Polyhexamethylene guanidine hydrochloride-based disinfectant: a novel tool to fight meticillin-resistant *Staphylococcus aureus* and nosocomial infections

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Polyhexamethylene guanidine hydrochloride (PHMGH), an antimicrobial biocide of the guanidine family, was tested for efficacy against quality-control strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, meticillin-resistant *S. aureus* (MRSA) and *Escherichia coli*. Bactericidal activity against *S. aureus*, *P. aeruginosa* and *Salmonella choleraesuis* was determined using the official methods of analysis of the Association of Official Analytical Chemists, with modifications as recommended by the Canadian General Standards Board. For MRSA and *E. coli*, the MIC and minimal bactericidal concentration were determined using the broth dilution technique. The experiments were carried out at 20 °C under a range of conditions including varying PHMGH concentration (0.001–0.1 %), contact time (0.5–10 min) and water type (distilled, tap and hard water). The phenol coefficient values determined with *S. aureus, Salmonella choleraesuis* and *P. aeruginosa* were 7.5, 6.1 and 5, respectively. No matter what type of water was used to make the dilutions, PHMGH killed MRSA and *E. coli* at concentrations as low as 0.04 and 0.005 % (w/v), respectively, within 1.5 min. The mode of action of PHMGH was elucidated by transmission electron microscopy: the cell envelope was broken, resulting in cell content leakage into the medium. The ultimate aim of this study was to show that PHMGH can be used as an odourless, colourless, non-corrosive and harmless disinfectant for hospital and household facilities.

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INTRODUCTION

Nosocomial infections increase morbidity among hospitalized patients and are a major cause of death. According to a survey conducted under the auspices of the World Health Organization in 55 countries, a mean of 8.7 % of patients had nosocomial infections and at any given time 1.4 million people worldwide suffer from hospital-acquired infections (WHO, 2002). The epidemic spread of nosocomial infections is likely to become even more prevalent as a result of increasing numbers and crowding of people, more frequently impaired immunity due to illness or ageing, inadequate or inappropriate therapy, new micro-organisms, a lack of general hygiene measures and increased bacterial resistance to antibiotics (Ducel, 1995). An increased incidence of nosocomial infections due to meticillin-resistant *Staphylococcus aureus* (MRSA) has been associated with the use of certain antibiotics, and has resulted in increased morbidity, mortality and cost of care (Mauldin *et al.*, 2008). 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 (Krebs *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

Abbreviations: AOAC, Association of Official Analytical Chemists; MBC, minimal bactericidal concentration; MRSA, meticillin-resistant *Staphylococcus aureus*; PC, phenol coefficient; PHMGH, polyhexamethylene guanidine hydrochloride; UD, used dilution.

variety of solid surfaces (Hiti *et al.*, 2002) and also in water treatment (Kusnetsov *et al.*, 1997). Polyhexamethylene guanidine hydrochloride (PHMGH) is a polymer with high solubility in water. It is odourless, colourless and non-corrosive (Kuznetsov, 2004), and is significantly less toxic and harmless than currently used disinfectants (Müller & Kramer, 2005) to humans and animals at a concentration $\leqslant$1 %. To demonstrate its potential use as an effective antibacterial agent that is less hazardous than currently used disinfectants, the antibacterial activity of PHMGH was tested against quality-control strains of *S. aureus*, MRSA, *Pseudomonas aeruginosa*, *Salmonella choleraesuis* and *Escherichia coli*.

METHODS

Test organisms. All test organisms were obtained from Oxoid. Stock cultures of *S. aureus* ATCC 6538, MRSA ATCC 3359, *Salmonella choleraesuis* ATCC 10708 and *E. coli* ATCC 33625 were maintained on nutrient agar slants for monthly transfers. *P. aeruginosa* ATCC 15442 stock cultures were maintained on cysteine–trypticase agar (BBL) by stab cultures incubated for 48 h at 37 °C and stored at 5 °C, with transfer every 30 days. New stocks of test organism transfers were incubated for 2 days at 37 °C.

Assessment of the disinfectant properties of PHMGH. The disinfectant properties of PHMGH were demonstrated by its phenol coefficient (PC) value determination using *S. aureus* ATCC 6538, *Salmonella choleraesuis* ATCC 10708, and *P. aeruginosa* ATCC 15442, as recommended by the Canadian General Standards Board (CGSB) and Health Canada. The PC method and the used-dilution (UD) method, based on the official methods of analysis of the Association of Official Analytical Chemists (AOAC) (Beloian, 2005) were used, with modifications as recommended by the CGSB.

PC method. A 5 % (w/v) phenol (Boreal Northwest) solution was standardized with 0.1 M $KBr/KBrO_3$ solution, 38 % HCl and 20 % KI solution. Titration was carried out with 0.l M $Na_2S_2O_3$ using a starch indicator. Dilutions in sterile hard water were made from 5 % (w/v) phenol and 1 % (w/v) PHMGH stock solutions. Test tubes containing 5 ml of each of the final dilutions of PHMGH and phenol, and a tube containing the 48 h test culture were placed in a water bath at 20 °C for 5 min. Next, 0.5 ml 48 h test culture was added to each of the test tubes at 30 s intervals. The tubes were agitated before being placed in the water bath at 20 °C. After 5, 10 and 15 min, one loopful was transferred to the subculture medium (nutrient broth without disinfectant). Subculture tubes were thoroughly agitated before incubating for 3 days at 37 °C. Samples from subcultures were streaked onto agar to confirm the absence of growth and contamination. Each test was performed in duplicate and repeated three times. The PC value was the highest dilution that killed the test organism in 10 min. This number was obtained by dividing the numerical values of the greatest dilution of PHMGH capable of killing the test organism in 10 min, by the greatest dilution of phenol showing the same results.

UD method. As described in section 955.14 C of the official methods of analysis of the AOAC (Beloian, 2005), sterile ring carriers were placed in multiples of 20 in a sterile 25 × 150 mm Pyrex test tube containing 20 ml 48 h nutrient broth test culture. After a 15 min contact period, carriers were removed and placed vertically in sterile Petri dishes matted with two layers of filter paper, covered and incubated at 37 °C for 40 min. Dilutions were made from the 5 % (w/v) phenol and 1 % (w/v) PHMGH stock solutions and one contaminated dried carrier was placed in each of the tubes of the used dilution. Immediately after placing the carrier in the tubes, the tubes were swirled three times before placing them back in the 20 °C water bath. After 5, 10 and 15 min intervals, each carrier was transferred to the subculture medium. Subculture tubes were shaken thoroughly before incubating for 48 h at 37 °C. Growth in tubes was checked by Gram staining to ensure that no contamination was present, and all positive tubes were confirmed by triplicate testing to ensure against false-positive tests. Each test was performed in duplicate and repeated three times. The highest dilution of a disinfectant that kills test organisms on carriers after 10 min represents the maximum presumed safe dilution for use for disinfecting hospitals, clinics and other places where pyrogenic bacteria are significant (Beloian, 2005).

Statistical analysis. Results were analysed using one-way analysis of variance and Student's *t*-test. The PC value of each test organism reported represents the mean of three tests performed in duplicate. Differences with a value of $P<0.05$ were considered statistically significant.

Determination of MIC and minimal bactericidal concentration (MBC) of PHMGH against MRSA and *E. coli*. Assessment was performed using the broth dilution technique as described by Soberón *et al.* (2007). MRSA and *E. coli* were used as test organisms and three types of water (hard, tap and distilled water) were used to prepare the growth medium. Serial twofold dilutions (0.001–0.1 %, w/v) PHMGH with a final volume of 5 ml were made in the growth medium in 15 × 150 mm test tubes. A 48 h bacterial culture 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 bacterial culture, respectively. Bacterial growth was indicated by turbidity and the absence of bacterial growth was interpreted as inhibitory activity. The MIC, expressed as a percentage, was the lowest concentration of PHMGH at which the test organism did not grow. To determine the MBC, a sample taken from each test tube where there was no growth in the MIC assay was inoculated in nutrient agar without PHMGH and incubated for 48 h at 37 °C. Controls included aliquots taken from control growth tubes. The MBC was the lowest concentration of PHMGH at which the test organism was killed. Each test was performed in duplicate and repeated three times.

Determination of time required for PHMGH bactericidal activity at the MBC. The assay was performed using MRSA and *E. coli* as test organisms, three types of water (hard, tap and distilled water) to prepare the nutrient broth, and solutions of PHMGH at the MBC values of 0.04 and 0.005 % (w/v), respectively, for MRSA and *E. coli*. For each test organism, 0.5 ml 48 h culture was added to a tube containing 5 ml PHMGH solution at the corresponding MBC. Tubes were agitated and placed in a water bath at 20 °C. One loopful of culture and PHMGH mixture was subcultured every 30 s for a period of 10 min. Individual subculture tubes were agitated thoroughly before being incubated at 37 °C for 2 days. Bacterial growth was indicated by turbidity and the absence of growth was interpreted as bactericidal activity. After incubation, 100 µl aliquots from each subculture tube were plated to confirm the results. Each test was performed in duplicate and repeated three times.

Transmission electron microscopy. The mode of action of PHMGH was assessed by transmission electron microscopy as described by Oulé *et al.* (2006). Cells centrifuged in a normal physiological solution at 3000 ***g*** for 10 min were included in an agar-gel medium. After treatment, cells were observed under a transmitting electron microscope (JEOL 1200 EX) at 80 kV.

RESULTS AND DISCUSSION

Determination of PHMGH PC values

To evaluate the disinfecting properties of PHMGH, its PC value was determined using the quality-control strains *S. aureus* ATCC 6538, *Salmonella choleraesuis* ATCC 10708 and *P. aeruginosa* ATCC 15442 in hard water. The PC and UD methods were both used. The UD method is applicable for testing disinfectants miscible with water, to confirm results from the PC method and to determine maximal dilutions effective for practical disinfection (Beloian, 2005). PHMGH is a disinfectant with broad-spectrum activity against Gram-positive and Gram-negative bacteria. The PHMGH PC values determined using *P. aeruginosa*, *Salmonella choleraesuis* and *S. aureus* were 5, 6.1 and 7.5, respectively. Table 1 shows the determination of PC values with *P. aeruginosa*. The concentration of disinfectant required is generally higher if micro-organisms are present on hard dry surfaces (Springthorpe, 2000). The tested disinfectant is therefore more effective if the PC value is the same using both methods or if it is higher when determined with the UD method (Beloian, 2005). For each test micro-organism, the PC value determined was the same for both the PC and UD methods, demonstrating that PHMGH possesses good disinfecting properties. These results also revealed disparities in the bacterial resistance to PHMGH as a disinfectant. Significant differences between test organisms were observed ($P<0.05$). As shown in Fig. 1, *P. aeruginosa* was found to be more resistant to PHMGH compared with *Salmonella choleraesuis* and *S. aureus*. Bacterial resistance to disinfectants and antiseptics can result from the nature and composition of the outer cell wall layers, which may act as a permeability barrier, and also from biofilm formation. This is an intrinsic resistance

Table 1. Determination of PHMGH PC value with *P. aeruginosa* using the PC and UD methods

PC value=400/80=5.0

Disinfectant	Dilution	Exposure time (min)		
		5	10	15
PHMGH	1:350	−	−	−
	1:400	+	−	−
	1:450	+	+	−
	1:500	+	+	−
	1:550	+	+	+
	1:600	+	+	+
	1:650	+	+	+
Phenol	1:70	−	−	−
	1:80	−	−	−
	1:90	+	+	+
	1:100	+	+	+

+, Bacterial growth in broth after exposure to PHMGH; −, no growth.

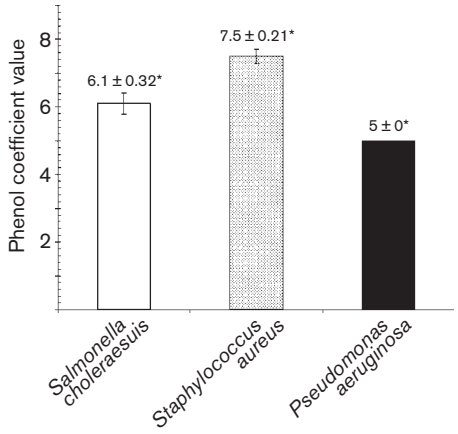

Fig. 1. PHMGH PC values for the three quality-control strains. Identical results were obtained with both the PC and UD methods. Results are from duplicate tests performed in triplicate. *, $P<0.05$.

(McDonnell & Russell, 1999; Russell, 1995). The cell wall of *S. aureus* is composed essentially of peptidoglycan and teichoic acid, which cannot act as an effective barrier to the entry of antiseptics and disinfectants. The sensitivity of *S. aureus* to PHMGH could arise from the fact that the cell wall of staphylococci is readily permeable to high-molecular-mass substances (McDonnell & Russell, 1999). In contrast, Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than Gram-positive bacteria, as their outer membrane acts as a barrier that reduces the entry of many antibacterial agents into the cytoplasm (Paulsen *et al.*, 1997). Gram-negative bacteria that show a high level of resistance to many antiseptics and disinfectants include *P. aeruginosa*, which is naturally resistant to many antimicrobial agents including disinfectants, antiseptics and antibiotics (Kumar & Schweizer, 2005), and is therefore a particularly dangerous pathogen. Its high resistance to antiseptics and disinfectants could be explained by the difference in its outer-membrane composition with respect to cations, lipopolysaccharides, proteins and polysaccharide contents, and phospholipid length and ramification, compared with *S. aureus* and *Salmonella cholereasuis*. Also, the tendency of *P. aeruginosa* to colonize surfaces in a biofilm form contributes to its high resistance (McDonnell & Russell, 1999). A biofilm is a complex aggregation of micro-organisms growing on a solid substrate. It is characterized by structural heterogeneity, genetic diversity, complex community interactions and an extracellular matrix of polymeric substances (Ishii *et al.*, 2008). McDonnell & Russell (1999) reported that biofilms could form an obstacle between the cells within the biofilm and the disinfectant. Also, there could be a chemical interaction between the disinfectant and the biofilm itself, and cells in the biofilm could produce degradative enzymes that neutralize chemicals or could exchange genetic material.

Determination of MIC and MBC values

Table 2 shows the antibacterial activity of PHMGH against MRSA and *E. coli*. Concentrations lower than 0.04 % (w/v) did not have any effect on MRSA growth. However, concentrations above 0.04 % (w/v) inhibited MRSA growth. For *E. coli*, the lowest concentration that inhibited growth was 0.005 % (w/v). The same results were obtained in distilled, tap and hard water, indicating that the type of water used to prepare culture media and disinfectant dilutions did not influence the effect of PHMGH on bacterial growth. As the lowest concentrations that inhibited MRSA and *E. coli* growth were 0.04 and 0.005 % (w/v), respectively, these concentrations can therefore be defined as the MICs for PHMGH against MRSA and *E. coli*. For each type of water, when aliquots from the tubes containing test organisms and PHMGH concentrations higher than the MICs ($\geqslant$0.04 % for MRSA and $\geqslant$0.005 % for *E. coli*) were inoculated in nutrient agar without PHMGH for 48 h at 37 °C, no growth was observed, indicating that PHMGH acts as a bactericide. The MBC of PHMGH was therefore 0.005 % (w/v) for *E. coli* and 0.04 % (w/v) for MRSA, the same values as the MIC for each. Hence, unlike the majority of disinfectants with bacteriostatic or bactericidal activities depending on their concentrations (McDonnell & Russell, 1999), PHMGH killed bacterial cells at the lowest concentration required to produce its effect. As shown in Table 3, the bactericidal effect of PHMGH at the MBC was achieved within 1.5 min and was independent of the type of water used. This demonstrates the rapid action of PHMGH, even when applied at low concentrations. As PHMGH is fast acting at low concentrations, it is incontestably a very effective disinfectant for the control of pathogens. Whilst MRSA and *E. coli* were both killed within 1.5 min of exposure to PHMGH, the MBC for *E. coli* was eight times lower than the MBC for MRSA, suggesting that *E. coli* is eight times more sensitive to PHMGH than MRSA. In fact, bacterial resistance to disinfectants and antiseptics can also be acquired by mutation or acquisition of plasmids or transposons (McDonnell & Russell, 1999; Poole, 2002). Although PHMGH is bactericidal against MRSA with an MBC corresponding to 0.04 % (w/v), this bacterium did exhibit a higher level of resistance compared with *S. aureus*, *P. aeruginosa*, *Salmonella choleraesuis* and *E. coli*. As Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than Gram-positive bacteria, the resistance of MRSA could be ascribed to plasmid-mediated resistance or to chromosomal mutations. Furthermore, acquired resistance to certain other types of biocides has been observed, and staphylococci are the only bacteria in

Table 2. Determination of PHMGH MIC values using the broth dilution technique

Identical results were obtained using distilled, tap and hard water. The MIC values for *E. coli* and MRSA are indicated in bold.

Concn of PHMGH (%)	Test organism	
	E. coli	MRSA
0.1	−	−
0.09	−	−
0.08	−	−
0.07	−	−
0.06	−	−
0.05	−	−
0.04	−	−
0.03	−	+
0.02	−	+
0.01	−	+
0.001	−	+
0.009	−	+
0.008	−	+
0.007	−	+
0.006	−	+
0.005	−	+
0.004	+	+
0.003	+	+
0.002	+	+
0.001	+	+

+, Bacterial growth in broth after exposure to PHMGH; −, no growth.

Table 3. Determination of the time required for PHMGH bactericidal activity at the MBC

Identical results were obtained using distilled, tap and hard water.

Exposure time (min)	Test organism	
	MRSA (MBC 0.04 %)	*E. coli* (MBC 0.005 %)
0.5	+	+
1.0	+	+
1.5	−	−
2.5	−	−
3.0	−	−
3.5	−	−
4.0	−	−
4.5	−	−
5.0	−	−
5.5	−	−
6.0	−	−
6.5	−	−
7.0	−	−
7.5	−	−
8.0	−	−
8.5	−	−
9.0	−	−
9.5	−	−
10	−	−

+, Bacterial growth in broth after exposure to PHMGH; −, no growth.

which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been described, with at least three genes including the *qac* genes involved (Sasatsu *et al.*, 1995). Generally, the resistance genes are multifunctional, which means that one gene can provide resistance to many chemical antibacterial agents such as antibiotics and disinfectants or antiseptics. The relative resistance of MRSA to PHMGH compared with the other bacteria tested in this work could be the result of an antibiotic-resistance gene carried by a plasmid or a chromosomal mutation.

Mode of action of PHMGH on bacterial cells

Fig. 2 shows the mode of action of PHMGH on bacterial cells. Before treatment, the cells were intact and the cell envelope and the cytoplasm appeared uniform (Fig. 2a). The cell envelope seems to be the principal target of PHMGH. As shown in Fig. 2(b), PHMGH appears to penetrate the cell envelope, attacking the cell wall and the membrane at the same time. This results in the cell envelope tearing and the cytoplasmic contents leaking out. The arrow shows a cell in which the envelope has cracked and the cytoplasmic contents have leaked out. The presence of nucleic acids in the medium, measured spectrophotometry as the optical density at 260 nm before and after treatment, confirmed the leakage of cytoplasmic contents. According to McDonnell & Russell (1999), guanidine derivates are rapidly attracted towards the negatively charged bacterial cell surface, with strong and specific adsorption to phosphate-containing compounds. The integrity of the outer membrane is impaired and the polymers are attracted to the inner membrane. Binding of guanidine derivates to phospholipids occurs, causing complete loss of membrane function. Gilbert & Moore (2005) reported that the interactions of polyhexamethylene biguanides and the membrane concentrates around such proteins, leading to a loss of their functions (such as loss of transport and biosynthetic and catabolic capability) by inflicting changes on their boundary phospholipids environment. Also, the adsorption of polyhexamethylene biguanides to the cell membrane leads to a sequestration of acidic phospholipids into domains composed of single rather than mixtures of the phospholipids (Broxton *et al.*, 1984). Each sequestered phospholipid will have different phase transition properties causing the membrane to fragment into fluid and liquid crystalline regions, leading to the cellular leakage (Broxton *et al.*, 1983).

On the other hand, as shown in Fig. 2(b) the material inside the cells appeared to be precipitated, suggesting a direct interaction between PHMGH and the cytoplasmic components. The penetration of PHMGH inside the cytoplasm would cause precipitation of proteins and nucleic acids, provoking cytosol coagulation (McDonnell & Russell, 1999). The contact between PHMGH and nucleic acids could impair gene expression. Allen *et al.* (2006) reported that polyhexamethylene biguanide caused alteration of a wide range of *E. coli* genes that are involved in transcriptional activity, and changed the expression of many genes. Chlorohexidine and polyhexamethylene biguanide are both members of the polymeric guanidine group, as is PHMGH. Their mechanisms against bacterial cells have been well documented. Chlorohexidine is used as an antiseptic and disinfectant. It acts on bacterial cells by binding to the fatty acids and phosphate groups of the cellular membrane, causing the leakage of cell contents and the precipitation of proteins and nucleic acids, resulting in cytosol coagulation (Hugo & Longworth, 1964). Polyhexamethylene biguanide is a cationic antimicrobial agent, and its germicidal function is attributed to the ability of the positively charged molecules to bind rapidly to the cytoplasmic membrane and to the lipopolysaccharides and peptidoglycan components of the cell wall. Bacterial cell death is caused by perturbation of sites of attachment, followed by disruption of the cell wall and consequent cell lysis (Broxton *et al.*, 1984; Gilbert & Moore, 2005). As shown in Fig. 2(b), PHMGH seems to act like other members of the polymeric guanidine family.

Conclusion

PHMGH is an innovative biocide of the polymeric guanidine group of disinfectants, with high solubility in water. Its effect is independent of the type of water used (distilled, tap or hard). It is bactericidal at very low

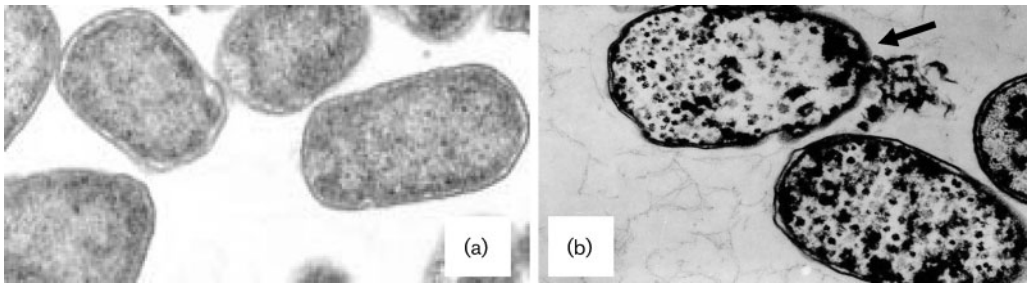

Fig. 2. Mode of action of PHMGH on *E. coli* cells revealed by transmission electron microscope: (a) untreated cells, (b) cells exposed to 0.05 % PHMGH for 10 min.

concentrations [MBC of 0.005 % (w/v) on *E. coli* and 0.04 % (w/v) on MRSA], destroying bacteria within 1.5 min. Its PC value was 5, 6.1 and 7.5, for *P. aeruginosa*, *Salmonella choleraesuis* and *S. aureus*, respectively. The mechanism of action of PHMGH has been clearly determined: it attacks the cellular envelope, causing leakage of cell contents and cytosol coagulation.

PHMGH is devoid of volatile organic compounds so it is odourless. It is colourless, does not stain, is non-corrosive, and is non-toxic for human and animal cells. PHMGH is also fast acting at low concentrations, and has broad-spectrum activity against Gram-positive and Gram-negative bacteria. All of these properties make PHMGH an ideal disinfectant for hospitals and household facilities.

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