ORIGINAL ARTICLE

Thermal inactivation of spores of *Bacillus atrophaeus*, *Bacillus anthracis*, *Bacillus cereus*, and *Clostridium difficile*

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ABSTRACT

There are inadequate evidence on the sporicidal effect of hot water. Therefore, we evaluated the efficacy of hot water against spores of *Bacillus atrophaeus*, *Bacillus anthracis*, *Bacillus cereus*, and *Clostridium difficile*. A portion (0.05 ml) of the spore suspension was used to inoculate 4.95 ml of sterilized distilled hot water. After standing for 1, 2, 5, 10, 30, and 60 min, 0.5 ml was added to 4.5 ml of physiological saline at room temperature (20°C–22°C). The spores of *B. atrophaeus* were the most resistant to hot water, followed by those of *B. anthracis*, *B. cereus*, and *C. difficile*. Disinfection of spores using hot water required contact at 100°C for 30 min for *B. atrophaeus* and *B. anthracis*, at 90°C for 30 min or at 100°C for 5 min for *B. cereus*, and at 90°C for 10 min or at 100°C for 2 min for *C. difficile*. All tested spores cannot be killed under the general conditions of use of hot-water washing machines (70°C–80°C, 10 min), but the spores of *C. difficile* can be killed under the general conditions of use of washer disinfectors (90°C–93°C, 10 min).

Key Words: Hot water, Disinfection, Spore, *Bacillus anthracis*, *Bacillus cereus*, *Clostridium difficile*

1. INTRODUCTION

In medical institutions in Japan, hot-water disinfection has become widespread, following the example of the Europe.[1,2] Washer disinfectors are used to disinfect metal instruments and hot-water washing machines to disinfect linens. Previous studies showed that these devices when used under conditions such as 80°C–93°C for 10 min and 80°C for 10 min, respectively, are effective against not only vegetative bacteria but also viruses.[1–8] However, there are inadequate data on the sporicidal effects of hot water on bacterial spores.[9] Therefore, we evaluated the sporicidal effects of hot water (80°C–100°C) on *Bacillus anthracis* as an important bioterrorism-related microorganism, *Bacillus cereus* as a common contaminant of linens, and *Clostridium difficile* as an important pathogen causing nosocomial infection.[10–12] The spores of *B. atrophaeus* is widely used as an indicator of sterilization.

2. METERIALS AND METHODS

Four bacterial strains were evaluated: *B. atrophaeus* ATCC6633, *B. anthracis* $34F_2$ (vaccine strain for horse and cattle, pXO1 positive, pXO2 negative; Kaketsuken, Kumamoto, Japan), *B. cereus* NIID-3, and *C. difficile* ATCC9689. For *C. difficile*, 2 clinical isolates (from 2 patients at Yamaguchi University Hospital) were also used. For spore preparation of *B. atrophaeus* and *B. cereus*, the

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previously reported preparation method for the spores of *B. atrophaeus* was employed.[13] For spore preparation of *B. anthracis*, a bacterial inoculum cultured on nutrient agar was suspended in saline. This suspension was inoculated into nutrient broth and cultured at 37°C for 10–17 days until more than 80% of bacteria had initiated spore formation. These spores were then resuspended in saline with 50 vol% glycerol added and heated at 65°C for 60 min to kill vegetative cells. A spore suspension containing 10^8 colony forming units (cfu)/ml was obtained. For spore preparation of *C. difficile*, the previously reported method was employed.[13]

A portion (0.05 ml) of the spore suspension was used to inoculate 4.95 ml of sterilized distilled hot water, which had been heated at different temperatures in a thermostat (Isotemp, Fisher Scientific, USA), and vortexed for 5 seconds. After standing for 1, 2, 5, 10, 30, and 60 min, 0.5 ml was added to 4.5 ml of physiological saline at room temperature (20°C–22°C) and vortexed for 10 seconds. The spores of *B. atrophaeus*, *B. anthracis*, and *B. cereus* were counted as previously reported for *B. atrophaeus*. The spores of *C. difficile* were also counted as previously reported for *C. difficile*, although the medium was replaced with chrom ID *C. difficile* agar (bioMérieux SA, France).[13] Experiments were performed three times, and the mean value was calculated.

3. Results

Table 1 displays the efficacy of hot water (80°C–100°C) against the spores of the 4 bacterial species. Hot water at 80°C did not kill the spores of *B. atrophaeus*, *B. anthracis*, or *B. cereus* even after contact for 60 min, but killed *C. difficile* spores after contact for 60 min. Hot water at 90°C did not kill the spores of *B. atrophaeus* or *B. anthracis* after contact for 60 min, but killed the spores of *B. cereus* after contact for 30 min and on those of *C. difficile* after contact for 5 min. Hot water at 100°C killed the spores of *B. atrophaeus* and *B. anthracis* after contact for 30 min, on those of *B. cereus* after contact of 5 min, and on those of *C. difficile* after contact for 1 min. Table 2 charts the efficacy of hot water against 2 clinical isolates of *C. difficile*. The clinical isolates were slightly more resistant to hot water than those of standard strain.

Table 1. Efficacy of hot water against spores of 4 bacterial species* in suspension test

Hot water temperature	Bacterial species	Spore count (cfu/mL) after contact time (minute) at						
		0	1	2	5	10	30	60
80 ℃	*Bacillus atrophaeus*	2.5×10^6	9.1×10^5	1.7×10^6	6.0×10^5	1.3×10^6	4.6×10^5	5.3×10^5
	Bacillus anthracis	1.1×10^6	1.1×10^6	9.3×10^5	7.8×10^5	7.5×10^5	5.8×10^5	2.6×10^5
	Bacillus cereus	8.3×10^4	8.6×10^4	7.8×10^4	4.2×10^4	5.7×10^4	9.7×10^3	1.7×10^2
	Clostridium difficile	4.8×10^5	1.7×10^5	8.0×10^4	2.5×10^4	5.3×10^3	33.3	< 5
90 ℃	*Bacillus atrophaeus*	2.0×10^6	9.2×10^5	1.2×10^6	1.3×10^6	8.6×10^5	1.1×10^6	8.6×10^5
	Bacillus anthracis	1.1×10^6	5.2×10^5	7.0×10^5	3.5×10^5	9.5×10^4	1.2×10^3	50
	Bacillus cereus	1.1×10^5	1.1×10^5	5.5×10^4	5.8×10^3	6.5×10^2	< 5	< 5
	Clostridium difficile	4.8×10^5	7.3×10^3	6.0×10^2	< 5	< 5	< 5	< 5
100 ℃	*Bacillus atrophaeus*	2.1×10^6	8.4×10^5	7.7×10^5	1.3×10^6	8.9×10^5	< 5	< 5
	Bacillus anthracis	1.1×10^6	4.5×10^5	1.2×10^5	4.8×10^2	1.0×10^2	< 5	< 5
	Bacillus cereus	1.1×10^5	8.7×10^2	1.2×10^2	< 5	< 5	< 5	< 5
	Clostridium difficile	5.0×10^5	< 5	< 5	< 5	< 5	< 5	< 5

Note. **B. atrophaeus* ATCC6633, *B. anthracis* $34F_2$, *B. cereus* NIID-3, *C. difficile* ATCC9689

Table 2. Efficacy of hot water against spores of 2 clinical isolates of *Clostridium difficile* in suspension test

Hot water temperature	Strain no.	Spore count (cfu/mL) after contact time (minute) at						
		0	1	2	5	10	30	60
80℃	1	5.4×10^6	1.8×10^6	2.4×10^6	7.5×10^5	7.3×10^5	7.5×10^4	4.7×10^2
	2	8.0×10^6	5.8×10^5	8.0×10^5	7.2×10^5	2.4×10^5	1.3×10^3	< 5
90℃	1	5.4×10^6	1.3×10^6	4.6×10^3	17	< 5	< 5	< 5
	2	8.0×10^6	5.2×10^4	4.3×10^2	< 5	< 5	< 5	< 5
100℃	1	5.4×10^6	17	< 5	< 5	< 5	< 5	< 5
	2	8.0×10^6	33	< 5	< 5	< 5	< 5	< 5

4. DISCUSSION

While hot-water disinfection requires careful attention to avoid burns, it has no residual toxicity associated with hazardous exposures. Indeed, disinfection with hot water is safer than that with chemical disinfectants. In addition, the effects of hot-water disinfection are reliable, and its operating cost is low. For such reasons, hot-water disinfection is the first choice for viruses and vegetative bacteria in medical institutions. However, bacterial spores are widely known to be resistant to hot water. Therefore, we evaluated the sporicidal effects of hot water on *B. anthracis*, *B. cereus* and *C. difficile*. *B. anthracis* is as an important bioterrorism-related microorganism, *B. cereus* tends to contaminate linens, and *C. difficile* is a major pathogen of nosocomial infection. We determined whether their spores are killed by hot-water disinfection using washer disinfectors or hot-water washing machines.

Among the 4 bacterial species evaluated in this study, the spores of *B. atrophaeus* showed the highest hot-water resistance, followed in order by *B. anthracis*, *B. cereus*, and *C. difficile*. Based on our results, the spores of *B. anthracis* and *B. cereus* cannot be killed under 80°C–93°C for 10 min, which is a setting commonly used for hot-water disinfection systems (see Table 1). A previous study showed that the spores of *B. anthracis* could be killed with hot water at 100°C for 5 min, but they could not be killed under these conditions in the present study, which might have been due to a difference in the bacterial strain.[11] On the other hand, the spores of a total of 3 *C. difficile* strains could not be killed after contact with hot water at 80°C for 10 min, but they are killed under hot-water conditions such as 90°C–93°C for 10 min (see Tables 1 and 2). Based on these results, methods using washer disinfectors at 90°C–93°C for 10 min can be also recommended for the disinfection of instruments contaminated with the spores of *C. difficile*.

5. CONCLUSIONS

The spores of *Bacillus atrophaeus* showed the highest hot-water resistance, followed in order by *B. anthracis*, *B. cereus*, and *Clostridium difficile*. Hot water at 90°C killed the spores of *C. difficile* after contact for 10 min.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare they have no conflict of interest.

REFERENCES

[1] Barrie D. How hospital line and laundry services are provided. J Hosp Infect. 1994; 27: 219-235. https://doi.org/10.1016/0195-6701(94)90130-9

[2] Barrie D. The provision of food and catering services in hospital. J Hosp Infect. 1996; 33: 13-33. https://doi.org/10.1016/S0195-6701(96)90026-2

[3] Cannon JL, Papafragkou E, Park GW, et al. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. J Food Protect. 2006; 69: 2761-2765. https://doi.org/10.4315/0362-028X-69.11.2761

[4] Collins BJ. Heat disinfection and disinfector machines. J Sterile Serv Manage. 1984; 3: 7-8.

[5] Dohmae S, Okubo T, Higuchi W, et al. Bacillus cereus nosocomial infection from reused towels in Japan. J Hosp Infect. 2008; 69: 361-367. PMid: 18602188. https://doi.org/10.1016/j.jhin.2008.04.014

[6] Ebner W, Eitel A, Scherrer M, et al. Can household dishwashers be used to disinfect medical equipment? J Hosp Infect. 2000; 45: 155-159. PMid: 10860692. https://doi.org/10.1053/jhin.1999.0720

[7] Gerding DN, Muto CA, Owens RC Jr. Measures to control and prevent Clostridium difficile infection. Clin Infect Dis. 2008; 46: S43-49. PMid: 18177221. https://doi.org/10.1086/521861

[8] Miles RS. What standards should we use for the disinfection of large equipment? J Hosp Infect. 1991; 18: 264-273. https://doi.org/10.1016/0195-6701(91)90032-4

[9] Nyström B. New technology for sterilization and disinfection. Am J Med. 1991; 91: 264S-266S. https://doi.org/10.1016/0002-9343(91)90379-C

[10] Oie S, Kamiya A, Tomita M, et al. Efficacy of disinfectants and heat against Escherichia coli O157:H7. Microbios. 1999; 98: 7-14. PMid: 10413874.

[11] Whitney EAS, Beatty ME, Taylor TH Jr, et al. Inactivation of Bacillus anthracis spores. Emerg Infect Dis. 2003; 9: 623-627. PMid: 12781999. https://doi.org/10.3201/eid0906.020377

[12] World Health Organization. Anthrax in Humans and Animals. Fourth Edition, WHO Global Alert and Response Report. Available from: http://www.who.int/csr/resources/publications/anthrax_webs.pdf

[13] Oie S, Obayashi A, Yamasaki H, et al. Disinfection methods for spores of Bacillus atrophaeus, B. anthracis, Clostridium tetani, C. botulinum and C. difficile. Biol Pharm Bull. 2011; 34: 1325-1329. PMid: 21804226. https://doi.org/10.1248/bpb.34.1325