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REVIEW ARTICLE

Assessing the activity of microbicides against bacterial spores: knowledge and pitfalls

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Summary

Bacterial endospores (spores) have a higher intrinsic resistance to microbicides as compared to other microbial forms, most likely due to their impermeable outer layers and low water content. Though structural differences between the spores of various bacterial species may account for observed variations in their resistance to microbicides, flaws in methods for testing the sporicidal activity of microbicides often exaggerate the differences. This has major implications when considering the selection of one or more surrogates to assess microbicides against clinically relevant spore-formers such as *Clostridium difficile*. The mounting significance of *Cl. difficile* as a pathogen is leading to a corresponding increase in the number of commercially available microbicidal formulations claiming activity against its spores without proper differentiation between the product's sporistatic and sporicidal actions. In this review we critically assess the situation and the implications of product claims on the field use of microbicidal products.

Introduction

When applied to surface disinfection treatments, the terms 'microbicidal' and 'microbistatic' relate to a chemical's ability to either kill or actively prevent the growth of a given micro-organism respectively. In reality however, the distinction between the two definitions is not so straightforward; many microbistatic treatments may exhibit a microbicidal activity depending on concentration, temperature and/or contact time. Conversely, microbicidal formulations may demonstrate 'static' activity at lower concentrations or under suboptimal conditions of exposure time or temperature (Maillard 2002; Maillard and McDonnell 2012; Pankey and Sabath 2004). The distinction between these two terms is further blurred when applied to bacterial spores, which are naturally under self-imposed 'stasis' or 'dormancy' without any exposure to microbicides. The transformation of a spore to an actively dividing vegetative form is a multistage process including germination, outgrowth and binary fission (Leggett *et al.* 2012).

Simply put, any sporicidal treatment must achieve a complete and permanent loss of the spore's ability to germinate and grow. In contrast, exposure to a sporistatic treatment may temporarily arrest its ability to germinate without affecting its viability. Owing to the relatively complex cascade of events taking place during the transformation of a spore to a vegetative cell (outlined below), both these definitions are open to misrepresentation/interpretation as they give no clear indication as to how, or at which stage of the transformation process a treatment inhibits the progression from spore to vegetative cell, or whether it is the vegetative cell growth itself which is inhibited (Russell 1982).

The life-cycle of a spore-forming bacterium can be described as a continuum from vegetative cell growth to dormant spore and back again via the processes of sporulation, germination and outgrowth. Germination can be further broken down into several defined stages (Setlow 2003) of which stage-I encompasses those events taking place prior to the degradation of the spore cortex, including the release into the surrounding medium of many of the spore core's constituents (various cations and the spore's large depot of dipicolinic acid (DPA) which is chelated with divalent cations, predominantly Ca^{2+}), and is accompanied by some core hydration, while stage-II sees the degradation of the spore's peptidoglycan cortex

Table 1 Examples of sporicidal chemicals

Chemical class	Chemical	Comments
Alkylating agents	Ethylene oxide (8·5–100%)	Gas which can be used alone or in combination with other carrier gases Articles need aeration following exposure
	Glutaraldehyde (2–3·5%)	Sporicidal activity requires 3 h or more at room temperature Raising of pH (activation) often required for a general enhancement in microbicidal activity
	ortho-phthalaldehyde (0·55%)	Requires 24–30 h at room temperature for sporicidal activity
	Formaldehyde (37%)	Can be used as gas (from paraformaldehyde) or liquid Can be used in combination with ethanol Articles need aeration following exposure
Oxidizing agents	Hydrogen peroxide (0·5–70%)	Can be used as liquid, vapour or gas plasma Sporicidal activity in liquid form requires acidic pH and addition of stabilizers and accelerants May be used in combination with other oxidisers such as peracetic acid
	Peracetic or peroxyacetic acid (0·05–1%)	A strong and fast-acting sporicidal chemical Can be generated inside certain types of automated endoscope reprocessors
	Chlorine dioxide (150 ppm)	Requires on-site generation by mixing citric acid with a solution of sodium chlorite
	Ozone	A powerful oxidizing gas Its activity is severely affected by organic matter, low temperature and relative humidity
Chlorine-releasing agents	Sodium hypochlorite (5·5–12%)	Commonly referred to as chlorine bleach Acidification can accelerate sporicidal action
	Sodium dichloroisocyanurate	Less susceptible to inactivation by organic matter Less corrosive than hypochlorites
	Chloramine-T	More stable than hypochlorite Efficacy probably linked to the release of HOCl following hydrolyses explaining a slow microbicidal action compared to hypochlorites
	Calcium hypochlorite	Calcium hypochlorite products are soluble in water and stable over long storage time

and further hydration and expansion of the core. This precedes the onset of outgrowth where metabolism and macromolecular synthesis are reinitiated, along with the degradation of the spores' DNA-protective small acid-soluble spore proteins (SASPs) and shedding of the spore coat, returning the bacterium to vegetative cell growth (Russell 1982; Setlow 2003; Leggett *et al.* 2012).

As discussed below, much of the confusion surrounding the characterization of a treatment as either sporicidal or sporistatic centres on the question, 'when is a spore no longer a spore?' This review presents the finer details of sporicidal or sporistatic treatments in order to clarify certain aspects of these definitions in the light of the more recent literature and discuss practical implications on testing of sporicidal formulations and on disinfection regimes.

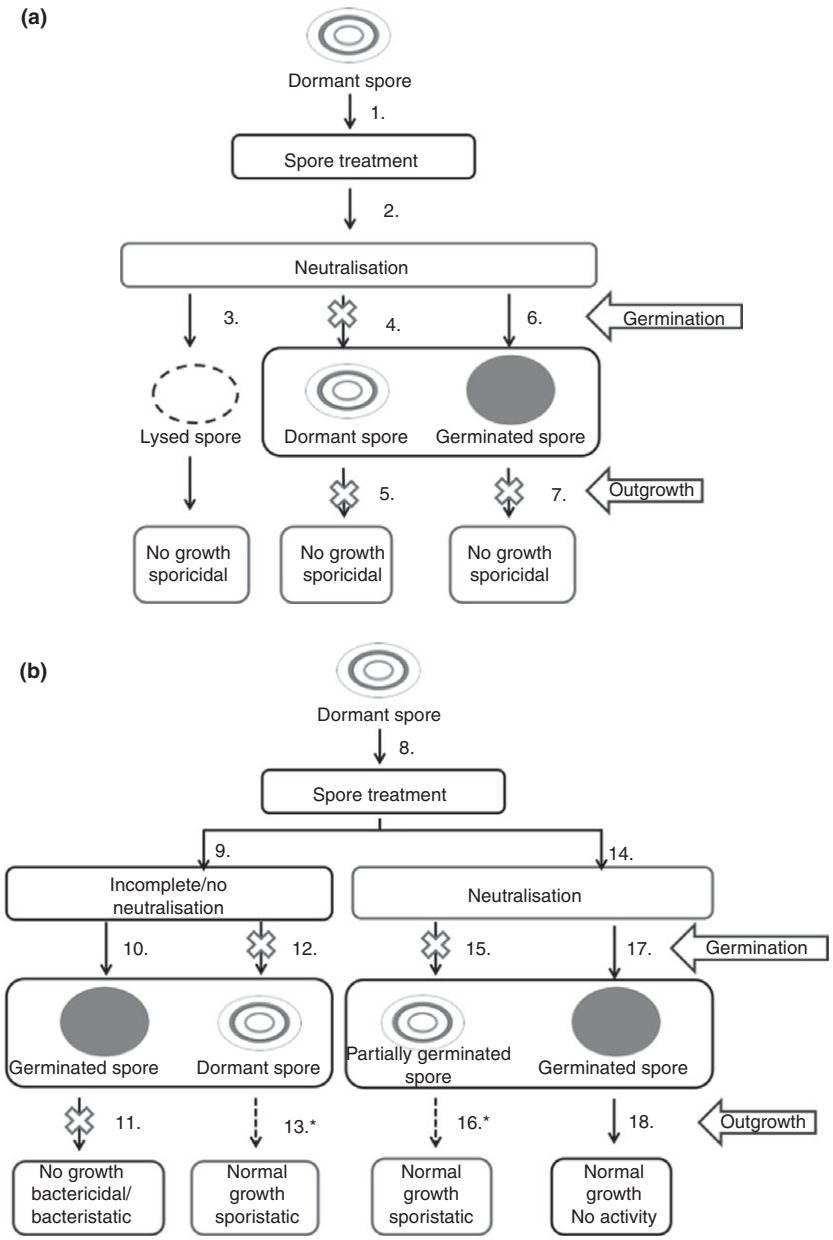
Sporicidal and sporistatic activity of microbicidal treatments

The usual microbicides with documented sporicidal activity are briefly listed in Table 1. It is not intended that this review should provide an exhaustive list of chemical classes and their activity against bacterial spores (readers wishing for such information are referred to McDonnell and Russell (1999) and Maillard (2011), but rather to discuss clarification of the terminology and its implications.

Sporistatic activity – inhibition of spore germination process

Sporistatic treatments should be defined as those that specifically prevent spore germination only (Fig. 1b). The spore remains dormant and viable and can, therefore, resume the germination process upon removal/neutralization of the inhibiting agent (see 'exception that proves the rule' below). In other words, 'sporistasis' is a transient and reversible state.

References to sporistatic activity in the literature are often somewhat confusing as they encompass treatments that prevent both spore germination (which does not require an assessment of microbial growth or colony formation) and/or outgrowth (most commonly assessed by colony formation/growth). The main element of confusion here is that outgrowth is not an intrinsic property of



the *dormant* spore, and therefore should not necessarily be associated with the prefix 'spori' at all, but should be referred instead as bactericidal or bacteristatic. Below are given some examples of various microbicidal treatments and an explanation of their classification according to our definition.

Several cationic microbicides, for example, the quaternary ammonium compounds benzalkonium chloride and cetylpyridinium chloride, or the bisbiguanide chlorhexidine, do not inhibit spore germination although they do prevent progression through outgrowth if not effectively neutralized and are commonly described as sporistatic in the literature (Fig 1b; legend scenario iv) (Russell *et al.* 1985; Shaker *et al.* 1986; Russell 1998). We suggest that such treatments not be classed as sporistatic as they do not inhibit any intrinsic property of the dormant spore. Indeed, it is commonly remarked in the literature that 'sporistatic' concentrations of such microbicides are very similar to those that inhibit vegetative cells (Russell 1990, 1998). Therefore, it would seem likely that such activity against spore outgrowth is bacteristatic or bactericidal but not sporistatic as often mentioned. It should be noted that under certain conditions, such as alkalinization, acidification and increased ionic strength, treatment with at least chlorhexidine can become sporicidal (Nerandzic and Donskey 2015; Nerandzic *et al.* 2015).

Figure 1 An illustration of the potential outcomes from a microbicide treatment of bacterial spores. Altogether seven scenarios can be presented. (a) Scenarios leading to a sporicidal activity. Scenario (i) The spore is treated with a microbicide/formulation (1), which is neutralized completely (2), and results in lysis of the spore (3). The microbicide/formulation is therefore sporicidal. Scenario (ii) The spore is treated with a microbicide/formulation (1), which is neutralized completely (2), but does not undergo or complete germination even with additional treatments (4). Consequently, the spore is unable to complete outgrowth and grow (5). The spore is inactivated. Scenario (iii) The spore is treated with a microbicide/formulation (1), which is neutralized completely (2), and then undergoes germination (6). However, the spore is unable to complete outgrowth (7) and thus is inactivated. Such a microbicide/formulation is sporicidal. (b) Scenarios leading to a sporistatic activity. Scenario (iv) The spore is treated with a microbicide/formulation (8) which is neutralized ineffectively (9) leaving residual microbicide in contact with the spore. The spore germinates normally (10) thus losing much of their enhanced resistance properties leaving them vulnerable to the residual microbicide resulting in killing of the organism which therefore cannot complete outgrowth or start dividing (11). Scenario (v) The spore is treated with a microbicide/formulation (8) which is not neutralized (9). In the presence of this microbicide, the spore is unable to germinate (12). This treatment is therefore sporistatic and upon complete removal of the microbicide (13) spores are able to complete germination and outgrowth, returning to vegetative cell growth. Scenario (vi) The spore is treated with a microbicide/formulation (8), which is neutralized completely (14), but the spore still fails to germinate (15). However, the treated spores can be revived by additional treatment (e.g. exposure to lysozyme), which allows the spore to complete germination (16) and outgrowth returning to vegetative growth. The microbicide/formulation is therefore sporistatic, although the spore, which remains viable, but unable to germinate completely under normal conditions, could fall under the viable but noncultivable (VNC) definition. Scenario (vii) The spore is treated with a microbicide/formulation (8), which is neutralized completely (14), and then undergoes germination (17) and outgrowth (18) as normal and resumes vegetative cell growth. Such a microbicide/formulation is neither sporicidal but may be sporistatic if the microbicide is not removed (scenario v). *Denotes the requirement for some additional agent (e.g. lysozyme) to resume germination.

While in the presence of some microbicides, bacterial spores are prevented from germinating but undergo no readily measurable damage, and remain in a dormant state. The spores are eventually able to return to vegetative growth following removal/neutralization of the microbicide (Fig. 1b; legend scenario v). Such a treatment has not compromised the viability of the spore and should therefore be considered sporistatic. Phenol and cresol are two examples of sporistatic treatments. Spores exposed to them undergo no detectable germination in broth (as measured by a decrease in optical density; OD), although they proceed through outgrowth if these chemicals are removed, by membrane filtration, for example (Parker 1969; Russell *et al.* 1985).

Sporicidal activity

Sporicidal treatments are those that result in the irreversible loss of spore viability, although the situation is more complicated than for bactericidal activity.

Some treatments (e.g. strong acids) cause spores to rupture, rendering them unable to germinate or form a colony on a plate regardless of any subsequent treatments, for example, neutralization of the acid or treatment with lysozyme (Fig. 1a; legend scenario i) (Setlow *et al.* 2002). Such a treatment is certainly sporicidal as spore viability is unquestionably compromised.

Oxidizing agents are commonly used as sporicides (Maillard 2011) and, given specific treatment conditions, can result in spore lysis as described above for strong acids (King and Gould 1969). However, treatment with oxidizing agents such as hydrogen peroxide, sodium hypochlorite and peracetic acid does not necessarily result in spore lysis. Following exposure to these oxidizing agents, spores are left unable to form colonies even after neutralization of the microbicide. A subsequent lysozyme treatment of such treated spores can often give apparent spore germination, but these germinated spores exhibit little or no metabolic activity and do not outgrow (Melly *et al.* 2002; Young and Setlow 2003; Setlow *et al.* 2013). Likewise Russell (1982) observed that the recovery of microbicide-treated spores was influenced markedly by some additions to recovery media, and also the recovery temperature(s). How then should such treatments be classified? Firstly, given that every effort was made to neutralize/remove the microbicide completely, the observed activity can neither be sporistatic as outlined above, nor can it be bacteristatic/cidal (i.e. from residual activity from any remaining microbicide) (Fig. 1b; legend scenario iv and v). Secondly, as the treated spores cannot be revived by treatment with lysozyme, the activity is not sporistatic as described below (Fig. 1b; legend scenario vi). Finally, spores are not lysed by the treatment, and yet are clearly inactivated. A compromised inner membrane may be the reason for spore inactivation (Shapiro and Setlow 2006). Such a treatment should therefore be considered sporicidal (Fig. 1a; legend scenario iii).

The exception that proves the rule

There is at least one example of a sporistatic treatment that does not fit our definitions, and yet is not truly sporicidal (Fig. 1b; legend scenario vi). Spores treated with sodium hydroxide (NaOH), followed by complete removal/neutralization do not form colonies on a medium that ordinarily supports their growth (Setlow *et al.* 2002); such a treatment would appear sporicidal at first glance. However, spores may be completely recovered if plated on a medium containing lysozyme, indicating no

loss in spore viability; this treatment is therefore not sporicidal. This is most likely a result of damage sustained to part of the spore's germination apparatus, the cortex lytic enzymes (CLE) which are required for degradation of the spore's thick peptidoglycan cortex during germination allowing the spore to swell and return to the vegetative state (Ishikawa *et al.* 1998; Setlow *et al.* 2001, 2002). In the absence of any functional CLE, the spore is trapped at Stage I of germination and cannot return to the vegetative state, but remains viable and may be recovered by lysozyme treatment (Popham *et al.* 1996; Setlow *et al.* 2001; Paredes-Sabja *et al.* 2009; Burns *et al.* 2010). In this instance, NaOH should be considered sporistatic, with the caveat that it does not conform strictly to our definition owing to the fact that such spores are able to partially germinate. Of course, this raises the question of what constitute reasonable recovery conditions.

Suitable methods of assessing sporicidal and sporistatic activities

Sporistatic activity

Historically, a microbicidal treatment would be assigned as sporistatic based on minimum inhibitory concentration (MIC) values determined using broth or agar dilution methods, where the lowest concentration of the microbicide preventing growth in broth is designated the MIC, or minimum sporistatic concentration for spores (Russell 1998). However, in reality, such a method is unsuitable for definitively assessing spore susceptibility, as no information can be gained as to which stage, germination, outgrowth/vegetative cell growth or all of these, is/are being inhibited. Consequently, the observed activity could be sporicidal, sporistatic, that is inhibiting germination, or bactericidal/static by inhibiting outgrowth/vegetative growth.

According to our definition, sporistatic treatments are those that specifically inhibit germination, and not outgrowth/vegetative growth. Therefore, any assessment of sporistatic activity cannot rely on microbial growth, and must be able to distinguish germination from outgrowth/vegetative growth. Several methods may be used to track spore germination, including direct observation of spore refractivity under a phase-contrast microscope (spore refractivity decreases during germination and can be observed as a transition from phase bright to phase dark spores), monitoring the optical density of a spore population (as the OD of a spore population decreases ~60% during germination) or by assaying for pyridine-2,6-dicarboxylic acid (dipicolinic acid – DPA) released during spore germination using a fluorometric analysis (Russell 1998; Hindle and Hall 1999; Yi and Setlow 2010). Spore germination requirements, and especially outgrowth can change after putative microbicide treatment, as treated spores often required very rich media, and are more sensitive to salt in plating media. Other, more intricate analyses can also monitor the germination of individual spores such as phase-contrast microscopy (or differential interference contrast microscopy) in combination with Raman spectroscopy to monitor DPA release (Kong *et al.* 2010; Zhang *et al.* 2010).

Following assessment of germination, spores must also be assessed for viability, as only those treatments, which *temporarily* prevent spore germination should be characterized as sporistatic, and upon removal of the inhibition (or following reasonable recovery conditions – see below) the spores should germinate normally, returning to vegetative growth. If spores do not return to vegetative growth then the process should be further investigated for sporicidal activity as outlined below. Note that a return to vegetative growth is dependent upon complete neutralization of any microbicide, and the presence of a growth-medium, and as such, would have to be assessed separately from the assessment of germination. Additionally, successful germination alone cannot be taken as a definitive indication of spore viability, as some treatments result in spores that germinate relatively normally, but do not outgrow and do not give rise to growing cells (Setlow *et al.* 2013).

Table 2 Common standard tests use to determine the sporicidal activity of a product

Test designation	Type of test	Organism(s) used
European Committee for Standardization (http://www.cen.eu/Pages/default.aspx; accessed September 2015)		
EN14347	Basic sporicidal activity – (phase 1) – suspension test	*Bacillus subtilis*
EN13704	Quantitative suspension test (phase 2, step 1)	*B. subtilis*
ASTM International (http://www.astm.org/; accessed September 2015)		
E2111	Glass vials – surface test	*B. subtilis* and *Clostridium sporogenes*
E2197	Stainless steel disks – surface test	*B. subtilis* and *Cl. sporogenes*
AOAC International (http://www.aoac.org/iMIS15_Prod/AOAC; accessed September 2015)		
AOAC International (996·04)	Porcelain cylinders and silk or Dacron suture loops – surface test	*B. subtilis* and *Cl. sporogenes*

Sporicidal activity

Sporicidal activity of microbicides is conventionally assessed using a suspension test, such as the BS EN 13704 standard efficacy test, where spores are exposed to a chemical for a given contact time after which the chemical is removed by membrane filtration and/or neutralized using an appropriate neutralizer and the colony formation resulting from the germination and outgrowth of viable spores enumerated on a growth medium (Humphrey 2011; Table 2). In North America only carrier tests are used for that purpose. They are based on the standards of either AOAC International or ASTM International (Humphrey 2011; Table 2). Whatever the standard sporicidal test, appropriate neutralization is essential in order to correctly characterize a sporicidal process, as any remaining microbicide could have a sporistatic activity on the surviving spore population (Fig. 1b; legend scenario v) or a bacteriostatic/cidal activity on the germinated or outgrowing spore (Fig. 1b; legend scenario iv), both of which would be mischaracterized as sporicidal under this test procedure.

Conclusions

This review aimed to refine the definition of sporistatic and sporicidal activity. One important question is whether preventing spore germination (sporistatic) or inactivating the spores (sporicidal) really matters in practice or not. Sporistasis remains a transient condition, whereby if the selective pressure is removed, the spore remains viable with the potential for outgrowth. In this review we mentioned the ability of lytic enzymes such as lysozymes to *resurrect* inactivated spores. When this principle is applied to *Clostridium difficile*, one can wonder if a viable spore that cannot germinate following a microbicidal treatment, could do so in the human gut, which is rich in lysozymes. Most protocols designed to cultivate *Cl. difficile* from the environment now utilize lysozyme in the growth media to promote recovery, but the use of lysozymes is not widespread in sporicidal standard efficacy tests.

Many products claiming sporicidal activity are based on one or more quaternary ammonium compounds (QAC) (Siani *et al.* 2011), which often makes their effective neutralization difficult (Zhang *et al.* 2010). This can result in a sporistatic or/and bacteristatic/cidal activity as mentioned in this review. But whether this is due to the action on the germinated spores or the process of outgrowth is most often not clear. Thus, an inhibitor of DNA replication would act only late in outgrowth, while a protein synthesis inhibitor would act to block outgrowth. Further research is clearly needed to ascertain how proper neutralization or removal of the active agent (s) can be achieved to ensure that claims for sporicidal activity are based on solid experimental data. At the same time, the practical application of sporistasis, notably with pathogens such as *Cl. difficile*, needs to be better understood and the pitfalls in use of any sporistatic agent need to be appreciated.

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None.

Conflict of Interest

None.

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