) (Block, 1991). PAA also decomposes into safe by-products (acetic acid and oxygen) but has the extra advantages of being not susceptible to decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (Lensing and Oei, 1984; Malchesky, 1993). Interestingly, its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (Crow, 1992; Malchesky, 1993). Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (SH) and sulfur (SS) bonds (Baldry and Fraser, 1988; Block, 1991).

In order to ensure the validity of data derived from a test, two parameters must be checked, namely: "accuracy" and "integrity" of the output readings. Minimum and maximum ranges for accuracy of plate count readings have been addressed previously (Sutton, 2011). A measurement that is clearly questionable because of a failure in the assay procedure should be rejected, whether it is discovered during the measuring or tabulation procedure. The arbitrary rejection or retention of an apparently aberrant measurement can be a serious source of bias. Each suspected potency measurement, or outlier, may be tested against a criterion which is based on the variation within a single group of supposedly equivalent measurements from a normal distribution (Pharmacopeial Forum Number 34, 2010).

This study aimed to evaluate and optimize a method of neutralizing peroxygen-based biocides with in-house made neutralizer for the recovery of environmentally and pharmaceutically important Gram-positive cocci and *C. albicans* (as an example of yeast) at two dilution levels. This aim would serve as a part of an improvement program for the detectability of selected microbial species in clean room environment in the presence of residual tested sanitizers, and to evaluate a recovery broth from disinfectants in biocidal agents' evaluation study. Such an optimized improvement program of quality could be applied in healthcare facility generally and a pharmaceutical plant specifically.

Experimental

Materials and Methods

Preparation of microbial suspension. Standard strains were purchased from the American Type of Culture Collection (ATCC; Manassas, Virginia) and handled according to standard procedure while bacterial environmental monitoring (EM) isolates were isolated and identified using miniaturized biochemical identifications kits of BBL™ Crystal™ Identification System purchased from BD (Becton Dickinson Microbiology Systems, Cockeysville, Md.). All media were purchased from OXOID (Basingstoke, Hampshire) and chemicals from Sigma-Aldrich (St. Louis, MO 63103). Table I shows the list of microorganisms used in the current study, source, family and general characteristics.

Standardized stable suspensions of test strains were used and prepared as stated by the supplier. Seed-lot culture maintenance techniques (seed-lot systems) were used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. All organisms were kept and preserved at –80°C in validated –86°C Ultra low temperature freezer (–86 Degree ULT Freezers, Qingdao, Shandong, China) in controlled cryogenic environment and reactivated only prior to conduction of the study using standard method determined by the supplier. All media were sterilized by autoclaving in steam sterilizer (FEDEGARI FOB3, Fedegari Autoclavi SpA, SS 235 km 8, 27010 Albuzzano (PV), Italy). All pH measurements and weighing procedures were done using Mettler-Toledo S20 SevenEasy™ pH Meter and XPE Analytical Balance respectively (Mettler-Toledo, LLC 1900 Polaris Parkway Columbus, OH 43240).

Suspensions were quantified by making serial dilutions and performing duplicate plate counts using conditions and media suitable for each tested microorganism

Table I
List of microorganisms challenged in neutralizer validation study with the source, family and general characteristics of them.

Challenged microorganisms	Source	Family	General characteristics
Staphylococcus aureus	ATCC6538	Staphylococcaceae	Gram-positive cocci and represent a large percentage of microorganisms isolated from environmental samples in pharmaceutical facilities
Staphylococcus capitis	EM isolate*		
Kucoria rhizophila	ATCC9341	Micrococcaceae	
Candida albicans	ATCC10231	Saccharomycetaceae	Diploid fungus (grows as yeast and filamentous cells)

* = Environmental Monitoring isolate identified mainly from air samples (active and passive) in clean area using BBL CRYSTAL GP miniaturized biochemical system kits.

to choose suspensions of concentration 300–1000 CFU/50–100 µl were chosen as working suspensions. Microbial test suspensions were used as soon as results of serial dilutions could be enumerated using digital colony counter (Digital Colony Counter Model: 361, Laxman Mahtre Rd. Navagaon, Dahisar West, Mumbai).

Neutralization validation study of peroxygen-based biocidal agents. The purpose of this study was to ensure that the assumed contact time is valid, *i.e.* the neutralizing agent can efficiently stop the action of the tested sanitizer after being mixed with it and to ensure that the neutralizing agent does not possess any inhibitory or toxic effect on microorganisms. It was suggested that two comparisons among three populations be performed. The first comparison is Neutralizer Efficacy (NE) which could 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 was Neutralizer Toxicity (NT); an aspect of neutralization that was determined by comparing survivors in the neutralizing medium without the biocide with the viability (growth) control (Sutton *et al*., 2002; Eissa *et al*., 2012).

Test solutions were freshly prepared and diluted under conditions simulating the actual usage environment of biocidal agents at the highest concentration of recommended by the manufacturer (5%; v/v). These commercial disinfectants were denoted Bixco (Hyrogen Peroxide/Ag^+), BafD 50 (Hyrogen Peroxide/Ag^+), Pury (Hyrogen Peroxide/Peroxyacetic Acid/Ag^+) and Mil (Hyrogen Peroxide/Ag^+). The peroxide concentration equivalents to H_2O_2 in the four disinfectants, was determined by conventional chemical titration method. Using neutralizing broth as a diluent 1:10 and 1:100 (v/v) dilutions of the test solution – *i.e.* disinfectant final concentrations per 10 ml of the neutralizer were 5% and 0.5% (v/v) respectively – were made at working concentration, then an aliquot of one ml of each dilution was transferred to each of a duplicate petri dishes; this was the test group. The neutralizer exposed group was prepared in parallel in the same manner as the test group but using sterile saline or buffer instead of the test solution. The viability control group was prepared using peptone water without the solutions or neutralizing broth. Organisms were prepared so that the required inoculums did not exceed 0.5–1.0% of the total volume in the tubes. In-house neutralizer of the study was double strength Fluid Thioglycollate Medium Thiosulfate (FTMT) which was supplemented with resazurine dye (redox indicator) to detect oxygen diffusion and used fresh or reheated once if there was a considerable diffusion of oxygen in the reservoir tube.

Inoculums of each used microorganisms were added to each of the above described tubes so that the final count per plate of positive control ranged between 30 to 100 CFU per plate. Then about 20 ml of the molten suitable medium at 45°C was added; allowed to solidify, then incubated at suitable temperature for 30–35°C for three days for bacteria and 20–25°C for at least three to five days for fungi in incubators (Hotpack 175 series; model 417532, Dutton Rd., Philadelphia, USA). After that, duplicate plate counts were done for the 3 groups. Negative control for each media containing the same volume of diluents or neutralizers was prepared and similarly treated to ensure the sterility of all used materials. All tests and control groups were performed in triplicate for each microorganism, disinfectant and dilution combination.

Testing of aberrant observation was done using the following formula: $G_1 = (y_2 - y_1) / (y_N - y_1)$ when N = three to seven. If G_1 exceeded the critical value of 0.987 for outlier measurements for the observed triplicate results, there was a statistical basis for omitting the outlier measurement. In this case, the test was repeated to replace the rejected values (Pharmacopeial Forum Number 34, 2010). At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of CFU recovered from the challenge product is not less than 70% of that recovered from the inoculum control (USP < 1227 >, 2014). Another criterion was selected from Sutton *et al.* (2002) for comparison which is the geometric means of results must not be less than 75%. If there was suspect results in the group,

Log_{10} transformation of recovered CFU were done then One-Way Analysis of Variance (ANOVA) was performed for transformed results groups to confirm the significance followed by Dunnett's Multiple Comparison Test at $p < 0.001$ which was used to confirm the success or failure of the test. Tukey's Multiple Comparison Test at $p < 0.001$ was used to perform comparisons between groups. Finally, all statistical analysis was performed using GraphPad Prism version 5.00.288 for Windows. Any interpretation of complex calculation and programming of equations was performed using Microsoft Excel 2007.

Results

NT study revealed that FTMT did not possess any adverse effects on the tested Gram-positive cocci and *C. albicans*, accordingly the neutralizing broth passed toxicity test at both USP and Sutton *et al.* (2002) criteria. The recovery ratio of the four microorganisms was equal or better than one and no statistical analysis was required. The currently applied procedure used to detect the presence of aberrant observations revealed that none of the results in each group was unusual and required omitting as $G_{1calc.} < G_{1tab.}$ either from NT or NE populations. This finding is illustrated in Table II in addition to the geometric recovery mean recovery ratio. Total number of samples that passed NE study was 21 and 23 per 32 for USP < 1227 > (2014) and Sutton *et al.* (2002) criteria respectively and all failing results came from 1:10 (v/v). The difference between both criteria was *S. aureus* recovery from BafD 50 and Mil 1:10 (v/v) dilutions which failed in the first and passed the second. Six results out of 32 did not show any microbial recovery from 1:10 (v/v) dilution of BafD 50, Mil and Pury with both *Staphylococcus capitis* and *C. albicans*. This finding is demonstrated in Table II and normally is evident in Fig. 2 and 4 as missing bars of the 3 previously mentioned biocidal agents. These two microorganisms were sensitive to the dilution of the 3 tested biocidal agents namely: BafD 50, Mil and Pury.

The concentration of peroxide equivalent to H_2O_2 for Bixco, BafD50, Mil and Pury was 23.0, 49.2, 49.2 and 32.8% (w/w) respectively. Table III shows that five results – all from 1:10 (v/v) – did not pass the NE tests, thus the total rate of microbial recovery success was 5 and 7 per 16 using USP<1227> (2014) and Sutton *et al.* (2002) criteria, respectively, while it was 16/16 with 1:100 (v/v) from all disinfectants in both criteria. If both acceptance criteria were taken into consideration the descending order of microbial recovery success was: *S. aureus* (14 successful recovery per 16) > *S. capitis* = *C. albicans* = *Kucoria rhizophila* (10 successful recovery per 16). However, the comparison in recovery in terms of recovered colonies in culture media regardless passing acceptance criteria or not gave the following order: *S. aureus* > *K. rhizophila* > *C. albicans* > *S. capitis*. This finding is demonstrated in Table II.

The total rate of neutralization success of the tested disinfectants was in the following decreasing order: Bixco > Pury > Mil = BafD 50. Both Tables II and III demonstrated these findings. Bar graphs of Fig. 1, 2, 3 and 4 illustrate the average recovery of Log_{10} transformed CFU with standard deviations and compared with both reference control of USP<1227> (2014) crite-

Table II
NT and NE ratios and G_1 calculated values for determination of outliers.

Organism	NT ($G_{1cal.}$)	NE ($G_{1cal.}$) of selected biocidal agents at 2 dilutions levels in FTMT neutralizer							
		Bixco		BafD 50		Mil		Pury	
		1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v
S. aureus	1.06 (0.20)	1.08 (0.97)	1.33 (0.89)	0.47* (0.62)	1.20 (0.94)	0.62* (0.88)	1.08 (0.84)	0.75* (0.45)	0.98 (0.17)
S. capitis	1.38 (0.42)	0.78 (0.82)	1.15 (0.68)	0.00	0.95 (0.70)	0.00	0.89 (0.50)	0.00	0.87 (0.86)
K. rhizophila	1.40 (0.22)	0.73* (0.13)	0.88 (0.50)	0.54‡ (0.50)	1.02 (0.75)	0.61‡ (0.11)	0.80 (0.79)	0.50‡ (0.33)	1.19 (0.97)
C. albicans	1.20 (0.32)	1.06 (0.74)	1.20 (0.75)	0.00	0.89 (0.84)	0.00	0.90 (0.78)	0.00	0.94 (0.41)

If $G_{1cal.}$ at ($P = 0.02$) < 0.987 (when N = 3) the suspect data are not aberrant results but if $G_{1cal.} \geq G_{1tab.}$ they are considered true outlier measurements.
* = Results that were subjected to One-Way ANOVA followed by Dunnett's test to confirm success or failure after being transformed to Log_{10} values and were found to pass the test at $P < 0.001$.
‡ = Results that were subjected to One-Way ANOVA followed by Dunnett's test to confirm success or failure after being transformed to Log_{10} values and were found to not pass the test at $P < 0.001$.
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. Statistical analysis was performed in all groups. Below geometric means between parentheses were G_1 computed to determine the relative gap by comparing the reference critical value with the calculated ones.

Table III
Rate of microbial recovery from the neutralization process and the ease of neutralization process of biocidal agents at the two dilutions level against the two selected reference criteria namely US Pharmacopoeia <1227> and Sutton *et al.*

Organism	Criteria of comparison	NT	Pass/Fail score according to test criteria for 4 selected Peroxygen/Ag^+-based disinfectants at 2 dilutions levels in FTMT neutralizer								Total rate of microbial recovery success
			Bixco		BafD 50		Mil		Pury		
			1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	
S. aureus	USP<1227>	+	+	+	–	+	–	+	+	+	6/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	8/8
		+	+	+	+	+	+	+	+	+	
S. capitis	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
K. rhizophila	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
C. albicans	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
Total rate of disinfectant neutralization success			4/4	4/4	0/4	4/4	0/4	4/4	1/4	4/4	21/32
			4/4	4/4	1/4	4/4	1/4	4/4	1/4	4/4	23/32

rion for acceptance. When statistical analysis was performed using One-Way ANOVA followed by Tukey's Multiple Comparison Test – to compare between different test groups was conducted at $p < 0.001$ ($F = 6.761$ – $R_{squared} = 0.765$) on data obtained from bar graphs – of all microorganisms the results showed that there is no significant impact of Bixco dilution from 1:10 to 1:100 (v/v) on microbial recovery from FTMT. None of the 1:100 (v/v) dilution of any tested disinfectant-microorganism combination differed significantly from one to another. The obvious failure (no CFU recovered) of both *S. capitis* and *C. albicans* with 1:10 (v/v) of BafD 50, Mil and Pury excluded them from any statistical analysis or comparison. *S. aureus* and *K. rhizophila* recoveries from BafD 50 and Pury 1:100 v/v respectively were significantly higher than that of *S. aureus* and *K. rhizophila* recovered from BafD 50 1:10 (v/v) and *K. rhizophila* recovered from Pury 1:10 (v/v). On the other hand, *S. aureus* recovery from Mil 1:100 (v/v) and Bixco 1:10 and 1:100 (v/v) was significantly different in recovery from that recovered from BafD 50 1:10 (v/v). While *S. aureus* recovered from BafD 50 1:10 (v/v) was

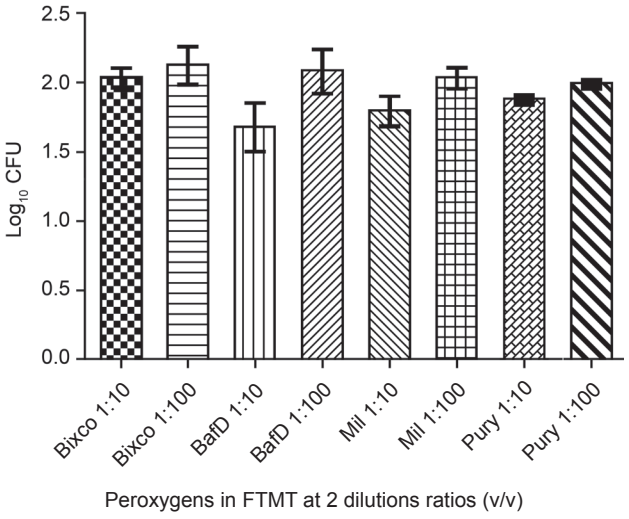

Fig. 1. *S. aureus* vs. 4 Peroxygens in FTMT. NE of FTMT on *S. aureus* (ATCC 6538) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

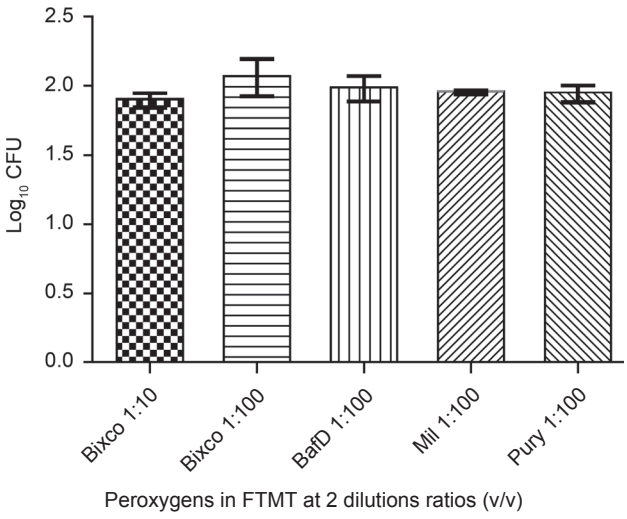

Fig. 2. *S. capitis* vs. 4 Peroxygens in FTMT. NE of FTMT on *S. capitis* (EM isolate) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

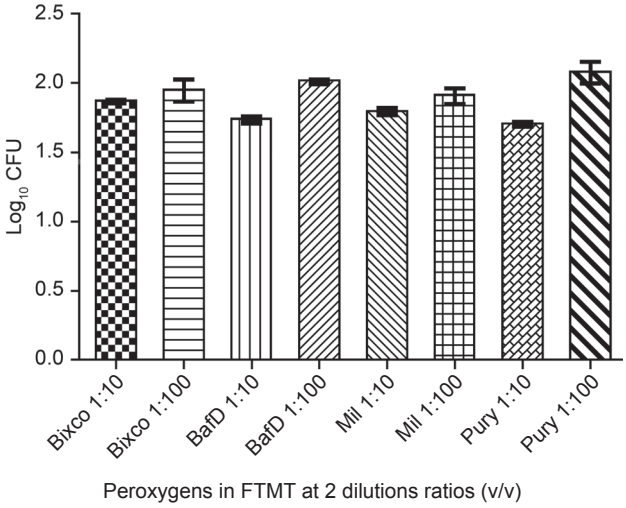

Fig. 3. *K. rhizophila* vs. 4 Peroxygens in FTMT. NE of FTMT on *K. rhizophila* (ATCC 9341) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

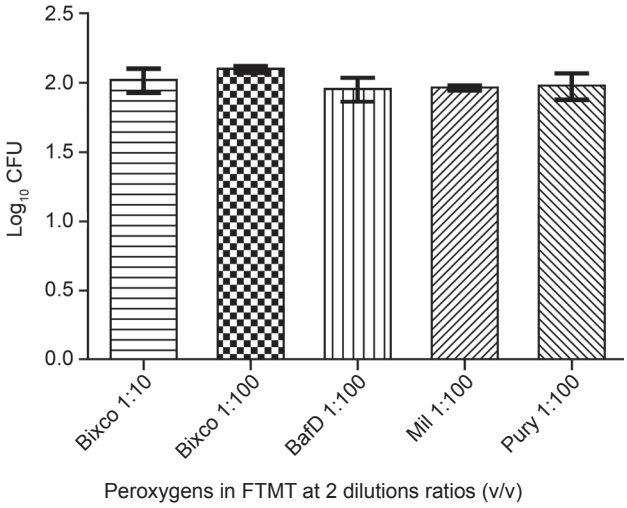

Fig. 4. *C. albicans* vs. 4 Peroxygens in FTMT. NE of FTMT on *Candida albicans* (ATCC 10231) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

significantly lower in recovery from *C. albicans* and *S. capitis* from Bixco 1:10 and 1:100 (v/v) respectively. In the same line, *K. rhizophila* recovered BafD 50 1:10 (v/v) was significantly lower than *C. albicans* and *S. aureus* from Bixco 1:10 and 1:100 (v/v) respectively. Finally, *K. rhizophila* recovery from Pury 1:10 (v/v) was considerably lower than *C. albicans* recovery from Bixco 1:10 (v/v), *S. aureus* and *S. capitis* recovered from Bixco 1:100 (v/v) and *S. aureus* from BafD 50 1:100 (v/v). Fig. 1, 2, 3 and 4 illustrate this difference.

Discussion

This study fulfilled two major aims as a part of sanitization validation program to test index microorganisms, either standard strains or selected environmental isolates, against certain commercially-available disinfectants to compare between their efficacies. The first aim was testing in-house neutralizer for suitability of recovering organisms from EM samples. As for the second aim, it was to provide proper chemical neutralization for commercial peroxygen-based disinfectants (which are usually fortified and stabilized with silver compounds) for assessment of their potency. Gram-positive cocci were chosen because they were generally found mainly in both active and passive air samples and *K. rhizophila* was found to contribute to large populations of aerially distributed microorganisms (Eissa, 2014). *S. aureus* and *C. albicans* are among objectionable microorganisms that should not be present in pharmaceutical products (USP<62>, 2014). *S. capitis* is an EM isolate and was subjected to testing in current validation program as being frequently isolated in different EM samples at critical processing points in clean area. Consequently, it could contribute to either environmental samples failure and/or compromising drug quality thus it was important to include it in risk assessment program.

Effective neutralization of a biocidal agent is critically important to the accuracy of the information obtained from any disinfectant efficacy study (Langsrud and Sundheim, 1998). The determination of NT and NE should be a comparison between a test and a control population. NT was determined 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 effect 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 success or failures (Sutton *et al*., 2002). Another criterion of comparison was USP < 1227 > (2014) of three independent replicates of recovery ≥ 70% in agar medium.

According to USP < 1072 > (2014): Biocidal activity reduction folds = (Dilution folds)$^{\eta}$, where η is the concentration exponent. Thus, the reduction of activity of hydrogen peroxide (η = 0.5) is about three to ten-fold the reduction in activity only for 1: 10 and 1:100 respectively while for $AgNO_3$ (η = 0.9 – 1) it is about eight and ten to 63 and 100. This indicates that for hydrogen peroxide, the dilution has minor effect in abolishing the antimicrobial properties although peracetic acid is neutralized effectively by dilution (Russell, 1990). It is the neutralizer capacity that played the role in the chemical neutralization as 1:100 (v/v) dilution passed the test

while all of the failure of neutralization process were from 1:10 (v/v), *i.e*. at lower dilution the neutralizer capacity was insufficient to scavenge the residual peroxides released from disinfectants. However, these values of concentration exponents were for each component alone and not in synergistic combinations. Further investigation is needed to determine if the concentration exponents are skewed in these formulae or not.

Another important consideration is the byproduct of the chemical neutralization reaction and its probable toxicity to microorganisms. Some investigators demonstrated that tetrathionate is produced from reaction of hydrogen peroxide and thiosulfate. Their work showed that at pH range six to seven (the range of neutralization in the current study) tetrathionate is produced during the course of the reaction till reaching a steady state while the thiosulfate is continuously depleted. Some researchers, also, showed that thiosulfate: tetrathionate at three-to-one ratio is inhibitory to many microorganisms at certain concentrations (Palumbo and Alford, 1970). If during the course of the reaction this ratio is attained at specific concentration, even transiently, possible toxicity to microorganisms could occur. This is a point that needs more investigation on the reaction mechanism in this neutralizer to elucidate if there is a true impact on microbial recovery or not from the residual biocidal agent declining in concentration and reaction by product accumulating.

Cystine $(SCH_2CH(NH_2)CO_2H)_2$ is the amino acid formed by the oxidation of two cysteine molecules that covalently link *via* a disulfide bonds and is cleaved rapidly at higher temperatures (Aslaksena *et al*., 2006) (such as these of media preparation and sterilization). Cystine is found in NIH Thioglycollate (NIH) in half amount of that in FTMT. Cysteine amino acid has reducing properties similar to thioglycollate (mercaptoacetate) with peroxygens. However, the autooxidation of the peroxide scavenging components in the neutralizing broth is expected on standing from the atmospheric oxygen in the head space of the reservoir tube thus neutralizing broth must be either prepared fresh, heated once directly before use and/or incorporation of thickening agent in the neutralizing broth (*eg.* small portion of agar to render the media thick but not solid) which retard atmospheric oxygen diffusion as sodium thioglycollate in the medium consumes oxygen (Madigan *et al*., 2010). The cysteine thiol group is nucleophilic, highly reactive and easily oxidized. Moreover, the reactivity is enhanced when the thiol is ionized, and has pK_a values close to neutrality, so are often in their reactive thiolate form (Bulaj *et al*., 1998). This property is more prominent in FTMT than NIH. Consequently, redox indicators such as resazurin is a good monitor for such situation to judge visually the presence and the degree of oxygen penetration into the media for either reheat or discard of.

Neutralizer toxicity study performed for FTMT used in this study revealed that it was non-toxic and could be used in the validation program. NIH investigated by other researchers gave similar outcome. NIH was non toxic or of low toxicity against microorganisms. In-house made neutralizer FTMT is in between DEB and NIH in composition. The other important subsequent aspect is NE. The combination of microorganism, neutralizer and disinfectant is unique and thus the success of one combination with one microorganism does not mean that same combination with other microorganisms will do accordingly (Sutton *et al*., 2002). This is in agreement with our findings in NE which showed different outcomes for microbial recovery from neutralization process.

Microorganisms may become sublethally injured after exposure to many chemical and physical stresses (Busta, 1976; Hurst, 1980). Injury may be measured by the difference in counts when stressed cells are simultaneously enumerated on selective and nonselective media. Only uninjured cells are recovered on selective medium, whereas the nonselective medium is assumed to recover both injured and uninjured cells (Busta, 1976; Hackney *et al*., 1979; Hurst, 1980). However, agents lethal to injured cells may be formed spontaneously in either selective or nonselective medium (Barry *et al*., 1956; Baird-Parker and Davenport, 1965; Carlsson *et al*., 1978). Media containing manganese, citrate, or both will autooxidize and form peroxides in concentrations lethal to bacterial cells stressed by ionization (Barry *et al*., 1956). Carlsson *et al*. (1978) reported the formation of superoxide radicals and hydrogen peroxide in anaerobic broth media exposed to oxygen. When thioglycollate is present in the media, H_2O_2 formation is inhibited but superoxide radicals are still formed. Supplementation of media with compounds which degrade H_2O_2 has been studied (Baird-Parker and Davenport, 1965; Martin *et al*. 1976; Rayman *et al*., 1978). On the other hand, Baird-Parker and Davenport (1965) reported that incorporation of pyruvate into selective media enhances the recovery of *S. aureus*. However, Martin *et al*. (1976) noted an improved recovery of injured *S. aureus*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Escherichia coli* when the media selective for these microorganisms are supplemented with either catalase or sodium pyruvate. Rayman *et al*. (1978) reported that the addition of pyruvate to nonselective tryptic soy agar greatly increases the recovery of heat-injured *Salmonella senftenberg*. Thus, it is recommended to include a supplement to scavenge superoxide from general non-selective media. This supplement is made of collection of wide range of "repairing compounds" to cover most types of injuries caused to a broad index of microorganisms. Then, this media are tested against standard unsupplemented media

to confirm the absence of toxicity to microbial cells. This will improve detectability of microorganisms and hence furtherly decrease the risk of getting false negative results (McDonald *et al*., 1983).

Although the commercially studied disinfectants were similar in the composition of the main active biocidal agents, the manufacturers have incorporated about 20 to 25 other constituents in their formulae that they keep confidential. Nevertheless, these components may have antimicrobial properties and thus may impact biocidal agent true activity and efficiency. Example of these components but not limited to are stabilizers, anticorrosives, surfactants, *etc*. The importance of disinfectant neutralization procedure validation generally resides on two facts: first of all it is considered important preliminary step in the determination of disinfectant true activity. The second aspect is that it could be incorporated in EM media such as contact plates and as broth for surface swabbing technique to detect low level of attenuated microbial contamination in hostile environment. A successful neutralization program is assessed by its ability to detect attenuated microorganisms – especially those having deleterious effect in the manufacturing process and product quality – in environmental samples in the presence of residual disinfectants. The last consideration is that if NE could not be estimated correctly the result will be improper biocidal agent potency determination which may be reflected in the failure of sanitization program of clean rooms and crippling microbes that may pass undetected in EM samples causing serious trouble in pharmaceutical manufacturing and other healthcare settings which impair patient health safety, cost companies huge financial loss and finally their reputability. The future plan of implementing of Rapid Microbiological Methods (RMMs) in this field is dependent to a large extent on intelligent approach of neutralization procedure so that the sensitivity of RMM instrumentation is not affected adversely. It can be concluded that in-house made neutralizing broth is a suitable candidate for neutralizing commercial peroxygen/Ag^{+}-based biocidal agents by combined chemical and dilution (at 1:100 (v/v)) inactivation to recover the tested environmentally important cocci and *C. albicans* as an objectionable yeast.

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