Akwaton, polyhexamethylene-guanidine hydrochloride-based sporicidal disinfectant: a novel tool to fight bacterial spores and nosocomial infections

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Bacterial spores are of continuing interest to the food and medical industries. In efforts to eliminate bacterial spore contamination, a number of sporicidal agents have been developed. Most of these compounds must be used carefully in very specific circumstances as they are toxic to humans. The sporicidal activity of Akwaton, a polyhexamethylene-guanidine hydrochloride (PHMGH)-based disinfectant, was tested against Bacillus subtilis spores. PHMGH is a colourless, odourless, non-corrosive and non-irritating antimicrobial biocide of the guanidine family. Spores suspended in distilled water and spores placed on solid surfaces (stainless steel and glass) were used to determine the log 10 reduction after exposure to varying concentrations of Akwaton. The minimum sporostatic concentration, the minimum sporicidal concentration and the time required for sporicidal activity corresponded to 0.06% (w/v), 0.08% (w/v) and 8.5 min, respectively. Disinfectant concentrations of 0.24% (w/v) and 0.44% (w/v) killed all spores suspended in distilled water within 3 min and 90 s, respectively. The sporicidal activity against suspended spores was linearly dependent with respect to the concentration of PHMGH and contact time ($y_{3\text{ min}} = 4x - 1.6$ and $y_{90\text{ s}} = 20x - 0.8$ thus $y_{3\text{ min}} = 2y_{90\text{ s}}$). Spores placed on surfaces were more resistant to the effect of the disinfectant and the positive linear correlation between the sporicidal activity and concentration was not observed. The concentration required to kill all spores placed on a surface (stainless steel or glass) corresponded to 0.52% (w/v) for 90 s of contact and 0.36% (w/v) for 3 min. This study demonstrated that PHMGH is an effective sporicidal disinfectant with great potential for use in hospitals, laboratories, food industries and households.

INTRODUCTION

Bacterial spores are of considerable interest due to their remarkable resistance to physical agents and regular antiseptics. Their tolerance to heat and the fact that they can survive a number of years in a dried state are of great importance as much in medicine as in food preservation. Very few disinfectants are able to destroy spores irreversibly (Huber, 1982). Their complex structure characterized by several concentric layers rich in disulfur bridges, the presence of calcium dipicolinate and their hydrated state are at the origin of their resistance to temperature change, desiccation, nutritional deficiencies as well as various chemical and physical agents (Russell, 1990; Oulé et al., 2010). Most chemical agents, including phenols, cresols, alcohols (Labbe et al., 1978), biguanides and quaternary ammonium compounds, often used as disinfectants or antiseptics are more sporostatic than sporicidal (Cook & Pierson, 1983; Russell et al., 1985). These chemicals generally prevent the germination of spores or the outgrowth of germinated spores, but are incapable of irreversibly killing spores (Russell, 1990). For example, while chlorhexidine, a biguanide biocide currently used as a disinfectant, is bactericidal towards both Gram-positive and Gram-negative bacteria at 0.05%, it does not kill bacterial spores at that concentration (Russell, 1990). However, chemicals can be sporicidal when they are used at very high concentrations or combined with other physical or chemical factors such as temperature, pH and treatment time. In effect, 0.05% chlorhexidine is sporicidal when it is applied at high temperature (Shaker et al., 1986; Gorman et al., 1987).
Chatauev & Peterson (2010) reported that disinfection by pulverizing an aqueous solution of chlorine dioxide (10 mg ml\(^{-1}\)) on surfaces only reduced *Bacillus anthracis* spores by 1 log\(_{10}\). However, when combined with 0.3 % sodium hypochlorite, the pulverized chlorine dioxide solution (10 mg ml\(^{-1}\)) completely destroyed the spores. A 1.5–4 % sodium hydroxide solution containing sodium hypochlorite (200 μg g\(^{-1}\)) produced a more rapid sporicidal effect than either sodium hydroxide or sodium hypochlorite when applied separately (Cousins & Allan, 1967). Iodine and hydrogen peroxide are microbicidal agents capable of killing bacterial spores, but their respective effects depend upon several factors (Russell, 1990). The sporicidal activity of iodine is strongly influenced by its concentration and pH (Balassa et al., 1979). The sporicidal activity of hydrogen peroxide is well documented (Bayliss & Waites, 1979; Baldry, 1983; Russell, 1990; Khadre & Yousef, 2001). At 6 % (v/v), hydrogen peroxide kills bacterial cells and is sporicidal at concentrations higher than 10 %. Aldehydes, especially formaldehyde and glutaraldehyde, are classed among the most efficient antimicrobials. Their microbicidal effect is due to the inactivation of proteins. Formaldehyde is sporicidal at 4–8 % (Russell, 1990) and its effect is accentuated when the temperature is above 40 °C (Trujillo & David, 1972). Glutaraldehyde, whose sporicidal effect is more rapid than that of formaldehyde (Power, 1997), kills bacterial spores at a concentration of 2 % (Power & Russell, 1990), its effect being more pronounced at alkaline pH (Gorman et al., 1980). Ethylene oxide is a sterilizing gas usually used in closed chambers. Because of its explosive nature, it is necessary to mix it with non-flammable substances such as CO\(_2\) or N\(_2\). Its use does not require high temperatures and as a result it is used in hospitals to sterilize medical material such as electronics and plastic objects. Its effect is influenced by a number of factors such as concentration, treatment time, temperature, relative humidity and the type of micro-organism (Reich, 1980; Russell, 1990).

For over half a century, cationic biocides have been prominent among other agents used to combat cross-infections and have contributed to the overall reduction in nosocomial infections (Gilbert & Moore, 2005). Correct application of these biocides plays a very effective role in the elimination of infection in veterinary, dental, domestic and hospital settings (McDonnell & Russell, 1999). Polyhexamethylene biguanide, a member of the polymeric guanidine family, has broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, yeasts (Müller & Kramer, 2005) and viruses, including human immunodeficiency virus (Kreib et al., 2005). It has been widely used for many years as an antiseptic in medicine and the food industry, as a mouthwash (Rosin et al., 2001), as a disinfectant for a variety of solid surfaces (Hiti et al., 2002) and also in water treatment (Kusnetsov et al., 1997). The bactericidal activity against *Escherichia coli* and meticillin-resistant *Staphylococcus aureus* (MRSA) and the mode of action of polyhexamethylene-guanidine hydrochloride (PHMGH) have been clearly demonstrated by Oulé et al. (2008). PHMGH is a polymer with high solubility in water. It is odourless, colourless and non-corrosive (Kuznetsov, 2004) and is significantly less toxic than currently used disinfectants (Müller & Kramer, 2005) to humans and animals at a concentration ≤1 %. To demonstrate the potential use of PHMGH as an effective sporicidal disinfectant that is less hazardous than currently used disinfectants, its sporicidal activity was tested against *Bacillus subtilis*.

**METHODS**

**Preparation of spores.** *B. subtilis* ATCC 6051 used in this study was acquired from the André Fréchette Laboratory in the Department of Biological Science, Faculty of Science, Université de Saint-Boniface. Spores were prepared using the method described by Kunihiko et al. (2010). Nutrient agar (NA; Difco) plates were inoculated with a *B. subtilis* culture in nutrient broth (NB; Difco) and incubated at 37 °C for 7 days. Spore formation (>90%) was verified by microscopic examination. The spores were collected by scraping and suspended in 10 ml distilled water and purified by repeated centrifugation for 10 min at 15 000 g. The spores were resuspended in 10 ml distilled water and incubated at 70 °C for 20 min to eliminate vegetative cells. The spores were then washed by centrifugation in sterile distilled water for 10 min at 15 000 g twice before treatment. All spore preparations used were free of sporing cells, cell debris and germinated spores. Spore resistance to HCl was tested following the method described by Setlow et al. (2002) with modifications. The spore suspension was added to a tube containing 5 ml 2 M HCl to achieve a final concentration of 10\(^{-5}\) c.f.u. ml\(^{-1}\). Samples were diluted 100-fold in 1 M KPO\(_4\) buffer (pH 7.5). Aliquots were then plated on NA before incubating for 48 h at 37 °C to determine spore viability. Spore suspensions were stored for no more than 3 weeks at 4 °C. Before each experiment, microscopic observation was performed to verify that spores did not germinate.

**Determination of the minimum sporicidal concentration (MSTC) and the minimum sporicidal concentration (MSDC).** Akwaton solutions containing 0.01–0.5 % PHMGH were prepared in sterile distilled water and stirred for 30 min. Assessment was performed using the broth dilution technique (Soborén et al., 2007; Oulé et al., 2008). Serial dilutions (0.01–0.15 %) of the disinfectant with a final volume of 5 ml were made in the growth medium [NB supplemented with yeast extract (0.3 %, w/v) and glucose (0.5 %, w/v)] in 15 x 150 mm test tubes. Spore suspension was added to each test tube to achieve a final inoculum of 10\(^{6}\) c.f.u. ml\(^{-1}\). The tubes were agitated before being incubated at 37 °C for 48 h. Positive and negative controls were growth medium with and without spore suspension. Spore germination and bacterial growth were indicated by turbidity; absence of bacterial growth signified inhibitory activity. The MSTC, expressed as a percentage, was the lowest concentration of the disinfectant at which there was no growth (absence of turbidity). The MSDC was the lowest concentration of the disinfectant at which spores were killed. To determine the MSDC, a sample taken from each test tube where there was no growth in the MSTC assay was inoculated in NB without disinfectant for 48 h at 37 °C, then plated on NA without disinfectant and incubated for 48 h at 37 °C. Controls included aliquots taken from growth control tubes. Each test was performed in duplicate and repeated three times.

**Determination of time required for complete sporicidal activity of PHMGH at the MSDC.** The assay was performed following the method described by Oulé et al. (2008) with modifications. A volume of 1.5 ml spore suspension (1 x 10\(^{6}\) c.f.u. ml\(^{-1}\)) was added to a tube...
containing 1.5 ml disinfecting solution at the MSDC (0.08 %). Tubes were agitated and placed in the water bath at 20 °C, then 0.1 ml spore suspension and disinfectant mixture was subcultured every 30 s for a period of 10 min. Individual subculture tubes were thoroughly agitated before being incubated at 37 °C for 2 days. Spore germination and bacterial growth were indicated by turbidity, and the absence of growth was interpreted as sporicidal activity. After incubation, 100 μl aliquots from each subculture tube were plated on NA without disinfectant and incubated for 48 h at 37 °C to confirm the results. Each test was performed in duplicate and repeated three times.

**Disinfectant assays**

**Spore suspension inactivation method.** The spore-killing power of PHMGH was demonstrated following the method described by Chatuev & Peterson (2010) with modifications. In the suspension–dilution method, 500 μl spore suspension (1 x 10⁶ c.f.u. ml⁻¹) was mixed with 500 μl of each disinfecting solution (0.08–0.8 %, w/v) for 90 s and 3 min. The spores were quickly separated from the disinfectant by diluting and washing with 10 ml sterile distilled water and centrifugation at 15000 g for 10 min. The survival of spores was assessed by serial dilution and plating on NA supplemented with yeast extract (0.3 %, w/v) and glucose (0.5 %, w/v). In the control, distilled water was used instead of disinfectant. Each test was performed in duplicate and repeated three times.

**Disc carrier method.** Carriers (stainless steel discs (18 mm diameter, grade 430, 18 gauge)) were brushed and washed with detergent in hot water, rinsed in distilled water and dried at 50 °C in a dry heat oven (Fisher Scientific, model DKN-810, no. 13-263-55). They were then wrapped in aluminium foil and sterilized by dry heat in an autoclave for 60 min. Each sterile disc carrier was inoculated by placing 50 μl spore suspension (1 x 10⁶ c.f.u. ml⁻¹) in the centre to avoid splashing or bubbles. Inoculated carriers were dried at 50 °C in an oven for about 25 min. After cooling to room temperature, the dried inocula on disc carriers were carefully covered with 100 μl disinfecting solution (0.08–0.8 %, w/v) for 90 s or 3 min in a class II biosafety cabinet. Immediately, each treated carrier was carefully placed in a sterile straight-side Pyrex test tube (20 x 150 mm) containing 10 ml sterile distilled water next to a flame. Tubes were vortex-mixed (American Scientific product, model 58223-1, no. 024662) for 3 min and the survival of spores was assessed by serial dilution and plating on NA. For the control, distilled water was used instead of disinfectant. Efficiencies for all spore extractions from carriers were 100 %. All spores were removed from the carriers in the control. Each test was performed in duplicate and repeated three times.

**Glass surface technique.** Pyrex brand rimless culture tubes (Fisher Scientific) with flat bottoms (25 x 100 mm) were washed with detergent in hot water, rinsed in distilled water and sterilized by dry heat in an autoclave. The bottom surface of each sterile tube was inoculated by placing 50 μl spore suspension (1 x 10⁶ c.f.u. ml⁻¹) in the centre to avoid splashing or bubbles, in a class II biosafety cabinet. Tubes containing spore suspensions were dried at 50 °C in an oven for about 25 min. After cooling to room temperature, the dried inocula on the flat bottom of tubes were carefully covered with 100 μl disinfecting solution (0.08–0.8 %) for 90 s or 3 min in a class II biosafety cabinet. Immediately after the end of the exposure time, 10 ml sterile distilled water was added to the tubes. Tubes were vortex-mixed for 3 min and the survival of spores was assessed by serial dilution and plating on NA. In the control, distilled water was used instead of disinfectant. Each test was performed in duplicate and repeated three times.

**Data analysis.** Reduction in the number of spores was expressed as the log₁₀ of the ratio between the number of viable spores initially present and the number of spores after exposure to the disinfecting solution [log₁₀ (N₀/N)]. N₀=number of viable spores before treatment and N=number of viable spores after treatment.

The results were statistically examined as mean values by one-way analysis of variance.

**RESULTS**

The hardness of the spores was determined using a HCl resistance test. This test showed that the spores were resistant to the action of 2 M HCl for a period of 5 min of exposure (Fig. 1), which confirmed that the spores used for testing the sporicidal activity of Akwaton for 90 s and 3 min were in good condition. Tests conducted to determine the sporicidal activity of Akwaton showed that a concentration of 0.06 % was the MSTC of the disinfectant. In fact, after 48 h incubation at 37 °C, the growth medium in the spore-containing tubes was turbid at less than 0.06 % disinfectant, indicating germination of the spores, outgrowth, and growth and development of vegetative cells. In these tubes (<0.06 %), the spores resisted the disinfectant. However, in the tubes having disinfectant at a concentration of 0.06 % or higher, the medium stayed clear, indicating the inhibition of vegetative cell formation. The concentration 0.06 % can be defined as the MSTC of the disinfectant. To determine the MSDC, aliquots taken from the tubes containing disinfectant at a concentration of 0.06 % or higher were inoculated on NA (plating) without disinfectant and incubated at 37 °C for 48 h. Spores from the tubes having 0.06 % disinfectant did in fact grow on NA, indicating that the disinfectant at these concentrations did not kill the spores, but merely prevented formation of vegetative cells and growth. At these concentrations (0.06 and 0.07 %, w/v), the disinfectant had a sporicidal effect on the spores. However, spores from tubes with disinfectant at a concentration of 0.08 % or higher did not grow, indicating that the spores were dead. Thus, a concentration of 0.08 % can be defined as the MSDC. At concentrations higher than or equal to 0.08 % (w/v), the disinfectant had a sporicidal effect. Tests conducted to determine the time required for PHMGH to achieve total sporicidal activity showed that after 8.5 min of exposure to the MSDC (0.08 %, w/v, of Akwaton), all the spores were killed. To determine the disinfectant concentration needed to kill all the spores in a short period of contact time, we subjected them to a series of concentrations starting at the MSDC (0.08 %) for 90 s and 3 min. At concentrations of 0.24 and 0.44 %, the disinfectant killed all the spores after contact for 3 min and 90 s, respectively. These two concentrations (0.24 and 0.44 %) can be defined as sufficient sporicidal concentrations (SSCs) for 3 min and 90 s contact times. As shown in Fig. 2, between the MSDC (0.08 %) and 0.24 % (SSC 3 min) for 3 min contact time and 0.44 % (SSC 90 s) for 90 s contact time, sporicidal activity is linearly dependent on the disinfectant concentration in terms of log₁₀ reduction. This positive linear correlation is expressed as follows:

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and Clostridium cells, but have no effect on spores (Shaker et al., 1986). The complex structure of bacterial spores and especially the presence of coats and some cortices are partly responsible for their resistance to antiseptics and disinfectants. This resistance develops during sporulation (Gorman et al., 1987). Spore resistance to phenols, quaternary ammonium compounds and chlorhexidine develops during cortex synthesis and is reinforced at the beginning of coat synthesis (Knott et al., 1995). Resistance to chloroform, as well as to phenol and alcohols, develops later during sporulation (Balassa et al., 1979). Much work has been done on the inactivation of bacterial spores, but the sporicidal effect of disinfectants has not been well studied. Most biocides, even powerful bactericides, are more sporostatic than sporicidal (Russell, 1990; Sykes, 1970; Gorman et al., 1984). Their sporostatic activity is due to inhibition of germination or outgrowth. Phenols and cresols inhibit spore germination (Russell & Hugo, 1987). Quaternary ammonium compounds and biguanides are strongly bactericidal and sporostatic. Their sporostatic activity manifests itself by the inhibition of outgrowth and not by the inhibition of germination (Russell et al., 1985). This study has shown that PHMGH is sporostatic from 0.06 to 0.07%. This activity functions by the inhibition of spore germination or of outgrowth or both at the same time. At concentrations higher than or equal to 0.08%, PHMGH is sporidical. Tennen et al. (2000) reported that the sporicidal activity of iodine against B. subtilis was not due to DNA damage but to the inactivation of corticolytic enzymes that are specific to spore germination or to the alteration of the cortex structure. The sporicidal activity of oxidizing biocides such as hypochlorite and chlorine dioxide against Bacillus rests on their ability to penetrate spore coats (Rogers et al., 2005; Young & Setlow, 2003) causing damage to the internal membrane and the cell wall (Rogers et al., 2005) and destabilizing structural proteins via interaction with disulfur bonds and thiols (Russell et al., 1997). The sporicidal activity of PHMGH at its MSDC (0.08%) is accomplished in 8.5 min. This time seemed

**DISCUSSION**

The work of Oulé et al. (2008) clearly established that the MIC and the minimum bactericidal concentration (MBC) for a PHMGH-based disinfectant solution were the same. This concentration corresponds to 0.04% (w/v) for MRSA and to 0.005% for E. coli. The PHMGH solution at the MBC (0.04% for MRSA and 0.005% for E. coli) killed all the cells in only 90 s. The preliminary work in this study effectively showed that 90 s of contact between B. subtilis cells and a 0.04% PHMGH solution killed all cells irreversibly (results not shown). At low concentrations, most biocides are bactericidal even against vegetative Bacillus subtilis and E. coli, and to 0.005% for MRSA. This concentration corresponds to 0.04% (w/v) for PHMGH-based disinfectant solution were the same.

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somewhat long, and so we determined the sporicidal concentrations of PHMGH for 90 s and 3 min contact times in different environments (spore suspensions in distilled water, on glass surfaces and on stainless steel).

The resistance of spores placed on a surface was also observed. Chatuev & Peterson (2010) attributed the resistance of spores placed on surfaces to the volatile character of the chlorine used in their work. PHMGH used in this work is not volatile. We think that the dry state of the spores on the surfaces was the reason for their resistance by slowing the sporicidal activity of the disinfectant. It is known that the resistance of spores to disinfectants is partly due to their dehydrated state. Spore suspensions, being hydrated, are more sensitive to the immediate effect of disinfectants. It is probable that the mechanism of inactivation of spores in the dry state on solid surfaces involves two steps. The first step consists of spore hydration by the water in the disinfectant and the second step consists of their inactivation by the active component in the disinfectant. The spores suspended in water are already hydrated and are inactivated by the disinfectant in one step. This explains the positive linear correlation between sporicidal activity and disinfectant concentration. The $R^2$ values (99.91 and 99.09 %) very close to 1 indicate the goodness of fit of this positive linear correlation. This also explains the relation $y_{3\text{ min}} = \frac{1}{2}y_{90\text{ s}}$, where $y_{3\text{ min}}$ represents sporicidal activity for 3 min contact time and $y_{90\text{ s}}$ the sporicidal activity for 90 s contact time. It is evident that the disinfectant’s sporicidal activity against dried spores on stainless steel and glass surfaces cannot be strictly proportional to concentration or contact time.
Conclusions

PHMGH is an innovative sporidical disinfectant. Its MSTC, its MSDC and the time required for complete sporidical activity correspond to 0.06 % (w/v), 0.08 % (w/v) and 8.5 min, respectively. Its sporidical activity against spores in suspension is linear with respect to concentration and contact time. Its sporidical activity against dehydrated spores on surfaces seems to occur in two steps: rehydration and contact time. Its sporicidal activity against dehydrated spores in suspension is linear with respect to concentration. PHMGH is devoid of volatile organic compounds so it is odourless. It is colourless, does not stain, and inactivation. PHMGH is an innovative sporicidal disinfectant. Its bactericidal, fungicidal and sporicidal activity, uses and mechanism of action of glutaraldehyde.

ACKNOWLEDGEMENTS

The authors are grateful to the Research and Development Committee of Université de Saint-Boniface for financial support, to Fosfaton-Akwaton International Ltd for providing Akwaton, to Dr Rokhaya Ndeye Gueye, Faculty of Sciences, Université de Saint-Boniface, for her guidance and helpful comments on the manuscript and also to the reviewers for their helpful comments on the manuscript.

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