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Impact of synthetic detergent on sporicidal activity in pharmaceutical facility

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ABSTRACT

A continuous validation programs for disinfectants are very important in controlling microbial bioburden of environmentally controlled area. Carrier test was chosen because it is more challenging than the usual suspension test. Materials used represent floor, wall, and stainless steel surfaces. This experiment showed that detergents such as sodium dodecyl sulphate improved sporicidal effect on microorganisms even with those who demonstrated relatively high resistance to the used antimicrobial. The used surfactant enhanced the sporicidal activity within five minutes contact time with most commonly found *Bacillus species* in the pharmaceutical facility. However, *Bacillus licheniformis* spores showed greatest tolerance among studied environmental isolates.

Keywords: Validation – biocidal agents - bioburden - carrier test - detergents - tolerance.

INTRODUCTION

Different types of microorganisms vary in their response to antiseptics and disinfectants. This is hardly surprising in view of their different cellular structure, composition, and physiology (McDonnell and Russell, 1999).

Bacterial spores of the genera *Bacillus* have been widely studied and are invariably the most resistant of all types of bacteria to antiseptics and disinfectants (Foegeding and Busta, 1983). Although *Bacillus species* are generally not pathogenic, their spores are widely used as indicators of efficient sterilization. Many biocides are bactericidal or bacteristatic at low concentrations for non sporulating bacteria, including the vegetative cells of *Bacillus species*, but high concentrations may be necessary to achieve a sporicidal effect (Shaker *et al.,* 1986).

Many of these reports of resistance have often paralleled issues including inadequate cleaning and incorrect product use which cannot be underestimated. Some acquired mechanisms have also been shown to be significant, but in most cases the results have been speculative. Increased MICs have been confirmed, in particular, for staphylococci (Alqurashi *et al.,* 1996).

The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acid. Neither of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high molecular weight substances can readily traverse the cell wall of staphylococci and vegetative *Bacillus* spp., this may explain the sensitivity of these organisms to many antibacterial agents (Russell and Russel, 1995).

Peroxyacetic acid is characterized by rapid action against all

microorganisms. Special advantages of peroxyacetic acid are that it lacks harmful decomposition products (i.e., acetic acid, water, oxygen, and hydrogen peroxide), enhances removal of organic material (Tucker *et al.,* 1996), and leaves no residue. The 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, peroxyacetic acid probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (-SH) and sulfur (S-S) bonds (Baldry and Fraser, 1988; Block, 1991).

The sterilant, 3.5% peroxyacetic acid when diluted to 0.2% with filtered water at 50°C had demonstrated excellent microbicidal activity (Tucker *et al.,* 1996; Bradley *et al.,* 1995; Duc *et al.,* 2001).

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. Of the mechanisms that have been studied, the most significant are clearly intrinsic, in particular, the ability to sporulate. In these cases, "resistance" may be incorrectly used and "tolerance," defined as developmental or protective effects that permit microorganisms to survive in the presence of an active agent, may be more correct (Cookson, 1994).

Surfactants inserting in the lipoidal layers disrupt it and create abnormal channels that alter permeability and cause leakage both into and out of the cell (Talaro, 2002). There have been reports of killing by high concentrations (0.2%) of sodium dodecyl sulphate (Baker *et al.,* 1941).

Our target is to examine relative sensitivity of microbial isolates to the disinfectant under study and to find the influence of surfactant on disinfection process. Peroxyacetic acid was chosen because of its safety for both environment and health in application in direct product contact area, wide applicability in many fields, and recommendation by guidelines.

MATERIAL AND METHODS

Methods used in preparation of microorganisms and validation of biocidal effect were adopted from Clontz (Clontz, 2008) with modification.

Preparation of Microorganisms

(A) Suspension of test microorganisms was prepared by growing each organism on a suitable medium and incubating at an appropriate temperature, and then a suspension was made for each isolate in 0.9% saline using loop to form stock solution.

(B) For spore forming microorganisms (*Bacillus species*) sporulation was insured by growing *Bacillus* isolates on suitable medium and at suitable temperature (30–35°C) for sufficient time to insure complete sporulation (5–7 days), then heat shock at 65–70°C for 30 minutes if necessary to achieve this purpose followed by ice bath at 0–4°C.

(C) Microorganism suspension was stored in refrigerator until ready to use. New suspension was made if microbial count decline significantly.

(D) Microbial suspension was quantified for each environmental isolates by making serial dilutions and plate count to determine suspension of concentration 10^5-10^6 CFU in specific volume to be applied on the surface coupon as working suspension.

Validation of Sporicidal Effect of Disinfectant w/o Synthetic Surfactant

(1) Using calibrated micropipette and microbial suspensions was added to the one of 3 surfaces (Floor materials, walls materials, or stainless steel).

(2) Peracetic acid 0.2% was applied to the microbial suspension over the coupon

and 5 minutes exposure was allowed on the surface.
(3) Contact time was done in the normal conditions of working in the facility in term of temperature, humidity, and light.
(4) The entire mixture was transferred after 5 minutes contact to a sterile tube containing suitable neutralizing broth determined from the previous neutralizer evaluation study.
(5) Proper series of dilutions and mixing were done in order to ensure covering maximum range of count.
(6) Plating in suitable agar media and incubation at appropriate temperature and time.
(7) Previous steps were repeated using the remaining test surfaces.
(8) Previous steps were repeated using the remaining challenge microorganisms.
(9) Previous steps were repeated using Peracetic acid 0.2% + Sodium dodecyl sulfate 0.1%.
(10) Previous steps were repeated, except using saline as the test solution. These are the positive plates that provide the base line inoculums concentration.
(11) The baseline inoculums for each microorganism were determined using control group showing at least 10^4 CFU present.
(12) 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.
(13) Significant difference in microbial count compared to the initial result, i.e., greater than a 0.3–0.5 log variation, which is defined as normal plating variability (Duc *et al.,* 2001) was chosen as true criteria of biocidal effect.

Plasmid Profile Analysis (Sambrook *et al.*, 1989; Zhou *et al.*, 2008)

This test was done to determine if the cause of great variability in tolerance to the used disinfectants is plasmid-related or not among Bacillus isolates. Purified plasmid DNA was obtained by alkaline lysis then horizontal agarose gel electrophoresis was carried out. The DNA was visualized by placing the gel on a UV light source.

RESULTS

Determination of the Disinfectant Effectiveness for PAA0.2% and PAA0.2% + SDS0.1%

Table 1 showed that *Bacillus licheniformis* followed by *Bacillus subtilis* had greater resistance to PAA and PAA+SDS than the other spore formers.

Effect of SDS on Biocidal Properties of the Used High-Level Disinfectant

After 5 minutes exposure to disinfectants, it was found that the impact of SDS on the biocidal activity of PAA *Bacillus circulans*, *Bacillus pumilus*, and *Bacillus licheniformis* was indistinguishable from that of plain disinfectant while for the remaining microorganisms SDS increased antimicrobial effect whether the acceptance level has been achieved or not with the exception of *Kocuria rhizophila* which was tested against PAA only. This finding is expressed in Table 2.

Table 1: Effect of PAA 0.2% and PAA 0.2%+ SDS 0.1% on microorganisms using stainless steel, wall and floor material. surface samples.

Surfaces	**Stainless steel**			**Wall**			**Floor**		
	Control*	**Test****		**Control***	**Test****		**Control***	**Test****	
Microorganisms	Positive control (CFUs)	PAA 0.2% (CFUs)	PAA 0.2% +SDS 0.1% (CFUs)	Positive control (CFUs)	PAA 0.2% (CFUs)	PAA 0.2% +SDS 0.1% (CFUs)	Positive control (CFUs)	PAA 0.2% (CFUs)	PAA 0.2% +SDS 0.1% (CFUs)
Bacillus cereus	$1.5x10^6$	544	8	$8.77x10^6$	535	3	$7.13x10^6$	540	2
Bacillus circulans	$1.35x10^6$	0	0	$1.3x10^6$	0	0	$1.3 x10^6$	0	0
Bacillus licheniformis	$2 x10^6$	$1.6x10^5$	$5.6x10^4$	$2.27x10^6$	$1.7x10^5$	$5.8x10^4$	$2.1 x10^6$	$1.5 x10^5$	$5.4 x10^4$
Bacillus pumilus	$3.45x10^6$	2	0	$1.5x10^6$	0	0	$2.7 x10^6$	1	0
Bacillus subtilis	$26.72x10^6$	$7.6x10^4$	170	$8.5x10^6$	$1x10^4$	189	$16.8 x10^6$	$2.7x10^4$	177
Kucoria rhizophila	$2.67 x10^5$	0	0	$2.1 x10^5$	0	0	$2.43 x10^5$	0	0

*: 0.1 mL of organism suspension and 0.1 mL of 0.9% saline on the test surface (base line inoculums concentration).

** : 0.1 mL of organism suspension and 0.1 mL of the double strength prepared sanitizing agent.

CFUs: colony forming units. PAA: peroxyacetic acid. SDS: sodium dodecyl sulphate.

Table 2: Effect of SDS on microbial population elimination expressed by logarithmic reduction (LR) for PAA with different surfaces used.

Surface/Biocide	Coupon surface material						Magnitude of potentiation of biocidal agent by the surfactant		
	Stainless steel		Wall		Floor				
Organism	PAA	PAA+SDS	PAA	PAA+SDS	PAA	PAA+SDS	Stainless steel	Wall	Floor
Bacillus cereus	3.44	5.27	4.21	6.47	4.12	6.55	1.83	2.26	2.43
Bacillus circulans	>6.13	>6.13	>6.11	>6.11	>6.11	>6.11	ND	ND	ND
Bacillus licheniformis	1.10*	1.55*	1.13*	1.59*	1.15*	1.59*	0.45	0.46	0.44
Bacillus pumilus	6.24	>6.54	>6.18	>6.18	6.43	>6.43	>0.3	ND	ND
Bacillus subtilis	1.55*	4.20	2.93	4.65	1.79*	4.98	2.65	1.72	3.19
Kocuria rhizophila	>5.43	NA	>5.32	NA	>6.20	NA	ND	ND	ND

* = Failure to meet acceptance criteria.

NA= Not applicable because *Kocuria rhoizophila* did not pass neutralizer efficacy test with PAA+SDS.

ND= Not determined.

Role of Plasmid on Biocidal Resistance of *Bacillus species*

Plasmids were extracted by alkaline lysis method (Sambrook *et al.*, 1989) and analyzed by horizontal agarose gel electrophoresis and revealed that the great variability in tolerance of *Bacillus species* to biocidal agents used w/o SDS is related to their intrinsic properties and not plasmid-linked. This finding was expressed in Fig. (1).

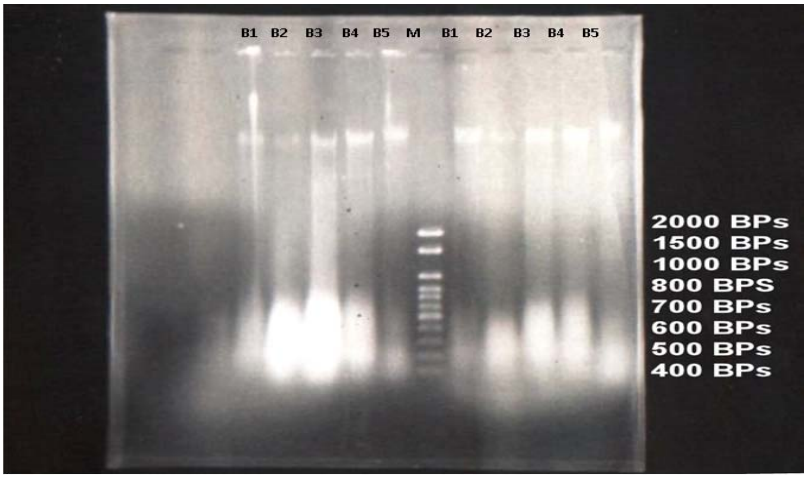

Fig.1: Agarose gel electrophoresis of plasmid DNA isolated from genus *Baci*llus M: marker, B1:*Bacillus subtilis,* B2: *Bacillus circulans,* B3: *Bacillus cereus,* B4: *Bacillus licheniformis,* B5: *Bacillus pumilus.*

DISCUSSION

The contact time between the disinfectant and the microorganisms on a carrier to attain a 6.5 log reduction in the number of viable cells was three times greater than when the cells were in suspension (Kunigk and Almeida, 2001). That is why using carrier test is more challenging than suspension test for disinfectant validation tests.

Bacterial resistance to biocides has long been reported with compounds such as: peroxygens (Greenberg *et al*., 1990; Dukan and Touati, 1996). The enzymatic transformation of biocides has also been described as a resistance mechanism in bacteria peroxygens (catalase, superoxide dismutasex, and alkyl hydroperoxidases) mopping up free radicals (Demple, 1991).

Our results showed that during 5 minutes contact time the ascending order of sensitivity of *Bacillus spp*. Spores was for both PAA and PAA+SDS: *Bacillus licheniformis* > *Bacillus subtilis* > *Bacillus cereus* > *Bacillus circulans* = *Bacillus pumilus*.

Results showed that the order of sensitivity of spores is the same in PAA and PAA+SDS but the magnitude of decrease due to surfactant is greater indicating beneficial effect of SDS on spore elimination if used with sporicidal agent.

Kocuria rhizophila being one of the most frequently isolated non-spore former microorganisms from the environment of pharmaceutical facility especially from the air samples was used for the reason of comparison the effect of biocidal agent against both vegetative and spore forms.

No doubt from previously reported marginal success of *Kocuria rhizophila* among other isolates of cocci in neutralizer efficacy study that this microorganism will not tolerate PAA and from its failure to be recovered from PAA+SDS that this combination will have greater killing effect much than PAA alone.

Organisms from the genus *Bacillus* are widely distributed in nature. However, their primary habitats are the soil, runoffs, dust, and infected plant materials. Spore forming organisms from the genera *Bacillus* are common isolates from pharmaceutical environments, including air and surfaces, often as a result of feet/wheel contamination. Some species are human pathogens, such as *Bacillus cereus*. Other species may be opportunistic pathogens: *Bacillus cereus, Bacillus subtilis, Bacillus circulans*, and *Bacillus pumilus* have all been associated with cases of meningitis, pneumonia, septicemia, and endocarditis. This make them good candidate for any sporicidal evaluation study.

Spore forming bacteria are highly resistant to adverse environmental conditions including dryness, heat, and poor nutrient supply. They are also highly resistant to chemical disinfectants, desiccation, and extremes in pH, temperature, pressure, and ultraviolet and ionizing radiation (Gould, 1977). Spores are highly refractile bodies, consisting of a central core surrounded by five layers; plasma membrane, germ cell wall, cortex, coats, and exosporium. The core, plasma membrane, and germ cell wall constitute a condensed cell which is self-contained and is protected by the outer integument. The complexity of the coat varies between different species (Warth, 1988). This difference is reflected on the great variability between *Bacillus species* spores to disinfectant especially within short contact time (5 minutes). Spores of different *Bacillus species* prepared in similar conditions differed markedly in their resistance to sporicidal agents and by exclustion of plasmid linked tolerance; intrinsic properties of each species play the role in determining proportions of the spore components that influence resistivity to biocidal agent.

Botzenhart and Jaax (1985) found that spores of *Bacillus licheniformis* were more resistant than spores of *B. cereus, B. subtilis*. Killing of spores by PAA, like that by H_2O_2, appeared not to require lysis. Clapp *et al.* (1994) also detected carbon-centered radicals, and it seems that radicals such as $CH_3C(=O)O–$ or $CH_3C(=O)$ may be involved in sporicidal actions of PAA.

The radicals could donate electrons to spore components. In fact, this sort of reduction may be reflected in the lytic phenomenon, which includes decoating. This decoating may include reductive cleavage of disulfide bonds. Decoating is not lethal for the spores, but the decoating reaction may allow peroxyl radicals to act as reducing agents rather than oxidizing agents.

It seems that among diverse bacterial spore response ranging from those highly susceptible to PAA and so SDS impact could not be sensed after 5 minutes exposure (*Bacillus circulans* and *Bacillus pumilus*) and those showing strong resistance in the same condition and time to PAA in which SDS effect is sensed but insignificant (*Bacillus licheniformis*) relay moderately affected spores (*Bacillus subtilis* and *Bacillus cereus*) in which the effect of SDS was pronounced and significantly present.

Surfactants may increase the wetting potential of the spore coat to such an extent as to allow greater penetration of oxidants and into the interior of the spore thus giving greater biocidal effect than disinfectants alone. Another effect of SDS that can assist the biocidal action of PAA on spores is its ability to break any lumps of hydrophobic spores thus increasing surface area of exposure and contact.

In the past few years, industry has moved progressively towards the use of less toxic compounds, such as surfactants (Lindman *et al.*, 1998). They are normally added to increase the washing effect of the sanitation practices because of their ability to alter the surface properties. Furthermore, they lower the surface and interfacial tensions of aqueous fluids, which comprise the ability to wet surfaces, penetrate soil and solubilise fatty materials (McDonnell and Russell, 1999; Glover *et al.*, 1999; Christofi and Ivshina, 2002; Van Hamme *et al.*, 2006).

The increased sensitivity of decoated spores to PAA suggests that the coats may act as a barrier to penetration by the agent or its radicals as they protect spores against other chemical agents. PAA may not move as readily through the integuments into intact spores.

It may be concluded that for routine sporicidal evaluation study one should perform surface challenge tests with the battery of environmental isolates spores and choosing the most resistant one based on this study to test any future sporicidal activity of newly introduced biocide which is in our case is *Bacillus licheniformis.* Moreover we think that in the field of pharmaceutical microbiology, process of spore preparation is needed to be standardized globally & to have universal guidelines since spores properties differ according to conditions of preparation. Finally one can add ingredients that could act in favor of disinfection process that could reduce contact time and concentration of biocidal agent used in pharmaceutical facility if this addition could be validated and proved to be effective and compatible with antimicrobial agent.

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ARABIC SUMMARY

تأثير المنظف الصناعى على النشاط المبيد للأبواغ فى مصنع الأدوية

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برامج التقييم المستمر للمطهرات هامة جداً في التحكم في المحتوى الحيوى الجرثومي فى تلك المنطقة التي المسيطر عليها بيئياً. تم اختيار اختبار الناقل لأنها تعتبر أكثر صعوبة من اختبار المعلق المعتاد. و المواد المستخدمة كسطوح تمثل الحائط، والجدار، والفولاذ المقاوم للصدأ. وأظهرت هذه التجربة أن المنظفات مثل كبريتات دوديسيل الصوديوم تحسن تأثير المبيد لأبواغ الميكروبات حتى مع أولئك الذين أبدوا مقاومة عالية نسبيا لمضادات الأبواغ المستخدمة. المركب المخفض للتوتر السطحى المستخدم عزز النشاط المضاد للأبواغ في غضون خمس دقائق من التعرض مع معظم الأنواع العصوية الشائع العثور عليها في المنشأة الصيدلانية. ومع ذلك أظهرت أبواغ ليتشينيفورميس العصوية أعظم مقاومة بين الميكروبات البيئية المعزولة.