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DEVELOPMENT OF METHODS FOR MICROBIAL RECOVERY: PHARMACEUTICAL DOSAGE FORMS INCLUDING DRUGS WITH ANTIMICROBIAL PROPERTIES (STUDY III)

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ABSTRACT

Bioburden determination and detection in the drug products is a crucial attribute in pharmaceutical manufacturing industry that affects both the patients and the reputation of business holders. Establishment and verification of the sensitivity for bioburden detection and enumeration in the dosage forms is critical in minimizing the potential hazard of delivering unsafe products with masked contamination to the market. In the present study, selected group of non-sterile dosage forms – including drugs with known antimicrobial properties - were tested for the ability to recover low level inoculums of pharmacopeial standard strains after applying specific neutralization and processing steps. The neutralization techniques included dilution, filtration, chemical neutralizers or combination of two or more of them together in a single procedure. Preliminary test was conducted to ensure non toxicity of the neutralization media or processes. The average combined microbial recovery of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans* (20–25°C), *C. albicans* (30–35°C) and *Aspergillus niger* (20–25°C) and *A. niger* (30–35°C) from all tested pharmaceutical dosage forms was 0.89±0.36, 0.85±0.27, 0.94±0.47, 0.85±0.28, 0.80±0.35, 0.95±0.40 and 0.85±0.32 respectively. The tested products passed the criteria of microbial enumeration and detection of specified microorganisms, with exception of one topical antimicrobial cream with fungi, oral Azithromycin capsule with bacteria, Sodium Risedronate tablet with *Pseudomonas aeruginosa* (in one neutralization trial without chemical neutralizer) and oral Nifuroxazide capsule with *Staphylococcus aureus*. Interestingly, microbial recovery of *B. subtilis*, *C.*

albicans and *A. niger* from tested products were significantly correlated with each other using Pearson correlation matrix.

KEY WORDS: Bioburden determination and detection, non-sterile dosage forms, standard strains, neutralization techniques, correlation.

INTRODUCTION

Microbial contamination has been on the list for top 10 reasons for FDA product recalls for the recent years with the most commonly detected organisms found in aqueous formulations being pseudomonads and other Gram-negative organisms. There are some examples of recent safety advisories and product recalls issued by the FDA Safety Information and Adverse Event Reporting System (AERS) due to contamination by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacteriaceae*, *Bacillus cereus*, *Burkholderia cepacia* and many other microorganisms.[1]

Certain manufactured goods, of which foodstuffs, cosmetics, and pharmaceutical products are the prime examples, can be contaminated with microorganisms during manufacture; this contamination can, at the best, cause spoilage and consequent rejection of the contaminated material and, at the worst, harm or even bring death to the consumer. The culprits are usually bacteria or fungi.[2]

Suitability demonstrates that the products tested do not exhibit inhibitory effects on the growth of microorganisms under the conditions of the tests. Neutralizing agents may be used to neutralize the activity of antimicrobial agents in products. The appropriate neutralizing agent should be added preferably before sterilization of the media. Include a blank control with neutralizer and without product to demonstrate efficacy and absence of toxicity for microorganisms.[3]

Due to the previously mentioned risks, the present study aimed to focus on the application of methods for the recovery of low level of bioburden from selected drugs. This would allow ensuring that any antimicrobial properties attributed to the medicinal products were neutralized and hence the microbial contents could multiply and proliferate in the culture media. This study was conducted as a part of large survey project that covered other medicinal dosage forms from the drug market.

MATERIALS AND METHODS

Standard strains were obtained from ATCC (American Type of Culture Collection**,** Manassas, Virginia) and handled according to the stated procedure by the supplier. All culture media and reagents were obtained from OXOID (Basingstoke, Hampshire) and Sigma-Alrich (St. Louis, MO 63103), respectively. Plastic 9 mm sterile plates were purchased from Sterilin Limited (solaar house, 19 mercers row, Cambridge, UK). Microbial suspensions were quantified by making serial dilutions and plating using conditions and media suitable for each organism and selecting dilutions of suitable microbial concentration as working suspensions. Microbial test suspensions were used once the results of serial dilutions could be quantified using digital colony counter (Digital Colony Counter Model: 361, Laxman Mahtre Rd. Navagaon, Dahisar West, Mumbai). All media were sterilized by autoclaving in steam sterilizer (FEDEGARI FOB3, Fedegari Autoclavi SpA, SS 235 km 8, 27010 Albuzzano (PV), Italy). All culture media used in the current study were subjected to growth promotion test as described in standard methods.

Neutralizing broth (NB) was prepared as described by Eissa *et al.*, 2014 with modification of adding Lecithin 7.0 g/L to NB. Tryptone Soya Broth (TSB) was supplemented with Lecithin and Tween 80 (5.0 and 40.0 g/L respectively).[4] This study was done without including product hold time with the test microorganisms which is required to be performed in a different experiments. The microbiological testing was done according to Pharmaceutical Microbiology Manual (PMM) with modification.[5] Preliminary microbiological cleanliness testing of the selected drugs was examined according to compenidal methods and negative control samples were included concurrently with the test.

Moreover, microbiological environmental monitoring (EM) samples from surfaces and air in the work area were taken according to Eissa, 2014 with every test group performed in biological safety cabinet (BSC) (Jouan MSC 9 Class II A2 BioSafety Cabinet, Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, California 95134) to ensure appropriate cleaning, sanitization and aseptic attitude under laminar air flow (LAF) conditions.[6] Bacterial visualization was enhanced using colorless Triphenyltetrazolium Chloride dye which turns red by viable cells. Cultures identification and freedom from contamination were done by methods stated by some investigators.[7,8] Acceptance criteria of the test results based on what is stated by Clontz, 2008.[1] Tested drugs were presented in Table (1). All statistical analysis and bar figure were demonstrated using GraphPad Prism version 6.01. Any

interpretation or complex calculation was illustrated using Microsoft Excel 2007. Box and Whiskers plot was generated using Minitab version 17.

Table (1): List of the tested non-sterile pharmaceutical dosage forms with their code names used, APIs, concentrations and excipients.

Dosage form	Code Name	APIs	Concentration	Other Ingredients*
Liquid Oral Products	PVT	Ascorbic Acid, Calcium Gluconate, Calcium Phospholactate, Panthenol, Nicotinamide, Vitamin B1, B2, B6, D3, A Palmitate, E Acetate	NA	Sorbitol Solution 70%, Glycerol, Evogran Grape Fruit, Evogran Flavor, Disodium EDETATE, Butylated Hydroxy Anisol, Citric Acid, Na Saccharine, Cremophor RH40, Glucose Syrup, K Sorbate, Xanthan Gum, Malt Extract
	BFL	Ibuprofen	20 mg/ml. suspension	Citric Acid, Sucrose, Sodium Saccharine, Sorbitol 70%, Methyl and Propyl Paraben, Avicel RC-591, HPMC 15 CP, Xanthan Gum, Cremophor RH40, Evogran Orange, Evogran Grape Fruit, Sunset Yellow, Purified Water USP
Semisolid Products	SPR	Bifonazole	10 mg/g cream	Cetostearyl Alcohol, Cetyl Palmitate, 2-Octyldodecanol, Sorbitan Monostearate, Tween 60, Benzyl Alcohol, Purified Water USP
	TPZ	Tioconazole	20 mg/g cream	Cetostearyl Alcohol, Cetyl Palmitate, 2-Octyldodecanol, Sorbitan Monostearate, Tween 60, Benzyl Alcohol, Citric Acid, Purified Water USP
	FCT	Fusidic Acid	20 mg/g cream	Propylene Glycol, Butylated Hydroxy Anisol, Liquid Paraffin Heavy, Cetostearyl Alcohol, Ethanol 96%, PEG-6 Stearate, Glyceryl Monostearate, K Sorbate, Sorbitan Monostearate
	TOP	Tolnaftate, Gentamycin Sulfate, Clioquinol, Betamethasone Dipropionate	10, 1.3, 10, 0.643 mg/g cream	Cetostearyl Alcohol, Tween 60, Cremophor RH40, Sorbitan Monostearate, Tween 60, Glyceryl Monostearate, petroleum Jelly White, Liquid Paraffin, Polyethylene Glycol Glyceryl Oleate, EDTA, Chlorocresol, Purified Water USP
	FLM	Nystatin, Neomycin Sulfate, Gramicidin,	29.54, 5.63, 0.36, 1	Cetostearyl Alcohol, Cetyl Palmitate, 2-Octyldodecanol, Sorbitan Monostearate, Tween 60, Methyl and Propyl Paraben, Ethanol, NaOH, Essence, Perfume,

		Triamcinolone Acetonide	mg/g cream	Purified Water USP
Hard Gelatin Capsules	ZTK	Azithromycin	500 mg/cap.	Lactose Anhydrous, Talc, Colloidal Silicone Dioxide, Magnesium Stearate, Sodium Lauryl Sulphate
	PPZ	Omeprazole	40 mg/cap.	Carrier Sustained Release Resin Pellets
	RBV	Ribavirin	200 mg/cap.	Avicel PH 101, Magnesium Stearate, Sodium Croscarmellose, Lactose
	LRL	Pregabalin	150 mg/cap.	Talc, Maize Starch, Lactose
	RVX	Rivastigmine Tartrate	6 mg/cap.	Avicel PH 101, Mg Stearate, Colloidal Silicon Dioxide, Hypromellose
	DIX	Nifuroxazide	200 mg/cap.	Talc, Povidone K-25, Magnesium Stearate, Lactose, Colloidal Silicon Dioxide
Tablets	CPB	Ciprofloxacin	750 mg/tab.	HPMC 15 CP, PEG 4000, Titanium Dioxide
	HPM	Paracetamol, DL-Methionine	500, 100 mg/tab.	Sodium Starch Glycolate, Colloidal Silicon Dioxide, Magnesium Stearate, PVP, Avicel PH 102, HPMC, PEG 4000 and 6000, Titanium Dioxide, Talc, Peppermint Oil
	RSL	Sodium Risedronate	40.11 mg/tab.	Avicel PH 102, Cross Povidone, Magnesium Stearate, Lactose, Aerosil 200, Opadry II
	KNZ	Losartan Potassium, Hydrochlorothiazide	100, 25 mg/tab.	Opaspray Yellow, Propylene Glycol, Lactose Anhydrous, Hypromellose, Colloidal Silicone Dioxide, Magnesium Stearate, Avicel PH 102, Cross Povidone
	GLC	Acarbose	50 mg/tab.	Microcrystalline Cellulose, Magnesium Stearate, Maize Starch, Colloidal Silicone Dioxide
	TRS	Trospium Chloride	20 mg/tab	Iron Oxide Yellow, Sodium Croscarmellose, Avicel PH 101, Hypromellose, Macrogols, Talc, Colloidal Silicone Dioxide, Lactose Monohydrate, Titanium Dioxide, Maize Starch, Stearic Acid, Povidone K25, Calcium Carbonate

NA= Not applicable as it was not detailed by the manufacturer.

RESULTS AND DISCUSSION

All culture media used in the current study passed growth promotion test. Initial assessments of bioburden quality of the tested non-sterile pharmaceutical products –using conventional methods- revealed that the tested products were clean microbiologically. EM samples which were taken during the study passed the acceptance criteria. Culture purity and identity for the standard strains was verified and confirmed. All neutralization methods were found to give

acceptable microbial recovery of >50% from the viability control group as demonstrated in Table (2). All negative control samples did not show any signs of microbial growth. Fig. (1) illustrates total corrected and logarithmically transformed microbial recovery of each standard strain from the neutralizer toxicity study. Interestingly, the highest and lowest recoveries were demonstrated by *Candida albicans* and *Aspergillus niger* respectively.

Table (2): Assessment of the toxicity of the neutralization procedure for standard strains used for testing pharmaceutical dosage forms samples collected from the market.

Product Codes	Microbial Recovery of Neutralizion Toxicity (NT) Group to Viability Group						
	Staphylococcus aureus	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Candida albicans***		***Aspergillus niger***	
	30-35°C	**30-35°C**	**30-35°C**	**20-25°C**	**30-35°C**	**20-25°C**	**30-35°C**
PVT	1.00	1.20	0.77	1.07	0.95	0.60	0.97
BFL	1.00	1.10	0.77	1.07	0.95	0.60	0.97
SPR	0.85	0.71	0.83	0.63	1.13	0.66	0.62
TPZ	0.79	0.83	0.77	0.63	1.13	0.60	0.97
FCT	0.94	0.98	1.10	1.07	1.06	0.68	1.00
TOP	0.85	0.71	0.63	1.00	1.13	0.66	0.62
FLM	1.16	1.16	0.63	1.19	0.93	0.88	0.68
ZTK	1.00	1.00	0.73	1.22	1.00	1.00	1.06
PPZ	1.00	1.23	0.77	1.07	1.34	0.60	0.97
RBV	1.00	1.00	1.00	0.76	1.16	1.00	1.06
LRL	0.94	1.00	1.10	1.07	1.06	0.60	1.00
RVX	0.85	0.71	0.63	1.00	1.13	0.66	0.62
DIX	1.05	1.12	0.92	1.02	0.96	1.08	1.04
CPB	1.00	1.26	1.00	1.00	1.00	1.00	1.06
HPM	1.00	1.20	0.77	1.07	0.95	0.60	0.97
RSL	1.00	1.26	1.00	1.00	1.00	1.00	1.06
KNZ	0.91	0.58	1.05	0.80	1.06	0.69	0.57
GLC	1.16	0.93	1.10	1.00	1.00	0.84	0.84
TRS	0.84	0.94	1.00	0.96	0.86	0.74	0.72

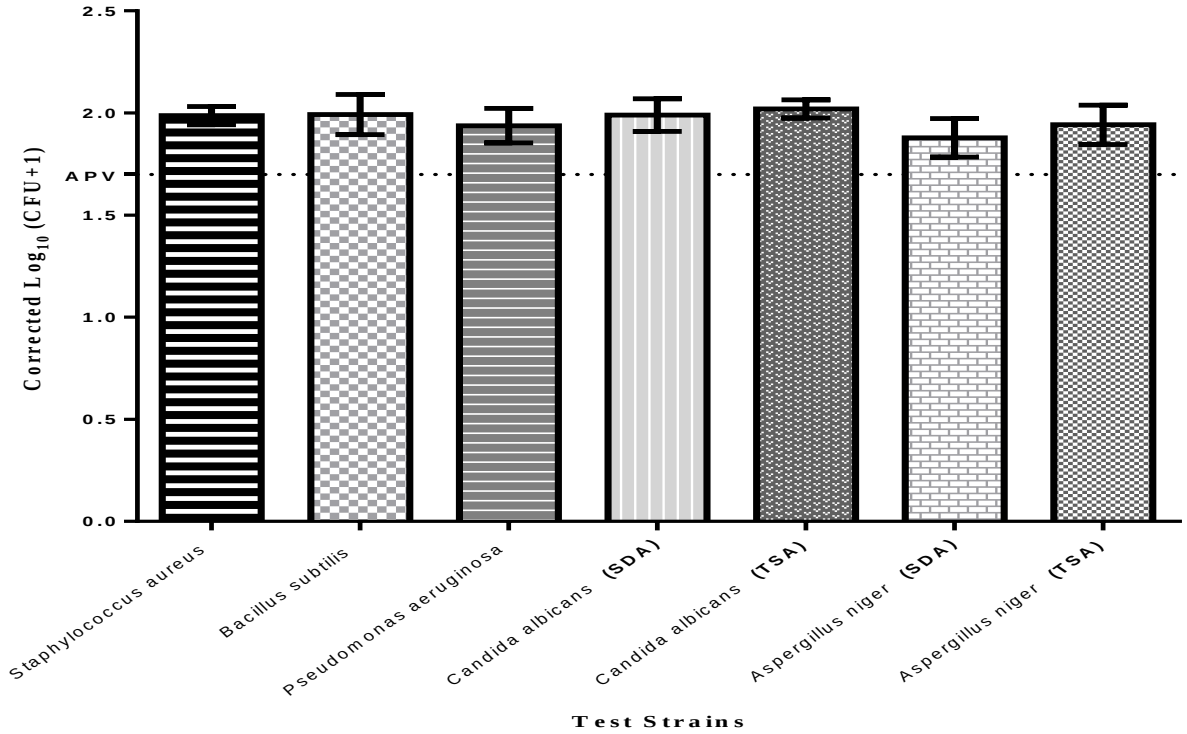

Figure (1): Bar graph showing corrected and transformed total averaged microbial count from the tested pharmaceutical products for each microorganism in NT study to Log_{10} scale with adjusted values for comparison ± S.D. with dotted horizontal line representing the minimum Acceptable Plating Variability (APV). (Graph was generated using GraphPad Prism version 6.01)

The average combined microbial recovery of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans* (20–25°C), *C. albicans* (30–35°C) and *Aspergillus niger* (20–25°C) and *A. niger* (30–35°C) from all tested pharmaceutical dosage forms was 0.89±0.36, 0.85±0.27, 0.94±0.47, 0.85±0.28, 0.80±0.35, 0.95±0.40 and 0.85±0.32 respectively. Standard strains were recovered successfully from the tested medicinal products with recovery >50% with the exception of topical antimicrobial cream (FLM) with fungi, Azithromycin capsule (ZTK) with bacteria, Sodium Risedronate film coated tablet (RSL) (MI) with *Pseudomonas aeruginosa* and Nifuroxazide capsule (DIX) with *Staphylococcus aureus* as demonstrated in Table (3). Box and Plot diagram illustrated in Fig. (2) demonstrates total microbial recovery from the tested products for each test strain with outliers and the distribution of data shown in the diagram. Table (4) shows that microbial recovery of *B. subtilis*, *C. albicans* and *A. niger* from tested products were significantly correlated with each other using Pearson correlation matrix. Product codes and the processing neutralization procedure for both microbial enumeration and detection are summarized in

Table (5). Dilution was observed to be the common method of neutralization and processing with all tested products. Interestingly, it was noted that combination of the three techniques of neutralization was required with both commonly known antimicrobials and some other products not intended to be used as antibiotics such as Sodium Risedronate tablet, Rivastigmine tartarate and Ribavirin capsules which indicated that these drugs may possess some antimicrobial activity, even their manufacturers indicate other uses. Although the first drug is pyridinyl bisphosphonate that inhibits osteoclast mediated bone resorption and modulates its metabolism. The second one treats mild to moderate dementia caused by Alzheimer's or Parkinson's disease. While the third drug is a guanosine (ribonucleic) analog antiviral used to stop viral RNA synthesis and viral mRNA capping.

Table (3): Assessment of the recovery of microorganisms from different types of non-sterile pharmaceutical dosage forms after applying the neutralization procedures for each one.

Product Code		Microbial Recovery of Product Test (PT) Group to NT Group						
		Staphylococcus aureus	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Candida albicans***		***Aspergillus niger***	
		30-35°C	**30-35°C**	**30-35°C**	**20-25°C**	**30-35°C**	**20-25°C**	**30-35°C**
PVT		0.95	0.76	0.97	0.86	1.02	1.45	1.10
BFL		0.82	1.01	0.98	0.91	1.02	0.86	1.10
SPR¥	MI	1.00	0.98	0.84	0.95	0.81	1.80	0.89
	MII	0.94	0.92	0.61	1.1	ND	0.50	ND
TPZ		1.03	0.96	1.13	0.98	1.11	1.07	1.17
FCT		0.40	1.16	1.19	1.14	0.78	0.89	0.73
TOP		0.96	0.60	0.76	0.79	0.78	0.89	1.05
FLM		0.76	0.14	0.69	0.00	0.00	0.00	0.00
ZTK		0.41*	0.42*	0.11*	1.24	0.56	0.89	1.00
PPZ		1.00	1.26	1.41	0.93	0.93	1.33	0.90
RBV		1.03	0.88	0.57	1.08*	0.66	1.55	1.00
LRL		0.80	0.90	0.57	0.58	0.71	0.56	0.65
RVX		1.91	1.05	1.91	0.95*	ND	0.85	ND
DIX		0.00*	0.70*	0.59	0.73	0.85	0.86	0.92
CPB		0.66	0.76	1.70	0.91	0.67	0.83	0.61
HPM		1.10	0.72	1.39	1.08	1.15	1.47	1.30
RSL¥	MI	0.93	0.83	0.03*	1.10	ND	1.05	ND
	MII	1.16	0.59	1.06*	0.86	0.56	0.65	0.50
KNZ		1.05	1.2	1.34	0.54	0.54	1.00	1.13
GLC		0.99	1.09	0.82	0.70	0.67	0.75	0.67
TRS		0.88	0.93	1.17	0.52	1.73	0.79	0.52

ND= Not determined due to unavailability of more samples for further testing.
**= Microorganism with the lowest recovery for pharmaceutical product that necessitated modification in the method of microbial recovery.*

¥= Two independent methods applied for enhancement of microbial recovery with chemical neutralization steps added to MII in SPR and RSL.

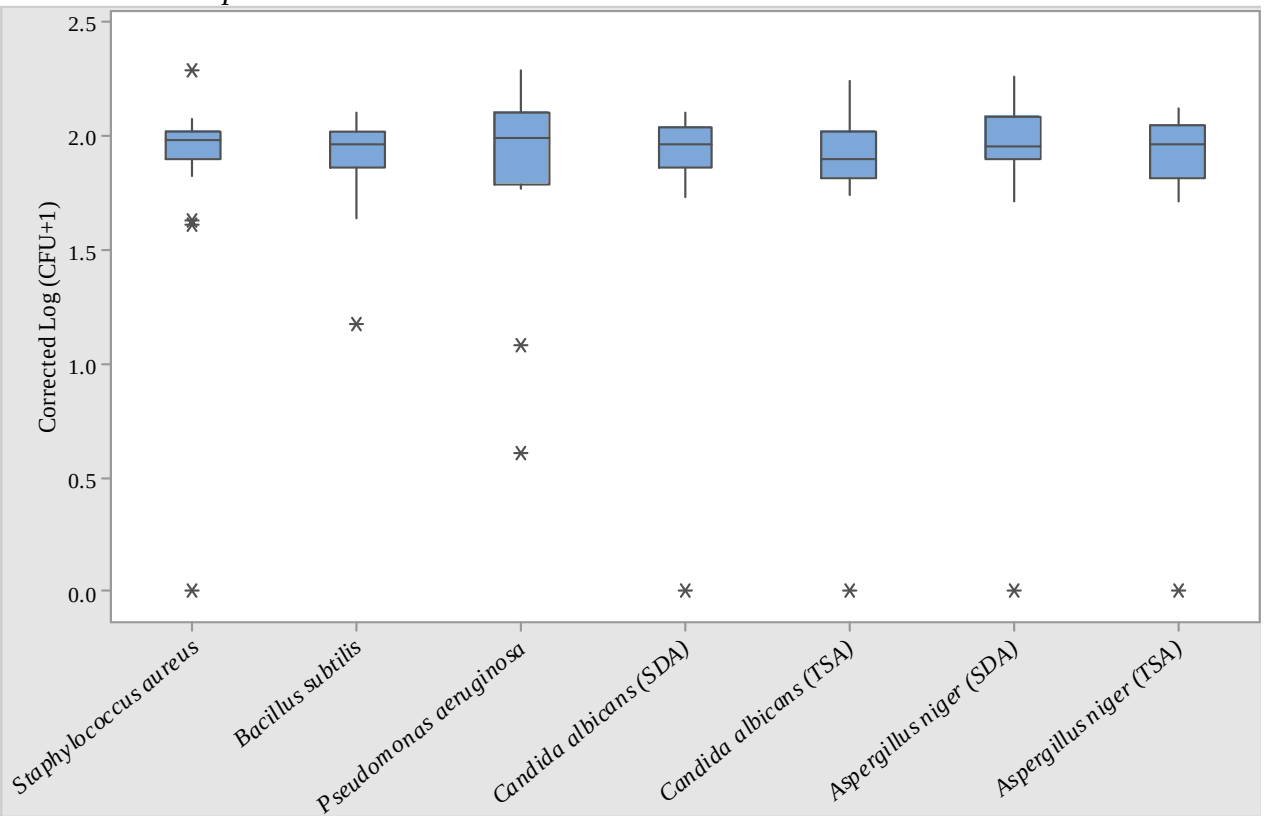

Figure (2): Box and Whiskers diagram showing general total microbial recovery count transformed to Log_{10} scale with adjusted values for comparison from each tested microorganism. (The plot was generated using Minitab version 17)

Table (4): Pearson correlation coefficient matrix at confidence interval 95% showing the relation of transformed and corrected microbial recovery from the neutralized pharmaceutical products of each standard strain with the others. (Matrix was generated using GraphPad Prism version 6.01)

Correlation Matrix	***Staphylococcus aureus***					
Bacillus subtilis	0.132	*Bacillus subtilis*				
Pseudomonas aeruginosa	0.158	0.255	*Pseudomonas aeruginosa*			
Candida albicans (SDA)	0.009	0.769*	-0.053	*Candida albicans* (SDA)		
Candida albicans (TSA)	-0.025	0.831*	0.183	0.929*	*Candida albicans* (TSA)	
Aspergillus niger (SDA)	0.022	0.803*	0.033	0.945*	0.941*	*Aspergillus niger* (SDA)
Aspergillus niger	-0.035	0.798*	0.054	0.962*	0.936*	0.973*

(TSA)						

*= *Very significant correlation.*

Table (5): Applied technique(s) in neutralization for each tested pharmaceutical product in both enumeration and specific microorganism detection tests.

Product Code	Neutralization for Enumeration			Neutralization for Detection of Specific Microorganisms		
	Dilution	Filtration	Chemical	Dilution	Filtration	Chemical
PVT	+	-	-	+	-	-
BFL	+	-	-	+	-	-
SPR	+	+	-	+	+	+
TPZ	+	+	+	+	+	+
FCT	+	+	+	+	+	+
TOP	+	+	+	+	+	+
FLM	+	+	+	+	+	+
ZTK	+	+	+	+	+	+
PPZ	+	-	-	+	-	-
RBV	+	+	+	+	-	-
LRL	+	-	-	+	-	-
RVX	+	+	+	+	-	-
DIX	+	+	+	+	-	-
CPB	+	+	+	+	-	-
HPM	+	-	-	+	-	+
RSL	+	+	+	+	-	-
KNZ	+	-	-	+	-	-
GLC	+	-	-	+	-	-
TRS	+	-	-	+	-	-

+= *Applied technique.* -= *Not required technique.*

Based on data of correlation matrix, the estimated microbial recovery from Tryptone Soya Agar (TSA) at 30-35°C for *C. albicans* from SPR(MII), RVX and RSL(MI) was 1.04, 0.91 and 1.04 respectively and for *A. niger* was 0.48, 0.78 and 0.95 respectively for the same products.

The method suitability test design can be customized to reflect the product specifications and the amount of product available for testing. [1] Meanwhile, the product specification and amount influenced the method used when using dilution technique. However, there are factors other than diminishing antimicrobial properties that were found to be favored by using higher dilutions of the products in diluents such as: ease of further handling and processing of the sample and ease of counting process in solid media due to clearer background matrix.

The principle of microbial recovery in the current test did not differ significantly from those used in the disinfectant or sanitizer validation study. The measurement of microbial kill requires the ability to measure the number of surviving microorganisms with time after exposure to the antimicrobial agent. Bioburden determinations have the same requirement as they depend on the ability to recover viable microorganisms in the presence of products or raw materials. However, carryover of residual disinfectant from the test could inhibit growth in the recovery medium, leading to poor microbial recovery. This potential residual activity must be neutralized and it is necessary to demonstrate the adequacy of neutralization for these tests.[8]

It should be noted however, that dilution affects sensitivity of the method used. So, improving sensitivity can be achieved by increasing plated volume per plate, keeping the same product to media ratio and/or increasing the number of plate replicates. Moreover, method suitability test must demonstrate that the chosen neutralization method is not harmful or toxic to microorganisms and that the test media are suitable for the recovery of specified organisms under the given test conditions. In addition, test-negative controls are performed alongside the challenge tests to verify absence of contamination in the media and in the materials used in the study. A product-negative control is performed to evaluate any inherent product bioburden that might interfere with the recovery challenge studies.[1]

Further investigations are required to improve the sensitivity of specific microorganisms' detections in case of topical antimicrobial products since the applied method of neutralization did not fit the requirements of **USP<62>, 2015**.[10] Although the method showed significant microbial recovery, yet the compendial requirements of 10 g of sample would be hard to be achieved for detection of microorganism such as *Salmonella spp.* The presence of antimicrobial properties in non-antibiotics pharmaceutical products is not strange in view of the finding of other researchers. [11]

Finally, standard strains were added to the product after processing and neutralization steps as the goal of the test was to demonstrate appropriate neutralization process and not to introduce another variable i.e. hold time study that is needed to be addressed in another separate investigation study. It is important to clearly define the goal of the method suitability. This goal is not to demonstrate the ability to recover microorganisms present in the product especially if the antimicrobial properties of the product are strong. In this case, the product could well kill off all challenge organisms before it was possible to plate the test organisms.

The goal of a microbiological method suitability test is to demonstrate that any residual antimicrobial properties of the product or the recovery method have been neutralized using the challenge microorganisms as a kind of biological indicator of neutralization.[12]

CONCLUSION

The current applied neutralization methods for the tested sample were effective. However, further studies are required to improve both the sensitivity and the recovery of injured microbial cells. In addition there some microorganisms that could not be recovered from antimicrobial products even after applying combination of the neutralization techniques (dilution, filtration and chemical neutralization).

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