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Proposal of a Simplified Technique for Staining Bacterial Spores without Applying Heat – Successful Modification of Moeller's Method

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Abstract

Background and Aimes: As the bacterial spores are difficult to stain, a number of staining techniques including their modifications have been proposed to date. Most of the conventional staining procedures unexceptionally contain the step of staining with steamed dye reagent in order to increase the stainability of the spores. We made an attempt to improve the conventional Moeller's methods for staining bacterial spores.

Methods: Spores of *Bacillus* species were stained with our modified Moeller's spore stain and evaluated for its staining properties. We investigated the stainability of both of the conventional and the modified Moeller's methods and the evaluation was made whether or not the step of steaming of Kinyoun's carbol-fuchsine dye reagent could be omitted by adding to aliquots of Tergitol 7, in place of the conventional dye solution steamed for some interval over hot blue flame of a Bunsen burner.

Results: We successfully omitted the heating step of steaming the Kinyoun's carbol fuchsine dye solution in the Moeller's method of bacterial spore stain, by the replacement of Kinyoun's carbol-fuchsine dye solution involving 2 drops of Tergitol 7, nonionic polyglycol ether surfactants type NP-7 (Sigma-Aldrich Japan, Tokyo, Japan) per 10 ml of Kinyoun's carbol-fuchsine dye solution. *Bacillus* spores stained pink to red and vegetative bacterial cells stained blue, although without applying any heating step during the whole course of staining processes including the fixation process. The novel staining method of our proposal resulted in far better satisfactory stainability in comparison with the conventional Moeller's method with the steaming dye solution.

Conclusions: The modified spore stain without applying any heating step using the Kinyoun's carbol-fuchsine dye solution with an addition of Tergitol 7 alliquots was demonstrated to be reproducible and yielded consistent and satisfactory stainability. This simplified staining procedure is rapid to perform and found to be applicable to detect the bacterial spores in routine clinical microbiology laboratories.

Key words: spore stain, Moeller's method, Wirtz-Conklin method, bacterial spore, without heat, *Bacillus cereus*

Introduction.

Spore-forming bacteria are highly resistant to adverse circumstances including dryness, heat, and poor nutrient conditions [1-5]. Bacterial spores are highly refractive bodies and are protected by the outer integument. In addition, bacterial spores are well known to be difficult to stain because of their low permeability to aqueous dye reagents [6], and some modifications of bacterial spore stain procedures have been proposed to date. Among the conventional methods, the representative Moeller's [7] and Wirts-Conklin [8] methods for staining bacterial spores incorporate exposure to heating circumstances for some interval. In fact, the former Moeller's method [7] involves exposure to heated staining solutions of carbol-fuchsine for a few minutes, and the latter Wirtz-Conklin method [8] includes disclosure to steamed staining reagent of malachite green for several minutes over the hot blue flame of a Bunsen burner.

In this study we evaluated the stainability by means of our modified spore stain of Moeller's method [7] against a clinical isolate of *Bacillus cereus* whether or not the method of our proposal should be useful and the straightforward method for detecting the bacterial spores.

Materials and Methods.

Bacterial strains investigated: A clinical strain of *B. cereus* isolate was used for the evaluation study. The strain was stored in Micro-Bank vials (Pro-Lab Diagnostic, Ontario, Canada) at -83 °C in a deep freezer in our laboratory. Prior to the experiment, the strain was inoculated onto sheep blood agar plate (Nippon Becton Dickinson, Co., Ltd., Tokyo, Japan), and incubated at 35 °C for 48 hrs.

Preparation of staining reagents: The absolute methyl alcohol was used for the fixation reagent. Chromic acid solution (5%) was prepared by dissolving 5 g of chromic acid in 100 ml of distilled water. Kinyoun's carbol-fuchsine solution [9] was made by dissolving 4 g of basic fuchsin and 8 g of phenol crystal with heating in 20 ml of absolute ethyl alcohol and the solution was made up by adding 100 ml of distilled water. In

addition, Kinyoun's carbol-fuchsine solution containing varying concentrations such as 2 drops, and 4 drops per 10 ml of Tergitol 7, a nonionic polyglycol ether surfactants type NP-7 (Sigma-Aldrich Japan, Tokyo, Japan). Sulfuric acid (2%) solution was prepared by adding 2 ml of concentrated sulfuric acid to 98 ml of distilled water. Methylene blue (0.1%) was made by dissolving 0.5 g in 500 ml of 0.01% potassium hydroxide solution.

Deposits of Bacillus cereus samples and their fixations with and without applying heat: Bacterial suspension was prepared adjusted to the concentrations of approximately 5×10^8 cells/ml from the lawns of *B. cereus* after incubation at 35 °C for 48 hrs, followed by keeping at room temperature for 1 day and 4 days, respectively. One drop of the suspension was deposited onto the slide-glass for making uniform smear preparations. They were air-dried for 15 to 20 min in an ambient air. Air-dried slide-glasses were then fixed both over blue flame of a Bunsen burner two or three times, and in absolute methyl alcohol solution for more than 1 min for comparison study.

Staining with Kinyoun's carbol-fuchsine dye solution containing Tergitol 7 without applying heat: Besides the conventional Moellller's method [7] of bacterial spore stain using the heated carbol-fuchsine solution over hot blue flame of a Bunsen burner, we made use of the method omitting the steaming step by adding 2 drops of Tergitol 7 per 10 ml of Kinyoun's carbol-fuchsine reagent. Remaining staining procedures were just the same as the original Moeller's method [7]. That is, the decoloration and the counterstaining were performed as the same procedures in Moeller's method (7) using completely the same reagents, decoloration for 2% sulfuric acid reagent and counterstaining for 0.1% methylene blue solution, respectively.

Reading smears and their evaluations: All of the stained glass-slide preparations were read microscopically (× 1,000; Olympus, Tokyo, Japan) and the staining conditions were compared for evaluation. That is, every slide-glass preparation was microscopically read blindly and counted for the numbers of spores stained pink to red per different 5 fields by the different three readers using Micro-Analyzer ver.1.1 (Nippon Poladigital Co. Ltd., Tokyo, Japan) and the reading findings were tabulated and considered for statistical evaluation.

Statistical Analysis: The data obtained were analyzed using Microsoft Excel. The significance of the obtained data were determined statistically by using the Student's *T* test between the numbers of percentages of spores stained pink to red among the *B. cereus* cells stained blue derived from different staining conditions and those obtained from the simplified procedures of our proposal (fixation in absolute methyl alcohol and stain at room temperature without heating by using the Tergitol 7 containing Kinyoun's carbol-fuchsine reagent). Intraobserver reproducibility was calculated for each of the three independent examiners on the basis of different consecutive measurements using an analysis of variance. *P* values (Student's *T* test) of less than 0.05 were considered to be significant.

Results

Microscopic examination of *B. cereus* spores stained with the modified Moeller's method for staining bacterial spores of our proposal demonstrated, as shown in Fig. 1, that the spores appeared as red to pink among the bacterial cells stained blue. Although the frequencies of spore-formation depend on the incubation time or on other circumstances, the majority of spores in this field (Fig. 1) were stained red to pink, although un-stained spores could also be observed among the cells. This modified Moeller's method revealed increasingly contrasted stainablility of spores in pink to red among the bacterial cells counterstained in blue, in comparison with the conventional method incorporating heating steps (fixation and/or staining condition).

Table 1 showed the intraobserver reproducibility

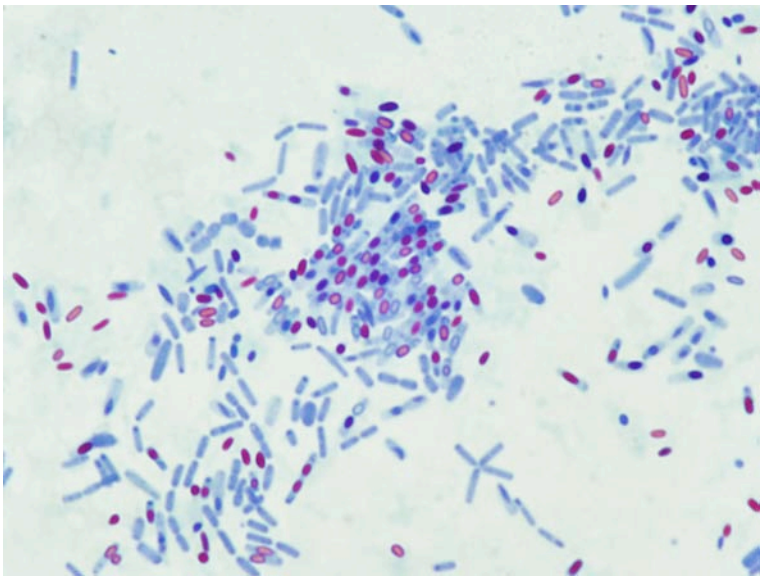

Fig. 1. Bacillus cereus spores stained with the simplified technique of Moeller's modification for staining bacterial spores without applying heat (× 1,000; Olympus, Tokyo, Japan). Spores in this field were stained red to pink and the vegetative cells in blue, with un-stained spores observed among the cells.

Table.1. Differences of LIs* for stain solution with and without addition of Tergitol 7 and for the fixation and staining conditions on the stainability of spores of *Bacillus cereus*.

Fixation	with methanol			over hot blue flame of a Bunsen burner	
Staining condition	at room temperature without applying heat				with steaming dye reagent
Additive amount of Tergitol 7 (drops) per 10 ml of Kinyoun's carbol fuchshine dye solution	0	2	4	2	0
LIs* of spores among the total No. of cells, mean % ±SD#	7.8±2.4	12.2±4.8	7.4±2.8	9.9±3.8	7.2±5.0

LIs*, labeling indices. SD#, standard deviation. Stainability with the dye reagent containing 2 drops of Tergitol 7 per 10 ml of Kinyoun's carbol-fuchsine solution against the preparation slide-glasses fixed with absolute methyl alcohol revealed significantly high LIs (12.2 ± 4.8) for stained spores ($p<0.05$ by Student's *T* test) among the total cell numbers per field.

counted with each of the respective three investigators by the following measurements. That is, by the difference of fixation methods (methanol and blue flame of Bunsenburner), and by the difference of staining temperature (at room temperature without applying heat of our proposal and the conventional method of steaming with the blue flame). As clearly demonstrated in Table 1, the numbers of mean percentages of spores stained pink to red according to the method of our proposal revealed remarkably high values in comparison with those of the results obtained by conventional Moeller's original procedures and the data were proved to be statistically significant, according to the Student's *T* test showing all of the *P* values, less than 0.05.

Discussions

In the field of clinical microbiology, it is extremely important to examine whether the isolate should be sporogenic or asporogenic in the identification of the causative isolates. However, bacterial spores are difficult to stain, because they are not permeable to aqueous dye reagents [6]. Many treatments are known which destroy the permeability barrier, such as severe heat fixation [10], acid hydrolysis [11], ultraviolet light [12], and mechanical rupture [13-15]. After such treatments, bacterial spores are easily stainable at room temperature. We tried to make the conventional Moeller's method [7] simpler and succeeded in modifying the staining procedures. We report in this paper on the successful development of simplified technique for staining bacterial spores without applying heat.

As clearly demonstrated in Table 1, no effect of heat fixation over hot flame of a Bunsen burner twice or thrice was observed on spore stainability. In addition, the method of fixation with absolute methyl alcohol scarcely influenced the stainability of the spores, thus indicating that the methyl alcohol fixation procedure can be utilized in place of the fixation with heating over a Bunsen burner adjusted to a hot blue flame.

Moreover, the most favorable findings was as follows; after fixation with absolute methyl alcohol, staining at room temperature with un-steamed Kinyoun's carbol-fuchsine dye reagent incorporating 2 drops of Tergitol 7, nonionic polyglycol ether surfactants, per 10 ml of Kinyoun's carbol-fuchsine solution, consistently brought the conspicuously distinctive stainability of *B. cereus* spores. In fact, the value of 12.2 ± 4.8 was proved to be significant as compared with any other values (p value of <0.05) obtained with various staining conditions, as demonstrated in Table 1.

A procedure for rapidly staining bacterial spores without applying any heating steps would be of great help to examine bacterial spores in routine clinical microbiology laboratories, especially for the beginners who may have some difficulty in procedures involving steaming dye reagents.

In consequence, we propose the simplified staining method of bacterial spores as follows:

1. Spread a small drop of the specimen on a slide, and allow it to air-dry at room temperature.
2. Fix in absolute methyl alcohol for 1-3 min.
3. Immerse in 5% chromic acid solution for 3 min.
4. Rinse thoroughly in running water.
5. Flood with un-steamed Kinyoun's carbol-fuchsine solution containing Tergitol 7, and stain for 3 min.
6. Rinse thoroughly in running water.
7. Differentiate with 2 % sulfuric acid for 5-10 sec.
8. Rinse thoroughly in running water
9. Decolorize with 80 % ethanol until removal of excess stain dye from the slides.
10. Rinse for 10 sec in running water.
11. Counterstain with 0.1 % Loeffler's methylene blue for 1-2 min.
12. Rinse with running water and allow it to air-dry.
13. Microscopic examination using oil objective lens (× 100)

Finally, we concluded that the rapid procedure for staining of bacterial spores at room temperature without involving steamed dye reagents of our proposal should yield consistent, satisfactory, and distinctive stainability as shown in Fig.1, and therefore would be of great help to detect the bacterial spores in routine clinical microbiology laboratories.

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