Recovery of spores of *Clostridium difficile* altered by heat or alkali

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Summary. The effect of heating or alkali-treatment on spore recovery in ordinary growth medium was examined for four strains of *Clostridium difficile*. Heating spores at 80°C for 10 min produced 95.50–99.95% decreases in the recovery rates. Treatment with 0.1 N NaOH for 15 min produced 99.47 and 99.83% decreases in spore recovery rates for two of the four strains. The influence of either addition of lysozyme after treatment with sodium thioglycollate (thioglycollate-lysozyme method) or addition of sodium taurocholate (taurocholate method) on recovery of heat- or alkali-treated *C. difficile* spores was also examined. Viable spores of all strains altered by heating at 90°C or 100°C for 10 min could not be recovered at all by the taurocholate method. Nor did this method allow recovery of alkali-altered spores treated with >0.2 N NaOH for 15 min. On the other hand, 10–47% of altered spores heated at 90°C for 10 min were recovered by the thioglycollate-lysozyme method, and alkali-altered spores treated with 0.1–0.3 N NaOH for 15 min were as completely recovered by this method as untreated spores. These results indicate that the thioglycollate-lysozyme method is more effective than the taurocholate method for recovery of the heat- or alkali-altered *C. difficile* spores.

Introduction

Clostridium difficile is a major cause of pseudo-membranous colitis and of many cases of antibiotic-associated diarrhoea (Bartlett *et al.*, 1978; George *et al.*, 1978; Larson *et al.*, 1978; Borriello and Larson, 1981). Environmental contamination has been suggested as a significant factor in the transmission of *C. difficile* (Fekety *et al.*, 1980; Larson *et al.*, 1980). Survival of *C. difficile* in an aerobic environment is possible because this obligate anaerobe can form aerotolerant spores; there are reports that *C. difficile* can survive for >5 months in aerobic conditions (Kim *et al.*, 1981). Although it is well known that *C. difficile* spores cannot be effectively recovered in ordinary growth medium (Raibaud *et al.*, 1980), their recovery is distinctly enhanced when they are treated with sodium thioglycollate and inoculated in lysozyme containing media (Ionesco, 1978; Nakamura *et al.*, 1985). It has also been reported that addition of sodium taurocholate to media resulted in good

spore recovery (Raibaud *et al.*, 1980; Wilson *et al.*, 1982) and sodium taurocholate-containing medium has been shown to be useful for detection of *C. difficile* spores, particularly when faecal specimens have not been handled optimally or when vegetative forms have lost viability after prolonged exposure to air (Buggy *et al.*, 1983, 1985; Wilson, 1983). It is possible that spores present in clinical specimens or on environmental surfaces may be altered by various kinds of physicochemical damage after aerobic exposure.

In the present study, the effects of heat or alkali treatment on *C. difficile* spores were examined and the influence of thioglycollate-lysozyme or sodium taurocholate on their recovery was assessed.

Materials and methods

Bacterial strains and preparation of spore suspensions

C. difficile strains KZ 1610, KZ 1628, KZ 1648 and KZ 1660 were isolated in this laboratory from faecal specimens of patients with antibiotic-associated diarrhoea (Nakamura *et al.*, 1980). Preliminary findings showed that strains KZ 1660 and KZ 1610 had exceptionally high spore-recovery rates, i.e., >10%, when assayed in ordinary GS-BHI medium (see below)

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after heating at 70°C for 10 min. In contrast, strains KZ 1628 and KZ 1648 had very low spore-recovery rates of <0.01%. Spores were produced in Brain Heart Infusion broth (BHI; BBL Microbiology System, Cockeysville, MD, USA) supplemented with Na₂HPO₄ 0.2% w/v (m-BHI) as described before (Nakamura *et al.*, 1985). Briefly, 0.1 ml of an 18-h culture of each strain in liver broth was inoculated into 10 ml of m-BHI and incubated anaerobically at 37°C for 5 days. Cultures were centrifuged at 3500 *g* for 5 min, and pellets resuspended in appropriate volumes of distilled water to yield (0.5–1.0) × 10⁷ spores/ml; these suspensions were heated at 70°C for 10 min to kill vegetative cells. For the experiment of recovery of heated spores at 60°C, the spore suspension was obtained by heating *C. difficile* culture at 60°C for 10 min.

Quantitative determination of spores and vegetative cells

Quantitative determinations of heat- or alkali-treated spores and vegetative cells were made with pour plates incorporating GS-BHI medium—BHI (Nissui, Tokyo, Japan) supplemented with glucose 0.8% w/v, soluble starch 1% w/v, L-cysteine-HCl 0.05% w/v and agar 1.3% w/v as described by Nakamura *et al.* (1985). A 0.1-ml amount of each 10-fold dilution of spore suspension in BHI broth supplemented with L-cysteine-HCl 0.05% w/v was pipetted into 9-cm diameter petri dishes and mixed with 20 ml of GS-BHI agar. Inoculated agar plates were incubated anaerobically at 37°C for 72 h.

Preparation of heat-treated spores

For heat-treatment, 3.0 ml of spore suspension in Pyrex tubes (9 × 150 mm) was heated at 60°, 70°, 75°, 80°, 85°, 90° or 100°C for 10 min. When heating at 90°C or 100°C, an oil bath was used. The tubes were cooled immediately in ice water and surviving spores enumerated as described above.

Preparation of alkali-treated spores

This was based on the method described by Duncan *et al.* (1972). Spore suspensions (3.0 ml) of each strain were treated with NaOH—0.05, 0.1, 0.2 or 0.3 N for 15 min at 4°C. Thereafter, spores were washed three times, and resuspended in an equal volume of cold distilled water. Quantitative determination of numbers of spores was made as described above.

Recovery of heat- or alkali-treated spores

Two different methods for recovery of heat- or alkali-treated *C. difficile* spores were employed. The first, the thioglycollate-lysozyme method, involves addition of lysozyme after treatment with sodium thioglycollate, and has been described before (Ionesco, 1978; Nakamura *et al.*, 1985). Three volumes of spore suspensions were

mixed with one volume of 2 M sodium thioglycollate, adjusted to pH 10.0 with 5 N NaOH and incubated at 50°C for 30 min. The treated samples were diluted and inoculated into GS-BHI agar medium containing lysozyme (egg white × 6 crystallised, Seikagaku Kogyo Co. Ltd, Tokyo) 10 µg/ml. The second method utilising sodium taurocholate was as described by Raibaud *et al.* (1980) and Wilson *et al.* (1982). Briefly, 0.1 ml of each 10-fold dilution of spore suspension was pipetted into 9-cm petri dishes and mixed with 20 ml of GS-BHI agar medium containing sodium taurocholate 0.1% w/v (guaranteed reagent grade, Nakarai Chemicals Ltd, Kyoto, Japan) and was prepared before use as described by Kamiya *et al.* (1987). The relative recovery rate (%) of spores was expressed as the ratio of the number of colonies recovered by either method after heat- or alkali-treatment to that of colonies recovered by the taurocholate method after heating at 70°C for 10 min, a technique which yields nearly 100% recovery (Kamiya *et al.*, 1987).

Results

Determination of temperature and concentration of NaOH altering *C. difficile* spores

By incubation of heat- or alkali-treated spores of *C. difficile* in ordinary GS-BHI medium, the temperature and concentration of NaOH which altered *C. difficile* were determined. With all strains there was a considerable decline in the relative recovery rates of their spores when heated at 80°C for 10 min (fig. 1A). Thus, the spores of strains KZ

Fig. 1. Recovery of heat- or alkali-treated spores of *C. difficile* strains in GS-BHI agar medium: A, heat-treated; B, alkali-treated. Strains: O, KZ 1660; ●, KZ 1610; △, KZ 1648; ▲, KZ 1628. ND = not detected.

1660, KZ 1610, KZ 1628 and KZ 1648 showed decreases in their recovery rates of 99.95, 98.84, 97.82 and 95.50% respectively. After heating at 85°C, the spores of strain KZ 1660 germinated with a very low recovery rate of 0.0001%, and those of the other strains could not be recovered at all. None of the strains germinated when the spores were heated at 90° or 100°C for 10 min.

When the spores were treated with 0.1 N NaOH for 15 min, the relative recovery rates of strains KZ 1610 and KZ 1660 decreased remarkably to 0.063 and 0.025% respectively (fig. 1B). In this experiment, strains KZ 1628 and KZ 1648 showed no considerable decreases in their relative recovery rates even when the spores were treated with 0.3 N NaOH. These results suggest that the determinative conditions of heating or alkali-treatment that alter *C. difficile* spores are 80°C or 0.1 N NaOH, respectively.

Recovery of heat- or alkali-altered spores by the taurocholate method

Recovery rates of heat- or alkali-treated spores were examined by the taurocholate method. When heated at 60°C, 70°C or 75°C for 10 min, the spores of strains KZ 1628 and KZ 1648 could be recovered as effectively as those of strains KZ 1610 and KZ 1660 (fig. 2A). It was also shown that the relative recovery rates of the spores were considerably decreased by heating at 80°C for 10 min to 2.9, 2.1, 0.73 and 0.05% for strains KZ 1628, KZ 1648, KZ

1610 and KZ 1660, respectively. Heating the spores at 85°C for 10 min resulted in further decreases in the relative recovery rates to 0.0015 and 0.0001%, for strains KZ 1610 and KZ 1660; the spores of the other two strains could not be recovered at all. After heating at 90° or 100°C for 10 min, none of the strains was recovered by this method. It was similarly noted that the alkali-treated spores could not be recovered effectively by the taurocholate method (fig. 2B). When treated with 0.1 N NaOH, the relative recovery rates of spores of strains KZ 1660 and KZ 1610 were decreased to 5.9 and 1.3%, respectively, although spores of strains KZ 1628 and KZ 1648 were relatively resistant. In contrast, treatment with 0.2 N NaOH for 15 min reduced remarkably the relative recovery rates of all strains to levels of 0.0034–0.074%. From these results, the taurocholate method was shown to be ineffective for recovery of heat- or alkali-altered spores of *C. difficile*.

Recovery of heat- or alkali-altered spores by the thioglycollate-lysozyme method

We next examined the effect of the thioglycollate-lysozyme method on recovery of heat- or alkali-altered *C. difficile* spores. The relative recovery rates of heat-altered spores at 80° or 85°C were not decreased considerably (fig. 3A). By heating at 90°C for 10 min, the relative recovery rates of the strains were 10.0–47.2%. Heating of the spores at 100°C for 10 min, however, reduced the relative recovery rates to 2.1, 1.8, 0.21 and 0.20% for strains KZ 1660, KZ 1648, KZ 1628 and KZ 1610, respectively. In contrast, complete recovery of the alkali-altered spores of *C. difficile* was demonstrated in the thioglycollate-lysozyme method (fig. 3B). There were no decreases in recovery rates of the spores of any strains even when the spores were treated with 0.3 N NaOH for 15 min. These results indicate that the thioglycollate-lysozyme method is effective for recovery of heat- or alkali-altered spores of *C. difficile*.

Discussion

The effect of heating or alkali-treatment on recovery of the spores of four strains of *C. difficile* was examined in ordinary GS-BHI medium. The determinative temperature which altered *C. difficile* spores was shown to be 80°C for all strains. This temperature was lower than that which altered *C. perfringens* spores (Duncan *et al.*, 1972). The spores of *C. perfringens* are heat-resistant at temperatures between 80° and 100° (Robert, 1968; Duncan *et al.*, 1972) and treatment of spores at ultrahigh temper-

Fig. 2. Recovery of heat- or alkali-treated spores of *C. difficile* by the taurocholate method: A, heat-treated; B, alkali-treated. Strains: ○, KZ 1660; ●, KZ 1610; △, KZ 1648; ▲, KZ 1628. ND = not detected.

Fig. 3. Recovery of heat- or alkali-treated spores of *C. difficile* by the thioglycollate-lysozyme method: A, heat-treated; B, alkali-treated. Strains: ○, KZ 1660; ●, KZ 1610; △, KZ 1648; ▲, KZ 1628.

ature, i.e., $>100^{\circ}\text{C}$, is required for their inactivation (Adams, 1973). It has also been reported that treatment of *C. perfringens* spores with 0.1 N NaOH for 15 min resulted in considerable decrease in germination of spores (Duncan *et al.*, 1972). In this study, similarly, the treatment of *C. difficile* spores with 0.1–0.3 N NaOH resulted in a remarkable decrease in the recovery rate of spores, although strains KZ 1628 and KZ 1648, which showed very low recovery rate even when untreated, did not show any decrease in spore recovery by alkali-treatment.

Two different methods, the thioglycollate-lysozyme method (Ionesco, 1978; Nakamura *et al.*, 1985) and taurocholate method (Raibaud *et al.*, 1980; Wilson *et al.*, 1982) have been reported to be effective in the recovery of *C. difficile* spores. The thioglycollate-lysozyme method performed in the present study contained two sequential procedures—treatment of the spores with sodium thioglycollate at 50°C for 30 min followed by addition of lysozyme at a final concentration of $10\mu\text{g}/\text{ml}$. The former was shown to rupture disulphide bonds in the spore coat allowing lysozyme to penetrate into the spore cortex, and the latter is thought to attack the underlying mucopeptide of the spore cortex releasing hexosamine peptides as end products (Gould and Hitchins, 1965; Gould and King, 1969). There have been no reports on the mechanism for the augmentation of outgrowth of *C. difficile* spores in the presence of sodium taurocholate but it is possible that it plays an important role as a detergent attacking the spore coat or cortex.

With regard to the efficiency of the recovery rate

of *C. difficile* spores, the thioglycollate-lysozyme method was shown to be slightly less effective than the taurocholate method for untreated spores (Kamiya *et al.*, 1987). However, in the present study, it was clearly shown that the heat- or alkali-altered *C. difficile* spores could be recovered by the thioglycollate-lysozyme method but not at all by the taurocholate method.

Kim *et al.* (1981) detected *C. difficile* on various hospital environmental surfaces (bedpans, toilet seats and floors) in both a patient room and a soiled utility room, not only in an adult intensive-care unit associated with cases of *C. difficile* colitis but also in a control unit. They also showed that 10^6 cfu of *C. difficile* inoculated on to the floor of an unused room could survive exposure for >5 months. Buggy *et al.* (1983, 1985) stated that addition of sodium taurocholate to a selective medium effected recovery of *C. difficile* spores from environmental surfaces. However, it is possible that *C. difficile* spores taken from different environmental surfaces might have suffered various kinds of physicochemical damage. It is possible, therefore, that the thioglycollate-lysozyme method would be more effective than the taurocholate method for recovery of these spores. Further investigations are required to clarify the mechanism(s) by which heat- or alkali-altered *C. difficile* spores can be recovered by the thioglycollate-lysozyme method but not by the taurocholate method.

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