

Flow Cytometric Analysis for Enterotoxin Exposed on *Clostridium Perfringens* Spores

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ABSTRACT. Flow cytometric method (FCM) with fluorescent-labeled anti-CPE antibody was applied to develop a rapid, specific, and convenient method to detect enterotoxin (CPE) exposed on the surface of spores of *Clostridium perfringens*. The results obtained indicate that FCM can specifically detect CPE exposed on *C. perfringens* spores for a short time. Thus, FCM is found to be a rapid, specific, and convenient assay method for detection of CPE exposed on *C. perfringens* spores, suggesting that it will be hopefully useful to diagnose food poisoning caused by *C. perfringens*. — **KEY WORDS:** *Clostridium perfringens*, enterotoxin, FCM.

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Clostridium perfringens is a common food bacterium distributed widely in human foods, especially meat and poultry products [20]. Type A strains of *C. perfringens* produce an enterotoxin (CPE) which is the causative factor of human food poisoning [19]. CPE is synthesized during sporulation of *C. perfringens* vegetative cells and the lysis of sporulated cells liberates CPE into the intestinal tract [7]. Since CPE involves in food poisoning outbreaks caused by *C. perfringens*, several methods for detection of CPE have been developed over the past few decades [5, 12, 18, 21]. Although enzyme-linked immunosorbent assay (ELISA) is one of the common methods to detect CPE at the present time, it is not useful to analyze CPE exposed on the surface of spores of *C. perfringens*. On the other hand, flow cytometry method (FCM) is an established method for analysis of different types of cells from bacteria to mammalian cells in terms of accuracy, sensitivity, and rapidity. FCM has recently been reported to be a useful method to analyze bacteria in the environment [1, 11, 16] and food samples [4, 8, 13]. Thus, we attempted to confirm whether or not FCM is applicable to identify and/or analyze CPE exposed on the surface of spores of *C. perfringens*.

Vegetative cells of *Staphylococcus aureus* strain FRI-722 were prepared by incubating in thioglycollate medium (TGC medium, Nissui Seiyaku, Tokyo, Japan) at 37°C for 12 hr followed by culturing in brain-heart infusion broth (Difco Laboratories, Detroit, USA) containing 0.05% Na-thioglycollate at 37°C for 10 hr. Spore of *C. perfringens* strain 8239 (H-3) were prepared by the methods described previously [6]. Vegetative cells and spores were harvested by centrifugation at 10,000 × g for 15 min. The precipitate was suspended in 0.6% formalin at 30°C for 2 days. Then, the formalin-treated vegetative cells and spores were washed 3 times in 0.15 M phosphate buffered saline, pH 7.2 (PBS).

Purification of CPE produced by *C. perfringens* strain 8239 and preparation of rabbit anti-CPE serum were performed by the methods described previously [22, 23].

Rabbit antiserum was purified by gel filtration on Sephacryl S-300 (Pharmacia, Uppsala, Sweden). Purified antibody was labeled by the methods of Williams and Chase [24]. Thirty µg of fluorescein isothiocyanate, isomer I (FITC, Wako Pure Chemicals, Osaka, Japan) was added to 1 mg of antibody and incubated at 25°C for 1 hr. Then the mixture was applied to gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden). Further purification of FITC-labeled antibody was carried out by ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman Paper Ltd, Maidstone, England).

Heat treatment of formalin-treated *C. perfringens* spores was carried out by incubating at 60°C for 30 min. The spores (10⁸) were also treated with 200 µl of rabbit anti-CPE serum by incubating at 37°C for 10 min. Binding of FITC-labeled anti-CPE antibody (40 µl) to either formalin-treated vegetative cells or spores (10⁶/ml) was done by incubating at 37°C for 10 min. After these treatment, the spores were washed in PBS by centrifugation at 10,000 × g for 10 min.

For flow cytometric assay, test samples were prepared as follows: 0.4 ml of formalin-treated vegetative cells or spores (10⁸/ml) was mixed with 50 µl of FITC-labeled purified rabbit anti-CPE antibody for 10 min at 37°C. FCM was performed in a FACStar™ (Becton Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.) equipped with 5 W argon ion laser (Coherent Innova 90) tuned at 488 nm and 0.2 W. At least 10,000 cells were determined by a measurement of forward angle light scatter (FSC). Green fluorescence (from FITC, through a 530/30 nm filter, Becton Dickinson) from stained cells was detected in the 90° light scatter, which was recorded and displayed in a cytogram. Subcellular debris were excluded from the analysis by setting a suitable threshold on the basis of FCS. Data were processed with Consort 30 Software in a Hewlett-Packard 900 series model 217 personal computer (Hewlett-Packard, Fort Collins, CO, U.S.A.). All other procedures were done according to the FACStar™ operation manual.

To study the specificity of FITC-labeled anti-CPE antibody, CPE exposed on *C. perfringens* spores was treated with unlabeled rabbit anti-CPE antibody prior to FITC-

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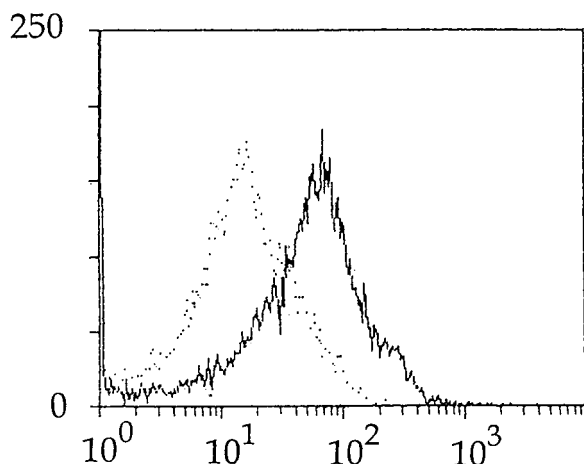


Fig. 1. Results of analysis of *C. perfringens* spores reacted with FITC-labeled anti-CPE antibody by FCM. *C. perfringens* spores treated with anti-CPE antibody. — *C. perfringens* spores untreated with anti-CPE antibody. Vertical axis indicates the number of cells. Horizontal axis indicates fluorescence intensity.

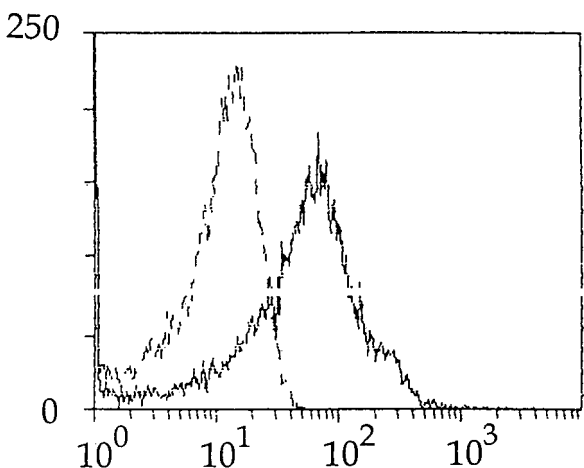


Fig. 2. Results of analysis of *S. aureus* and *C. perfringens* spores reacted with FITC-labeled anti-CPE antibody by FCM. — *C. perfringens* spores, *S. aureus* vegetative cells. Vertical axis indicates the number of cells. Horizontal axis indicates fluorescence intensity.

labeled anti-CPE antibody. After the treatment, the reactivity of the treated spores was analyzed by FCM. As shown in Fig. 1, the spores did not react with FITC-labeled anti-CPE antibody, indicating that CPE exposed on the surface of *C. perfringens* spores reacted with anti-CPE serum. To further study the specificity of FITC-labeled anti-CPE antibody, the reactivity of *C. perfringens* spores and *S. aureus* vegetative cells were analyzed by FCM. As shown in Fig. 2, the peak channel of CPE exposed on *C. perfringens* spores was found to be different from that of CPE-nonproducing *S. aureus*. These indicated that FITC-labeled anti-CPE antibody was a highly specific reagent to detect CPE exposed on the surface of *C. perfringens* spores.

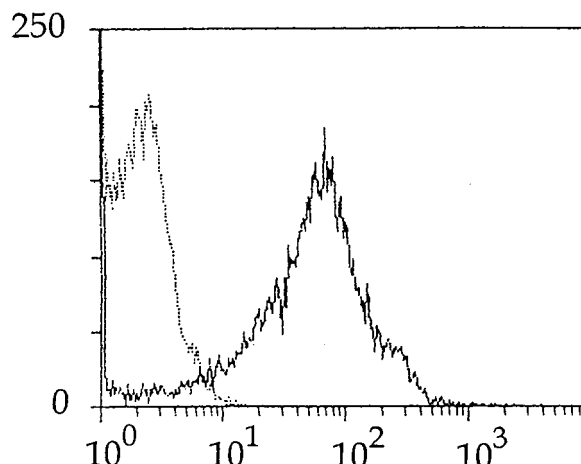


Fig. 3. Results of analysis of formalin-treated *C. perfringens* spores reacted with FITC-labeled and -unlabeled anti-CPE antibodies by FCM. — *C. perfringens* spores reacted with FITC-labeled anti-CPE antibody. *C. perfringens* spores reacted with FITC-unlabeled anti-CPE antibody. Vertical axis indicates the number of cells. Horizontal axis indicates fluorescence intensity.

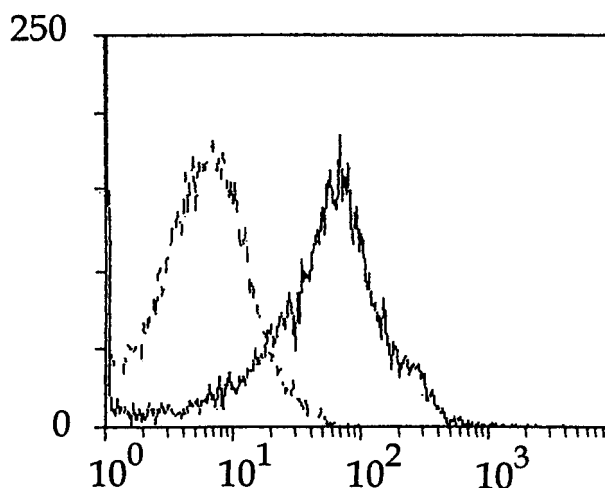


Fig. 4. Results of analysis of heat-treated *C. perfringens* spores reacted with FITC-labeled anti-CPE antibody by FCM. — unheat-treated *C. perfringens* spores, heat-treated *C. perfringens* spores.

Formalin-treated spores of *C. perfringens* were also analyzed by FCM after incubation with FITC-labeled and unlabeled rabbit anti-CPE antibodies. As shown in Fig. 3, CPE exposed on the surface of the spores appeared in a single peak. On the other hand, FITC-unlabeled spores appeared at the different position, showing that formalin treatment at 0.6% was found to be ineffective to the antigenicity of CPE exposed on *C. perfringens* spores.

To study the antigenicity of CPE exposed on the surface of *C. perfringens* spores after heat treatment at 60°C for 10 min, the reactivity of *C. perfringens* spores before and after the treatment were analyzed by FCM. As shown in Fig. 4, the peak channel of *C. perfringens* spores before the

treatment was found to be different from that after the treatment, indicating that the heat treatment at 60°C for 30 min destroyed the antigenicity of CPE exposed on the surface of *C. perfringens* spores. This is supported by the previous report [17] that CPE is a heat-labile protein.

The food industry needs an accurate, sensitive, and rapid method to detect bacteria in food samples since the faster the microbiological detection is carried out, the sooner perishable foods can be released for sale. They also need the method to reduce false negative results to permit the release for sale of contaminated foods. To overcome such problems, FCM is a useful method which will be automation for in a factory environment. As described previously [2, 3, 15], the minimum concentration of bacteria detectable by FCM has been reported to be 10^3 – 10^4 bacterial cells/ml. With two species of salmonellas, the minimum detectable concentration by FCM has been to be 10^3 /ml in pure cultures [9], and 10^3 – 10^4 /ml in milk and egg [10, 14]. In all cases, the total analysis time was about 30 min. Thus, FCM is suggested to be more rapid method to detect and/or analyze salmonellas than polymerase-chain reaction (PCR).

From the present findings, FCM is found to be a rapid, specific, convenient method to detect and/or analyze CPE on the surface of spores of *C. perfringens*. Thus, it will be a useful method to detect food stuffs contaminated with *C. perfringens* for prevention of food poisoning.

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