Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences ,Department of Entomology ,Faculty of Sciences Ain Shams University .

Microbiology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers related to the research across the whole spectrum of the subject. These including bacteriology, virology, mycology and parasitology. In addition, the journal promotes research on the impact of living organisms on their environment with emphasis on subjects such a resource, depletion, pollution, biodiversity, ecosystem…..etc

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 6(1): 17 – 27 (2014)

Egyptian Academic Journal of Biological Sciences
G. Microbiology

ISSN: 2090-0872

www.eajbs.eg.net

Study of Antimicrobial Power of Amphoteric Disinfectants of Tego Series Used in Pharmaceutical Industry

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ARTICLE INFO

Article History
Received: 25/11/2013
Accepted: 5/1/2014

Keywords:
Amphoteric
Tego Sanitization program
Carrier test
Shelf life
Bioburden.

ABSTRACT

Disinfectant validation study of 2 amphoteric disinfectants of Tego series–were introduced in sanitization program of clean room in pharmaceutical facility–has been done in order to apply them in sanitization process. Carrier test for both disinfectants was done on different surface material coupon samples representing stainless steel, wall, floor, and curtain found in classified area in the plant and compared with other non-sporicidal alcoholic disinfectant (Isopropyl alcohol 70%) after 1 and 5 minutes exposure against 3 different representative microorganisms, namely, vegetative form of *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), and *Candida albicans* (ATCC 10231). After 1 minute, results exceeded 3 log reduction but after 5 minutes no microbial recovery was detected with all biocidal agents with the four types of surfaces examined. Shelf life study of Tego disinfectants in comparison with Isopropyl alcohol 70% had been performed –in addition to zero point 7 and 18 days points were selected and storage was in normal facility conditions. Results showed that all prepared and diluted biocidal agents (to the use concentration) in this study were able to reduce microbial bioburden effectively after 18 days storage of diluted disinfectants within 1 and 5 minutes contact time. Both Tego 51 and 2000 could be used effectively in disinfection program besides Isopropyl alcohol 70% in controlled manufacturing area in drug manufacturing facility.

INTRODUCTION

Pharmaceutical products are subject to microbiological contamination that can represent a health hazard to the consumer and cause product spoilage, aesthetic changes, and possible loss of drug efficacy.

Microbial contamination may originate from the raw materials and excipients or may be introduced during manufacture (operators and contaminated equipment, environment, and packaging materials), storage, and use. Most raw materials used in pharmaceutical manufacturing, including water, may contain several types of microorganisms. Depending on the type of the manufacturing process, these contaminants may be reduced or eliminated. However, care must be taken not to further increase the potential for introducing microorganisms during an uncontrolled manufacturing process (Clontz, 2008).

As reported in the Pakistan Journal of Scientific and Industrial Research (Obuekwe *et al*., 2002), various types of tablets, both coated and noncoated, were found to be contaminated with bacteria such as *Bacillus cereus* and *Pseudomonas aeruginosa*. Fungi were also isolated from the samples tested and those included *Aspergillus flavus* and *Candida albicans*.

The relationship between surface activity and germicidal action was aroused by the important development with quaternary ammonium germicides by Domagk in 1935, and investigation of the amphoterics soon followed. A group of related amphoteric disinfectants under the trade name Tego was produced by Th. Goldschmidt AG (Schmitz, 1952, 1954). These were based on the ampholyte dodecyl-di (aminoethyl)-glycine. The composition of these products is given in Table 1. Tego 2000, the latest addition to this family, is a mixture of an amphoteric and a cationic amine surfactant (Block, 1991).

Table 1: Active components and composition of Tego series of disinfectants.

Tego	Active ingredient	Composition	pH
103S	$RNH(CH_2)_2NH(CH_2)_2NHCH_2CO_2H{\bullet}HCl$	15% aqueous solution	~ 7.7
103G	$RNH(CH_2)_2NH(CH_2)_2NHCH_2CO_2H{\bullet}HCl+[RNH(CH_2)_2]_2NCH_2CO_2H{\bullet}HCl$	10% aqueous solution	~ 7.7
51	$RNH(CH_2)_2NH(CH_2)_2NHCH_2CO_2H+ RNH(CH_2)_2NHCH_2CO_2H$	9% aqueous solution	~ 8.2
51B	$RNH(CH_2)_2NH(CH_2)_2NHCH_2CO_2H+ RNH(CH_2)_2NHCH_2CO_2H$	22.5% aqueous solution	~ 8.2
2000	$RNH(CH_2)_2NHCH_2CO_2H + RNH(CH_2)_2NH_2$	20% aqueous solution	~ 8.0

There are three main types of studies performed in this category, and all are carried out in a laboratory setting because the test methods call for challenging the chemical agents with live cultures: the AOAC Hard Surface Carrier Test Method (AOAC, 1999), Surface Challenge Tests (USP36-NF31, 2013), and Use-Dilution Tests (AOAC, 1999).

Our concern will be in the development of practical application of these disinfectants in pharmaceutical plant sanitization program so that they can be used routinely in classified area to control microbial bioburden. Tego 51 and 2000 were selected to be used in the testing and qualification procedures. This study was intended to be done in complementation with sporicidal agents validation study in order to have complete system for sanitization and disinfection program for clean room in manufacturing facility especially critical processing area that have direct impact on drug manufacturing quality.

MATERIALS AND METHODS

1. Preparation of Test Strains (Clontz, 2008)

Standardized stable suspensions of test strains were used or prepared as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

(1) Each of the bacterial test strains [*Bacillus subtilis* (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 9027)] was grown separately in containers containing casein soya bean digest broth or on

casein soya bean digest agar at 30–35°C for 18–24 h. The test strain for *Candida albicans* was allowed to grow separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20–25°C for 2–3 days.

(2) Buffered sodium chloride-peptone solution (sterile, pH 7.0, TS) or phosphate buffer (sterile pH 7.2, TS) was used to make heavy test suspensions by inoculating it with harvested microbial suspension (with sterile loop or swab).

(3) Suspensions were quantified by making serial dilutions and plate counts using conditions and media suitable for each microorganism to choose suspensions of concentration 10^6–10^7 CFU/0.1 mL as working suspensions. Microbial test suspensions should be used as soon as results of serial dilutions could be enumerated.

2. Preparation of Sanitizing Agent

(1) *Preliminary Study*: Disinfectanting agents were prepared as per production procedure and/or supplier guideline so that the test solution is made to its final dilution using USP purified water pH 5.0–7.0 from the facility distribution water system.

(2) *Antimicrobial Effectiveness Test*: Disinfectants were prepared as step (1) but during test they were diluted to 90% of the working concentration with definite volume of previously settled microbial suspension during test as a matter of challenge to account for dilution error and variability during actual situation of biocidal agent preparation.

3. Validation Method

The entire evaluation process of antimicrobial potency of biocidal agents must ensure that true efficiency of disinfectants is represented without any over or under estimation in the working environment and conditions.

3.1 Preliminary Study (Eissa *et al.*, 2012)

Purpose of this study is to ensure that the assumed contact time is valid, that is, the neutralizing agent can efficiently stop the action of the tested sanitizer after mixing with each other and at the same time the neutralizing agent should not have any inhibitory or toxic effect on microorganisms. It is suggested that two comparisons among three populations are performed. 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.

(1) Using neutralizing broth as diluent make 1:100 dilution of test solution at working concentration, then 1 mL is transferred of this dilution to each of duplicate petridishes, this is test group.

(2) Neuralizer exposed group is prepared in parallel in the same manner as test group but using sterile saline or buffer instead of test solution.

(3) Viability control group is prepared using saline or buffer without test solutions or neutralizing broth.

(4) Inoculums of range from 30 to 100 CFU of used microorganisms were added to each duplicate of 90 mm sterile petri plates.

(5) About 20 mL of molten suitable medium was added; allowed to solidify, then incubation at appropriate conditions for each microorganism.

(6) Duplicate plate count was done and used as a positive control. Negative control for each media with the same volume of diluents or neutralizers added was performed to ensure sterility of all used materials.

(7) The percentage variance (%V) will be calculated as follows:

$\%V = (A - B)/A \times 100$

A = positive control count

B = test article count.

Notes:

1- Negative percentage (%) variance will be recorded zero.
2- For NT A = Viability group. B= Neutralizer exposed group.
3- For NE A = Neutralizer exposed group. B= Biocide exposed group.

3.2. Surface Challenge Test (Clontz, 2008; USP36-NF31, 2013)

The challenge test was modified so wet application method of microorganisms to coupon surfaces was adopted as dryness will act in favor of disinfectant which may over estimate its true effectiveness.

(1) Using calibrated micropipette, 0.02 mL of one of the suspensions was added to the surface of one of the tested materials. The microbial suspension drop should not reach the edge of the material coupon. Microbial suspension was left on the surface for at least 30 seconds but should not be allowed to dry.
(2) The prepared Isopropyl alcohol 70% was added gently in 0.18 mL volume on the microbial suspension and allowed exposure on the surface for 1 and 5 minutes.
(3) The entire 0.2 mL mixture was drawn up and transferred to a sterile tube containing 19.8 mL of suitable neutralizing broth; this tube is 2×100^{-1} dilution of the original mixture.

N.B: Drawing the mixture & transferring it should take the minimum applicable time, as this time is not calculated.

(4) The mixture was homogenized well, and then 1 mL of broth was transferred to a second sterile tube containing 9 mL of neutralizing broth and vortex well. This is the tube of 10^{-3} dilution.
(5) One mL broth aliquots were transferred from tube in (4) to one pair of 9 mm sterile petridishes, and 0.1 mL broth aliquots to a second pair of plates and suitable molten agar media was added to each plate and swirled gently. These are the plates of the 10^{-3} and 10^{-4} dilutions, respectively.
(6) Step (5) was repeated using tube from (3) to generate duplicate plates of 10^{-1} and 10^{-2} dilution using 0.2 mL and 2 mL aliquots, respectively.
(7) Steps (1) through (6) were repeated using Tego 2% 51 and 2000.
(8) Steps (1) through (7) were repeated using each remaining test surfaces.
(9) Steps (1) through (8) were repeated using remaining challenge organisms.
(10) Steps (1) through (9) were repeated, except using 0.9% saline as the test solution, and use a 1 and 5 minutes exposure for each surface. These are the positive plates that provide the base line inoculum concentration.
(11) The plates were incubated at appropriate conditions for each microorganism. Plates were examined and plate counts were performed on readable plates.
(12) The count for each readable dilution level was read then multiplied the average by the reciprocal of the dilution level.
(13) The base line inoculum was determined for each organism and disinfectant that should show at least 10^4 CFU present and then calculation of log reduction of microorganisms exposed to disinfectants for each contact time from this base line.
(14) The entire test procedure was repeated after 7 and 18 days in order to establish antimicrobial effectiveness over a reasonable period of time in situation that simulated actual storage conditions: room temperature, well closed container, normal day, and florescent light.

All statistical analysis was performed by *t*-test and ANOVA using GraphPad Prism version 5.0 for Windows.

RESULTS

Both neutralizers (Fluid Thioglycolate Medium (FTM) and Buffer Lecithin Tween (BLT)) had passed NT in disinfectant validation preliminary study where both neutralizers could be used with each microorganism. However, in NE screening study FTM failed with *Bacillus subtilis* which was replaced with BLT as our second alternative to be tested and gave recovery 92.68% which passed NE test.

In disinfectant validation study, although all biocidal agents were able to reduce microbial count effectively after 5 minutes and 1 minute, that is, more than 3 log reduction, a small difference in microbial recovery was observed after 1 minute in the following decreasing order: Isopropanol 70% (8 times) > Tego 51 (6 times) > Tego 2000 (4 times).

It was found in general that *Bacillus subtilis* (representative of gram positive microorganisms) in its vegetative form showed relatively greater tolerance to the used disinfectants (recovered 12 times after 1 minute) followed by *Pseudomonas aeruginosa* (representative of gram negative microorganisms) (recovered 5 times after 1 minute) then *Candida albicans* (representative of fungi). *Candida albicans* recovered once only after 1 minute from PVC curtain coupon sample material.

Table 2: Neutralizer toxicity screening study for used disinfectants against index microorganisms.

Microorganism	Neutralizer	Agent plate count (CFU)	Control plate count (CFU)	% Variance
Bacillus subtilis	FTM[(a)]	68	50	0.00
	BLT[(b)]	50	50	0.00
Pseudomonas aeruginosa	FTM[(a)]	79	100	21.00
	BLT[(b)]	93	100	7.00
Candida albicans	FTM[(a)]	42	56	25.00
	BLT[(b)]	56	56	0.00

(a) = Fluid Thiogllycolate Medium.
(b) = Buffer Lecithin Tween.

Table 3: Neutralizer efficiency screening study for used disinfectants against index microorganisms.

Microorganism	Disinfectant[(a)]	Neutralizer	Agent plate count (CFU)	Control plate count (CFU)	% Variance
Bacillus subtilis	IPA 70%[(e)]	FTM[(b)]	81	95	14.74
Pseudomonas aeruginosa		FTM[(b)]	79	100	21.00
Candida albicans		FTM[(b)]	76	78	2.56
Bacillus subtilis[(c)]	Tego 51 1%	FTM[(b)]	5	36	86.11
		BLT[(d)]	38	41	7.32
Pseudomonas aeruginosa		FTM[(b)]	30	36	16.67
Candida albicans		FTM[(b)]	31	39	20.51
Bacillus subtilis	Tego 2000 1%	FTM[(b)]	80	95	15.79
Pseudomonas aeruginosa		FTM[(b)]	67	100	33.00
Candida albicans		FTM[(b)]	72	78	7.69

(a) = Disinfectant to the final neutralizing diluent ratio (v:v) 1:100.
(b) = Fluid Thiogllycolate Medium.
(c) = Failure of FTM in neutralizer efficacy study for *Bacillus subtilis* replaced by BLT for Tego 51, 1%.
(d) = Buffer Lecithin Tween.
(e) = Isopropyl alcohol.

In terms of surfaces, recovery of microorganisms was in the following decreasing order: Floor (vinyl) 9 times, wall (epoxy-coated gypsum) 6 times, curtain (PVC) 3 times, and finally stainless steel (316L) no recovery at all. All recovered bacteria were after 1 minute only and non after 5 minutes.

After storage of Isopropanol 70%, Tego 51 and Tego 2000 for 7 and 18 days they were able to reduce all representative microorganisms by more than 3 log reduction.

Table 4: Disinfectant validation study of Isopropyl alcohol 70% against index microorganisms on different surface samples at different time intervals.

Disinfectant shelf life	Contact time	Microorganism	Wall (Epoxy coated Gypsum)			Floor (Vinyl)			Stainless steel (316L)			Curtain (PVC)		
			+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]
0 Day	1 minute	*Bacillus subtilis*	$1.4x10^5$	<10	>4.15	$1.3x10^5$	25	3.72	$2.5x10^5$	<10	>4.40	$1.3x10^5$	<10	>4.11
		Pseudomonas aeruginosa	$1x10^6$	<10	>5.00	$9.4x10^5$	$1.2x10^2$	3.89	$9.6x10^5$	<10	>4.98	$1x10^6$	<10	>5.00
		Candida albicans	$6.2x10^5$	<10	>4.79	$1.6x10^5$	<10	>4.20	$8.4x10^5$	<10	>4.92	$2.3x10^5$	<10	>4.36
	5 minutes	*Bacillus subtilis*	$1.4x10^5$	<10	>4.15	$1.3x10^5$	<10	>4.11	$2.5x10^5$	<10	>4.40	$1.3x10^5$	<10	>4.11
		Pseudomonas aeruginosa	$1x10^6$	<10	>5.00	$9.4x10^5$	<10	>4.97	$9.6x10^5$	<10	>4.98	$1x10^6$	<10	>5.00
		Candida albicans	$6.2x10^5$	<10	>4.79	$1.6x10^5$	<10	>4.20	$8.4x10^5$	<10	>4.92	$2.3x10^5$	<10	>4.36
7 Days	1 minute	*Bacillus subtilis*	$2.4x10^5$	<10	>4.38	$2 x10^5$	40	3.70	$4.6x10^5$	<10	>4.66	$2.2x10^5$	<10	>4.34
		Pseudomonas aeruginosa	$6.2x10^5$	<10	>4.79	$4.8x10^5$	80	3.78	$2.1x10^5$	<10	>4.32	$1.2x10^5$	<10	>4.08
		Candida albicans	$5x10^5$	<10	>4.7	$6.2x10^5$	<10	>4.30	$4x10^5$	<10	>4.60	$8.9x10^5$	<10	>4.94
	5 minutes	*Bacillus subtilis*	$2.4x10^5$	<10	>4.38	$3.1x10^5$	<10	>4.49	$7.5x10^5$	<10	>4.88	$4.3x10^5$	<10	>4.63
		Pseudomonas aeruginosa	$2x10^5$	<10	>4.3	$4.5x10^5$	<10	>4.65	$2.4x10^5$	<10	>4.38	$5.7x10^5$	<10	>4.76
		Candida albicans	$5x10^5$	<10	>4.3	$6x10^5$	<10	>4.78	$3.8x10^5$	<10	>4.58	$6.1x10^5$	<10	>4.79
18 Days	1 minute	*Bacillus subtilis*	$4.6x10^5$	80	3.76	$4x10^5$	100	3.60	$7.7x10^5$	<10	>4.89	$1.9x10^5$	<10	>4.28
		Pseudomonas aeruginosa	$2x10^5$	100	3.3	$8x10^5$	$2x10^2$	3.60	$2.4x10^5$	<10	>4.38	$2x10^5$	<10	>4.30
		Candida albicans	$5.4x10^5$	<10	>4.73	$4.6x10^5$	<10	>4.66	$3.9x10^5$	<10	>4.59	$5.4x10^5$	<10	>4.70
	5 minutes	*Bacillus subtilis*	$4.6x10^5$	<10	>4.66	$5.9x10^5$	<10	>4.77	$7.4x10^5$	<10	>4.87	$7x10^5$	<10	>4.85
		Pseudomonas aeruginosa	$6.2x10^5$	<10	>4.79	$3.8x10^5$	<10	>4.58	$2.3x10^5$	<10	>4.36	$4.3x10^5$	<10	>4.63
		Candida albicans	$5.4x10^5$	<10	>4.73	$4.1x10^5$	<10	>4.61	$3.5x10^5$	<10	>4.54	$8x10^5$	<10	>4.90

(a) = Positive control of microbial suspension in saline in contact with specific surface.
(b) = Test preparation of microorganism with disinfectant in contact with specific surface.
(c) = Logarithmic reduction of microbial bioburden from baseline inoculum (the relative number of live microbes eliminated from a surface by disinfecting).

Table 5: Disinfectant validation study of Tego 51 against index microorganisms on different surface samples at different time intervals.

Disinifectant shelf life	Contact time	Microorganism	Wall (Epoxy coated Gypsum)			Floor (Vinyl)			Stainless steel (316L)			Curtain (PVC)		
			+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]	+ve[(a)]	Test[(b)]	Log R[(c)]
0 Day	1 minute	*Bacillus subtilis*	$2.4x10^5$	30	3.90	$1.6x10^5$	20	3.90	$8x10^5$	<10	>4.90	$6x10^5$	20	4.48
		Pseudomonas aeruginosa	$2x10^5$	<10	>4.30	$6.2x10^5$	<10	>4.79	$2.6x10^5$	<10	>4.41	$8x10^5$	<10	>4.90
		Candida albicans	$4.8x10^5$	<10	>4.68	$4.2x10^5$	<10	>4.62	$4.1x10^5$	<10	>4.61	$5x10^5$	<10	>4.70
	5 minutes	*Bacillus subtilis*	$2.4x10^5$	<10	>4.38	$1.6x10^5$	<10	>4.20	$8x10^5$	<10	>4.90	$6x10^5$	<10	>4.78
		Pseudomonas aeruginosa	$2x10^5$	<10	>4.30	$6.2x10^5$	<10	>4.30	$2.6x10^5$	<10	>4.30	$8x10^5$	<10	>4.30
		Candida albicans	$4.8x10^5$	<10	>4.68	$4.2x10^5$	<10	>4.68	$4.1x10^5$	<10	>4.68	$5x10^5$	<10	>4.68
7 Days	1 minute	*Bacillus subtilis*	$2.1x10^5$	50	3.62	$3.2x10^5$	50	3.81	$7x10^5$	<10	>4.85	$6.9x10^5$	60	4.06
		Pseudomonas aeruginosa	$1.3x10^5$	<10	>4.11	$4.8x10^5$	<10	>4.68	$2.1x10^5$	<10	>4.32	$7.8x10^5$	<10	>4.89
		Candida albicans	$4.7x10^5$	<10	>4.67	$6.2x10^5$	<10	>4.30	$4x10^5$	<10	>4.60	$4.5x10^5$	<10	>4.65
	5 minutes	*Bacillus subtilis*	$1.7x10^5$	<10	>4.23	$3.1x10^5$	<10	>4.49	$7.5x10^5$	<10	>4.88	$4.6x10^5$	<10	>4.66
		Pseudomonas aeruginosa	$1.5x10^5$	<10	>4.18	$4.5x10^5$	<10	>4.65	$2.4x10^5$	<10	>4.38	$3.8x10^5$	<10	>4.58
		Candida albicans	$4.1x10^5$	<10	>4.61	$6x10^5$	<10	>4.78	$3.8x10^5$	<10	>4.58	$1.5x10^5$	<10	>4.18
18 Days	1 minute	*Bacillus subtilis*	$2.3x10^5$	80	3.46	$4x10^5$	100	3.6	$7.7x10^5$	<10	>4.89	$6x10^5$	50	4.08
		Pseudomonas aeruginosa	$2x10^5$	<10	>4.30	$8x10^5$	<10	>4.90	$2.4x10^5$	<10	>4.38	$2.8x10^5$	<10	>4.45
		Candida albicans	$4.6x10^5$	<10	>4.66	$4.6x10^5$	<10	>4.66	$3.9x10^5$	<10	>4.59	$3.5x10^5$	<10	>4.54
	5 minutes	*Bacillus subtilis*	$2.1x10^5$	<10	>4.32	$5.9x10^5$	<10	>4.77	$7.4x10^5$	<10	>4.87	$6x10^5$	<10	>4.78
		Pseudomonas aeruginosa	$1.9x10^5$	<10	>4.28	$3.8x10^5$	<10	>4.58	$2.3x10^5$	<10	>4.36	$3.8x10^5$	<10	>4.58
		Candida albicans	$4.2x10^5$	<10	>4.62	$4.1x10^5$	<10	>4.61	$3.5x10^5$	<10	>4.54	$4.3x10^5$	<10	>4.63

(a) = Positive control of microbial suspension in saline in contact with specific surface.
(b) = Test preparation of microorganism with disinfectant in contact with specific surface.
(c) = Logarithmic reduction of microbial bioburden from baseline inoculum (the relative number of live microbes eliminated from a surface by disinfecting).

After 18 days of storage, microbial recovery was observed after 1 minute only with the following decreasing order: Isopropanol 70% (4 times) > Tego 51 (3 times) > Tego 2000 (no recovery at all). Although this order was different after 7 days of storage: Isopropanol 70% (2 times) > Tego 2000 (only 1 time) > Tego 51 (no recovery at all).

Table 6: Disinfectant validation study of Tego 2000 against index microorganisms on different surface samples at different time intervals.

Disinifectant shelf life	Contact time	Microorganism	Wall (Epoxy coated Gypsum)			Floor (Vinyl)			Stainless steel (316L)			Curtain (PVC)		
			+ve[a]	Test[b]	Log R[c]	+ve[a]	Test[b]	Log R[c]	+ve[a]	Test[b]	Log R[c]	+ve[a]	Test[b]	Log R[c]
0 Day	1 minute	*Bacillus subtilis*	$2.8x10^4$	20	4.14	$2.9x10^5$	<10	>4.46	$2.6x10^5$	<10	>4.41	$5.1x10^5$	<10	>4.71
		Pseudomonas aeruginosa	$7.5x10^5$	10	4.88	$2.5x10^5$	<10	>4.40	$1.7x10^5$	<10	>4.23	$1.3x10^5$	<10	>4.11
		Candida albicans	$2.2x10^5$	<10	> 4.34	$3.7x10^5$	<10	>4.57	$9.5x10^5$	<10	>4.98	$3.2x10^5$	35	3.96
	5 minutes	*Bacillus subtilis*	$2.8x10^4$	<10	> 4.45	$2.9x10^5$	<10	>4.46	$2.6x10^5$	<10	>4.41	$5.1x10^5$	<10	>4.71
		Pseudomonas aeruginosa	$7.5x10^5$	<10	>4.88	$2.5x10^5$	<10	>4.40	$1.7x10^5$	<10	>4.23	$1.3x10^5$	<10	>4.11
		Candida albicans	$2.2x10^5$	<10	>4.34	$3.7x10^5$	<10	>4.57	$9.5x10^5$	<10	>4.98	$3.2x10^5$	<10	>4.51
7 Days	1 minute	*Bacillus subtilis*	$2.3x10^5$	<10	>4.36	$3.2x10^5$	50	3.81	$7x10^5$	<10	>4.85	$6.9x10^5$	<10	>4.84
		Pseudomonas aeruginosa	$9.8x10^5$	<10	>4.99	$4.8x10^5$	<10	>4.68	$2.1x10^5$	<10	>4.32	$7.8x10^5$	<10	>4.89
		Candida albicans	$5.8x10^5$	<10	>4.76	$6.2x10^5$	<10	>4.30	$4x10^5$	<10	>4.60	$4.5x10^5$	<10	>4.65
	5 minutes	*Bacillus subtilis*	$2.3x10^5$	<10	>4.36	$3.1x10^5$	<10	>4.49	$7.5x10^5$	<10	>4.88	$4.6x10^5$	<10	>4.66
		Pseudomonas aeruginosa	$9.8x10^5$	<10	>4.99	$4.5x10^5$	<10	>4.65	$2.4x10^5$	<10	>4.38	$3.8x10^5$	<10	>4.58
		Candida albicans	$5.8x10^5$	<10	>4.76	$6x10^5$	<10	>4.78	$3.8x10^5$	<10	>4.58	$1.5x10^5$	<10	>4.18
18 Days	1 minute	*Bacillus subtilis*	$2.3x10^5$	<10	>4.36	$4x10^5$	100	3.6	$7.7x10^5$	<10	>4.89	$6x10^5$	<10	>4.78
		Pseudomonas aeruginosa	$4.2x10^5$	<10	>4.62	$8x10^5$	<10	>4.90	$2.4x10^5$	<10	>4.38	$2.8x10^5$	<10	>4.45
		Candida albicans	$5.9x10^5$	<10	>4.77	$4.6x10^5$	<10	>4.66	$3.9x10^5$	<10	>4.59	$3.5x10^5$	<10	>4.54
	5 minutes	*Bacillus subtilis*	$2.3x10^5$	<10	>4.36	$5.9x10^5$	<10	>4.77	$7.4x10^5$	<10	>4.87	$6x10^5$	<10	>4.78
		Pseudomonas aeruginosa	$4.2x10^5$	<10	>4.62	$3.8x10^5$	<10	>4.58	$2.3x10^5$	<10	>4.36	$3.8x10^5$	<10	>4.58
		Candida albicans	$5.9x10^5$	<10	>4.77	$4.1x10^5$	<10	>4.61	$3.5x10^5$	<10	>4.54	$4.3x10^5$	<10	>4.63

(a) = Positive control of microbial suspension in saline in contact with specific surface.
(b) = Test preparation of microorganism with disinfectant in contact with specific surface.
(c) = Logarithmic reduction of microbial bioburden from baseline inoculum (the relative number of live microbes eliminated from a surface by disinfecting).

DISCUSSION

Tego had, in addition to its bactericidal properties, the ability to form a tight unimolecular film and thereby reduce the transmission of bacteria. Another interesting property of the Tego compounds is their ability to adsorb onto solid surfaces and leave film that resists removal by running water. This can be shown by a clear zone on seeded agar plates around pieces of material which had been put in 1% Tego solution for 5 minutes and thoroughly rinsed with water. Similar adsorption to the bacterial cell is no doubt functional in germicidal action (Block, 1991). This characteristic made it disinfectant of our choice for long term protection of clean room especially in situations of heavy work load and/or activities of personnel to maintain low bioburden level on surfaces.

The newest addition to the Tego line is Tego 2000, a mixture of an amphoteric surfactant and a cationic. This product is a general disinfectant and sanitizer for the food and beverage industry, but is also used in hospitals and medical applications. This product is a general disinfectant and sanitizer for the food and beverage industry, but is

also used in hospitals and medical applications (Block, 1991).

According to Edelmeyer and Laqua (1978), Tego 51 is a safe, suitable disinfectant for use in the processed edible gelatin industry. The minimum inhibitory concentration of Tego 51 in gelatin was found to be 90 ppm. The nontoxic effect level was said to be 300 ppm, and an acceptable daily intake was 3 ppm or 9 mg per person per day.

In the "EC Guide to Good Manufacturing Practice, Revision to Annex 1" (EC., 2003), it is stated that "where disinfectants are used, more than one type should be employed. Monitoring should be undertaken regularly in order to detect the development of resistant strains." The practice of rotation of disinfectants is also mentioned in the FDA guide for aseptic processing (FDA Guidance for Industry, 2004) and in the USP Chapter <1072>. Our disinfection program is based on rotation between

Tego 103G is also recommended by the manufacturer for disinfection of surgical instruments and rubber articles (1% solution for 10 minutes' minimum contact) and for prophylactic foot baths (1% solution for 5 minutes' minimum contact). For disinfection of operating theaters, wards, dick rooms, toilets, and equipment, he related Tego 103G is recommended. Frisby (1959) employed it for such purposes in a hospital and recorded his findings. An operating theater was sprayed weekly and the walls and floors were mopped more often with 1% 103G. Our aim was to implement Tego but of 51 and 2000 series in pharmaceutical industry.

Traditionally, acceptable microbial variability has been defined as 0.5 log variation in microbial counts. However, in the harmonized USP Chapter <61>, the definition has been changed to a factor of 2 (0.3 log). If the difference is less than 0.3 log harmonized, any of the evaluated methods should be considered suitable for the application (Clontz, 2008). This is the criteria for deciding acceptance or failure of neutralization method for biocidal agents.

Surface challenge tests are customized procedures based on the AOAC method for germicidal spray products and designed to evaluate the effectiveness of a disinfectant against standard and environmental isolates when applied to representative surfaces found in a manufacturing facility. This test has become the preferred disinfectant qualification method by the regulatory agencies. It is quantitative and demonstrates log reduction of the test organism upon exposure to the selected disinfectant concentration as it is used by a company during a cleaning procedure. In order to not deliberately contaminate the manufacturing areas, surface challenge tests are performed in a laboratory setting and using representative surfaces (referred to as coupons) that are scaled down to a size of about 2 × 2 in (Clontz, 2008).

Acceptance criteria were chosen to be 3 log reductions for vegetative microorganisms while for spores must not be less than 2 log reduction for successful disinfection (Eissa *et al*., 2013). This was the basis of our decision for the biocidal efficacy of antimicrobial agents used in this study.

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 (Russell *et al*., 1979; Cremieux *et al.*, 1983; USP <1227> Validation of Microbial Recovery from Pharmacopoeial Articles, 2013).

NT study performed for the 2 neutralizers (FTM and BLT) used in this study revealed that both of them are non-toxic and could be used in the validation program also both neutralizers did not differ significantly from each other (at $P < 0.05$). The other important subsequent aspect is NE. The scheme followed was using FTM as primary neutralizer (supported by previous work) and if it had failed BLT was tested as an alternative neutralizing agent. Sutton *et al.* (2002) presented 2 similar neutralizers

NIH thiogllycolate (close in composition to FTM) and TAT (close in composition to BLT). Both were non toxic or of low toxicity against microorganisms but could not effectively recover some of them from disinfectants under study. 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.

FTM proved to be effective for both Isopropyl alcohol 70%, Tego 51 1% and Tego 2000 1% with exception of *Bacillus subtilis* with Tego 51% in which FTM failed in NT study. FTM was replaced with BLT and managed to recover *Bacillus subtilis* effectively from the selected disinfectant.

According to USP <1072> DISINFECTANTS AND ANTISEPTIC chapter, effect of dilution can play major or minor role in the decrease of antimicrobial properties depending on biocidal concentration exponent (η) so for dilution error challenge 90% of the use concentration aliphatic alcohols may range from 1.88 to 3.81 times reduction of antimicrobial activity occurs as they have high concentration exponent that ranges from 6 to 12.7. While for amphoteric compound such as Tego 2000 according to Th. Goldschmidt AG Suspension Test (Block, 1991) concentration exponent above transition concentration of 0.05% differs from that below it. In the lower part of the curve (below transition concentration) there is relatively rapid change in antimicrobial activity (av. = 1.83 LR/1% increase in concentration) in contrast to that above transition concentration (av. = 0.21 LR/1% increase in concentration). About 9 times decrease in the effect of concentration on biocidal activity.

Antimicrobial Activity of Tego 2000 was studied by Th. Goldschmidt AG on *Staphylococcus aureus* and *Pseudomonas aeruginosa* revealed that after 5 minutes contact with tego 2000 at concentration ≥ 0.1% gave 4 logarithmic reduction or more after 5 minutes. Moreover, his finding showed that at concentration 0.25% there is no significant difference in activity for 5 minutes contact time from that after 30 or 60 minutes. That is why we have chosen this time as maximum contact time and our results agreed with this assumption. Also Th. Goldschmidt AG antimicrobial activity for Tego 2000 is in agreement with our results which gave us more than 3 log reduction with both bacteria. This advantage makes Tego suitable in surface disinfection and sanitization as it is affected little by dilution when it is used at 1% concentration (10 times above minimum effective concentration during 5 minutes in the use-dilution test).

At 0.01 to 0.8% Tego 51 showed high bactericidal activity against *E.* coli, *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Mycobacterium phlei,* and *Oospora lactis* (Andriasyan, 1983). At 0.5% it displayed good detergency and inhibited bacterial contamination by 99.8%. He suggested its use as a detergent and disinfectant for the sanitation of dairy equipment. Our results revealed that Tego 51 at 1% concentration was very effective after 1 minute against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*.

Bactericidal test data on a long list of microorganisms given by Goldschmidt showed that most organisms killed by 1% aqueous solutions of the Tego compounds in 1 minute. This finding is in agreement with our finding with both Tego 51 and 2000.

It is expected that a relationship exists between the surface activity and the biocidal activity of Tego compounds according to Yet Yamada (1968), maximum surface activity (100%) was attained with different Tego compounds concentrations ranges from 0.3-0.6%.

Storage tanks and tankers used to transport orange juice and other food are often made of stainless steel, because of its corrosion resistance and antibacterial properties. In this study stainless steel was the only surface from which non of the used microorganisms was recovered thus it seems that it is at least presents unfavorable

environment for survival of settled microbial cells and/or its surface properties acts in favor of the used biocidal agents in this study.

The heat and light stability of eight sanitizers was investigated by Gelinas and Goulet (1982). Solutions at 40°C at use concentration were exposed to florescent light For 6 days. Tego 51 retained full activity for the 6 days. This finding matches our result of storage for both Tego series especially at temperature 20-25°C in tightly closed and opaque stock container in which degradation will be lower thus extending shelf life of biocidal agent.

In general it could be concluded that in addition to Isopropanol 70% Tego 51 and 2000–diluted to 1% and stored for up to 18 days–could be used in the process of sanitization and disinfection effectively in the pharmaceutical industry but in rotation with effective and validated sporicidal agent. We think that the use of this biocidal agent could be extended from the field of food industry to the healthcare facilities as safe and good sanitizer and disinfecting agent.

The described disinfectant validation study provided good guideline for evaluation biocidal agents whether they are sporicidal or not. The same procedure was adapted to other antimicrobial agents to find their value in the process of surface disinfection to be used in sanitization program in a continuous effort to take control over microbial contamination that represents a unseen threat to pharmaceutical products.

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