MONOCLONAL ANTIBODY BASED FLOW CYTOMETRIC METHOD FOR THE DETECTION OF ENTEROTOXIGENIC *CLOSTRIDIUM PERFRINGENS*

R.H.PIYANKARAGE and T.UEMURA*
*Department of Veterinary Paraclinical Studies, Faculty of Veterinary Medicine, University of Peradeniya, *Department of Veterinary Public Health, College of Agriculture, University of Osaka Prefecture, Osaka, Japan.*

ABSTRACT

An assay combining flow cytometry (FCM) and *C. perfringens* enterotoxin (CPE) specific monoclonal antibody (MAb) was developed for the detection of enterotoxigenic *Clostridium perfringens*. Biotin conjugated anti-CPE MAb and fluorescent avidin was used to study the sporangial cells of *C. perfringens*. which were collected from 6-8 hour sporulation cultures. Using this assay. eight strains of *C. perfringens* which possessed the enterotoxin gene and belonged to distinct Hobb's types were analyzed.

FCM profiles of *C. perfringens* sporangia harvested from 6-8 hr sporulation cultures in Duncan and Strong (DS) medium revealed positive reaction for CPE. whereas vegetative cells and heat-treated sporangia of the same strain produced a negative reaction. Among eight different strains. high CPE producing strains showed high peak channel number and poor CPE producers demonstrated low peak channel numbers under FCM analysis.

The flow cytometric method was found to be rapid. specific. accurate and reproducible. and can be used effectively in the detection of enterotoxigenic *C. perfringens*. Furthermore it might be of immense use in the diagnosis of food poisoning outbreaks.

INTRODUCTION

Enterotoxigenic *C. perfringens* is a common cause of human food poisoning that ranks as one of the leading food borne disease in industrialized countries (Bean & Griffen, 1990). During food poisoning outbreaks, detection of CPE from affected fecal samples provides the conclusive evidence for the involvement of *C. perfringens* (Labbe, 1991). However, CPE is excreted only during diarrheal stage of an infection (Itoh *et al.*, 1979, Tsukamoto *et al.*, 1981) and sometimes it is difficult to obtain adequate fecal samples for CPE detection (Saito *et al.*, 1992). An alternative method for the diagnosis is to isolate *C. perfringens* from fecal samples of affected individuals. But occurrence of *C. perfringens* in normal fecal flora of humans, and its ubiquitous distribution in the environment hinders the laboratory identification of *C. perfringens* etiology (McClane, 1996). Thus during an outbreak, the demonstration of an elevated level of *C. perfringens* in respective samples and confirmation of their enterotoxigenicity deemed necessary (Saito, 1990). To determine the enterotoxigenicity, either gene detection methods or immunological assays could be employed. Among these, serological assays are commonly used. To detect CPE by serology, CPE should be produced *in vitro* by culturing the specific strain. However, some strains produce very low toxin levels

and the percentage of sporulation fluctuate from experiment to experiment even under similar culture-conditions. When considering the properties of CPE, it is not an actively secreted protein and accumulates in the sporangia until the completion of the sporulation process (Labbe, 1989). In fact, prior to lysis, the sporangia of many food poisoning strains contain extremely high level of the toxin (McClane, 1997). Thus, analysis of such sporangia for CPE seems to be ideally suited to confirm the enterotoxigenicity of *C. perfringens*. Nillo (1977) applied fluorescence microscopy to the smears of *C. perfringens* to detect the presence of intracellular CPE. Other than that, this subject has not been discussed elsewhere in the literature. In the present study, attempts were made to develop a specific, rapid, sensitive, accurate and reproducible FCM method for the detection of enterotoxin producing *C. perfringens*.

MATERIALS AND METHODS

Bacterial strains

C. perfringens type A, NCTC 8797 (Hobb's type 1), NCTC 8238(H-2), NCTC 8239(H-3), NCTC 8679(H-6), NCTC 8235(H-8), NCTC 8798(H-9), NCTC 10240(H-13), NCTC 10611(H-14) maintained in Cooked Meat Medium (CMM, Difco, MI, USA) and *Escherichia coli* were used.

Preparation of *C. perfringens* sporangia and vegetative cells for FCM

From the respective stock cultures, each strain of *C. perfringens* was inoculated into CMM treated at 80°C for 25 min, and incubated for 24 hr at 37° C. Thereafter, 0.5 ml of cooked meat culture was inoculated into 10 ml of sodium thioglycollate medium, (TGC, Difco) and incubated at 37°C for 12 hr after applying the heat treatment as above. This was followed by sub-culturing in TGC and incubating at 43°C for 4 h. The cultured TGC medium was inoculated into freshly prepared Duncan and Strong (DS) medium (Duncan *et al.*, 1972). Following 6-8 h incubation at 37°C, the sporangia were harvested by centrifugation at 10,000 g for 20 min. The sporangia were treated with 0.8% formalin at 30°C for 30 min, washed three times in phosphate buffered saline (PBS) and suspended as 1×10^7 cells/ml in PBS. A portion of sporangial suspension of *C. perfringens* strain NCTC 8239 (H-3) was heat treated for 30 min at 75°C and then formalinized as above. The 12 hr incubated TGC culture mentioned above, was inoculated into brain heart infusion broth (Difco) containing 0.05% TGC and then incubated for 10 h at 37° C to prepare vegetative cells. The harvested vegetative cells were washed and treated with formalin as described above.

Preparation of biotin labeled MAb

MAb (3C3) against purified CPE was prepared and sub isotyped as described previously (Piyankarage *et al.*, 1999). IgG fraction from mouse ascitic fluid was separated by $(NH_4)_2SO_4$ precipitation. One milligram of purified IgG in 1ml of PBS was mixed with 20 μl of biotinyl N-hydroxy succinimide ester (50 mg/ml in dimethylformamide; E-Y laboratories, Inc, USA). The mixture was incubated for 3 h at room temperature and the labeled MAb was separated from free biotin by dialysis in PBS for 24 h at 4°C. This biotinylated MAb was stored at -20°C until use. As a negative control, anti-feline calci virus MAb (gift received from Dr. T. Tajima, University of Osaka Prefecture, Japan) was used after confirming the reactivity against CPE.

Flow cytometry

Five hundred microliters of formalinized *C. perfringens* sporangia/vegetative cells were washed twice with PBS and suspended in PBS containing 0.05% bovine serum albumin (BSA). This cell suspension was mixed with 200 µl of biotin labeled MAb 3C3 (100 µg/ml) and incubated at 37°C for 30min. The reaction products were washed 3 times in PBS to remove any unbound antibody. Finally, a 200 µl of FITC conjugated streptavidin (Zymed Laboratories, Inc. USA) diluted 1:40 with PBS containing 0.05% BSA was added to the cells and incubated at 37°C for 30min. At the end, samples were washed twice in PBS to remove any unbound FITC and the cell pellet was dissolved in 1 ml of PBS and analyzed by FCM. Heat-treated sporangial cells and sporangia incubated with nonlabeled MAb 3C3 prior to biotin labeled MAb 3C3 were also stained in the same manner. The fluorescence of stained cells was analyzed using FACStar^{TM} flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The light source used was water cooled argon ion laser (Coherent Innova 90, Coherent, Palo Alto, CA) tuned at 488 nm to give an output of 200 mW and filtered distilled water driven under constant pressure was used as the sheath fluid. Fluorescence data were acquired through 530 nm filter (Becton Dickinson) for 10,000 cells from each sample. Cellular debris and clumps were excluded by setting a gate on the basis of forward light scatter (FSC). Signals from the detectors were processed and digitized by custom electronics and analyzed by consort 30 software in a Hewlett-Packard 9000 series model 217 personal computer (Hewlett Packard, Fort Collins, CO.). Flow cytometer was calibrated daily using chicken erythrocytes.

Polymerase chain reaction for cpe gene

For the preparation of template DNA, incubation of DS cultures were continued up to 48 h. Then 20µl portion of each culture was mixed with the same volume of sterile distilled water (SDW), boiled for 5min and centrifuged at 12,000 rpm for 10 min. From the supernatant 5 µl was added into the PCR mixture. The primers CPE-1, CPE-2 and positive control template were supplied from TaKaRa biomedicals (Shiga, Japan). PCR amplifications were carried-out using Perkin Elmer DNA thermal cycler. The conditions adopted were 35 cycles of 1 min at 94°C, 55°C and 72°C. Following electrophoresis on 3% agarose gel, PCR amplified products were visualized with ethidium bromide (0.5 µg/ml) and photographed under UV light.

RESULTS

After 8 hr incubation, DS culture inoculated with *C. perfringens* NCTC 8239 was examined under phase contrast microscope. More than 80% of the cells contained refractile fore spores as shown in Fig. 1. Flow cytometric profile of the vegetative cells of *C. perfringens* NCTC 8239 exhibited significantly low fluorescence as in the panel A of Fig. 2, whereas intense staining of formalin fixed sporangial cells of *C. perfringens* NCTC 8239 produced a positive fluorescent profile with a unimodal sharp peak at 200 fluorescence channel level (Fig. 2B) Similarly a bright fluorescence was also observed under fluorescence microscope (Fig. 3). In addition, sporangial cells reacted with PBS instead of MAb, produced a clear negative fluorescence pattern (Fig. 2C). Further, heat treated sporangial cells of *C. perfringens* NCTC 8239, produced a well-defined negative fluorescence pattern (Fig. 2D). When the sporangia were reacted with control MAb instead of 3C3 a negative fluorescence profile was observed (Fig. 2E). Apart from this, the fluorescence peak produced by the sporangial cells that were incubated with non labeled MAb 3C3 prior to biotin labeled MAb 3C3, moved to the left accompanying with a reduction of peak channel number (Fig. 2F).

Fig. 4 shows the FCM pattern produced by 8 different strains. Intense staining of *C. perfringens* NCTC 8239 sporangia produced a sharp peak at 209 fluorescence channel number (Fig. 4d). Whereas the sporangia of *C. perfringens* NCTC 8238 revealed a biphasic fluorescence pattern with high intensity peak at 98 (Fig. 4c). Moderate degree of staining of *C. perfringens* NCTC 8235 and NCTC 8798 demonstrated peak fluorescence intensity of 51 and 62 respectively (Figs. 4f & g). The fluorescence intensity of *C. perfringens* NCTC 8797, 8679, 10240, 10611 stayed at low levels (peak < 20, Fig. 4 b, e, h and i) and unstained cells of negative control produced a peak at 6 (fig. 4 a). According to the manual of Takara, cpe gene is detected as a 456 bp band following amplification by PCR. The positive control template gave well-amplified DNA band (data not shown). Templates of all 8 strains examined showed a 456-bp band (fig. 5 lane 2 to 9) but the template of *E. coli* did not show such a band (Fig. 5 lane10).

DISCUSSION

Roper *et al.* (1976) reported the presence of high CPE concentration in the mother cell compartment of *C. perfringens* sporangia. Moreover, accumulation of CPE during sporulation process of some food poisoning strains, result in the formation of paracrystalline inclusion bodies in the cytoplasm of many sporulating cells (Labbe, 1989). In addition *C. perfringens* NCTC 8239 has been identified as a heavy CPE producing strain (Stelma *et al.*, 1985) and thus the sporangia separated from 6 - 8 h sporulation cultures of *C. perfringens* NCTC 8239 was used to testify the potency of FCM in the detection of enterotoxigenic *C. perfringens*. FCM profiles produced by the sporangia of *C. perfringens* NCTC 8239 further clarify their heavy CPE producing capability and demonstrate the possible application of sporangial cells for flow cytometry.

A negative fluorescence pattern produced by *C. perfringens* vegetative cells clarifies the absence of CPE at this stage of growth. It is consistent with the results of McClane (1998) who demonstrated the inability of CPE expression in the absence of sporulation for any CPE positive *C. perfringens* isolate. In contrast Granum *et al.* (1984) demonstrated very low levels of CPE from vegetative cultures of *C. perfringens*.

CPE was identified as a heat sensitive protein that denatures at 55°C (Granum & Skjelkvale, 1977). In the present study, heat-treated sporangia were also analyzed and the results were found to be compatible with the established reports. A clear negative fluorescence profile produced by sporangia when incubated with control MAb and PBS indicated the specificity of MAb 3C3. All these observations indicate the suitability of applying FCM in the detection of enterotoxigenic *C. perfringens*. Use of biotin labeled probes were also found to be better for FCM assay of CPE as it reduces the occurrence of non-specific fluorescence and further improves the accuracy of assay.

The applicability of this FCM method was tested using eight strains that were cultured simultaneously under similar laboratory conditions. Fluorescence histograms obtained by FCM revealed a wide variation in the level of CPE produced by these strains. However, the PCR showed that all strains possess the cpe gene and have the potential to produce CPE. This phenotypic variation should be considered in the analysis of outbreaks because gene amplification methods alone might produce an inaccurate picture regarding enterotoxigenicity. Thus, to confirm an outbreak of *C. perfringens*, detection of CPE

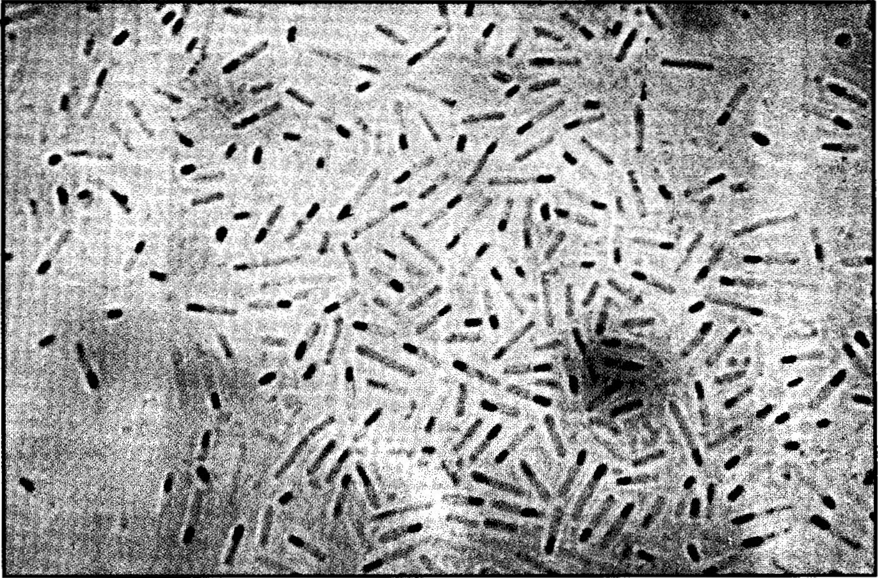

Fig. 1. ***C. perfringens*** **NCTC 8239 sporangia harvested from sporulation media after 6-8 hr incubation.**

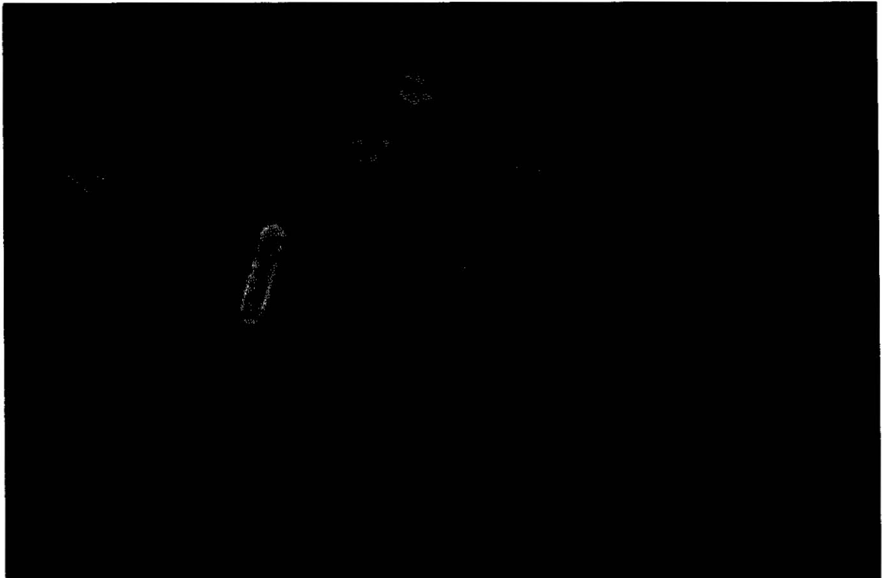

Fig. 3. Fluorescence microscopic appearance of ***C. Perfringens*** **NCTC 8239 sporangia incubated with biotin labeled MAB 3C3.**

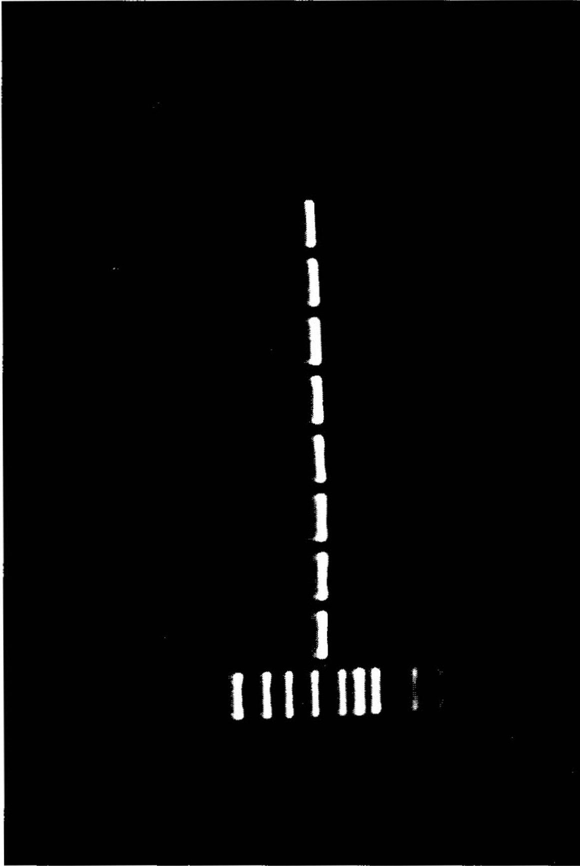

Fig. 5. Agarose gel electrophoresis pattern of PCR products of different strains of *C. Perfringens* for its enterotoxin gene.

Lane 1, Market (1,057 bp, 770,612,495, 392, 345, 341, 335, and 297 from the top) 2; Strain NCTC 8239, NCTC 8238, 4; NCTC 10611, 5; NCTC 8679, 6; NCTC 8235; 7; NCTC 8798, 8; NCTC 10240, 9; NCTC 8797, 10; E. Coli (negative control)

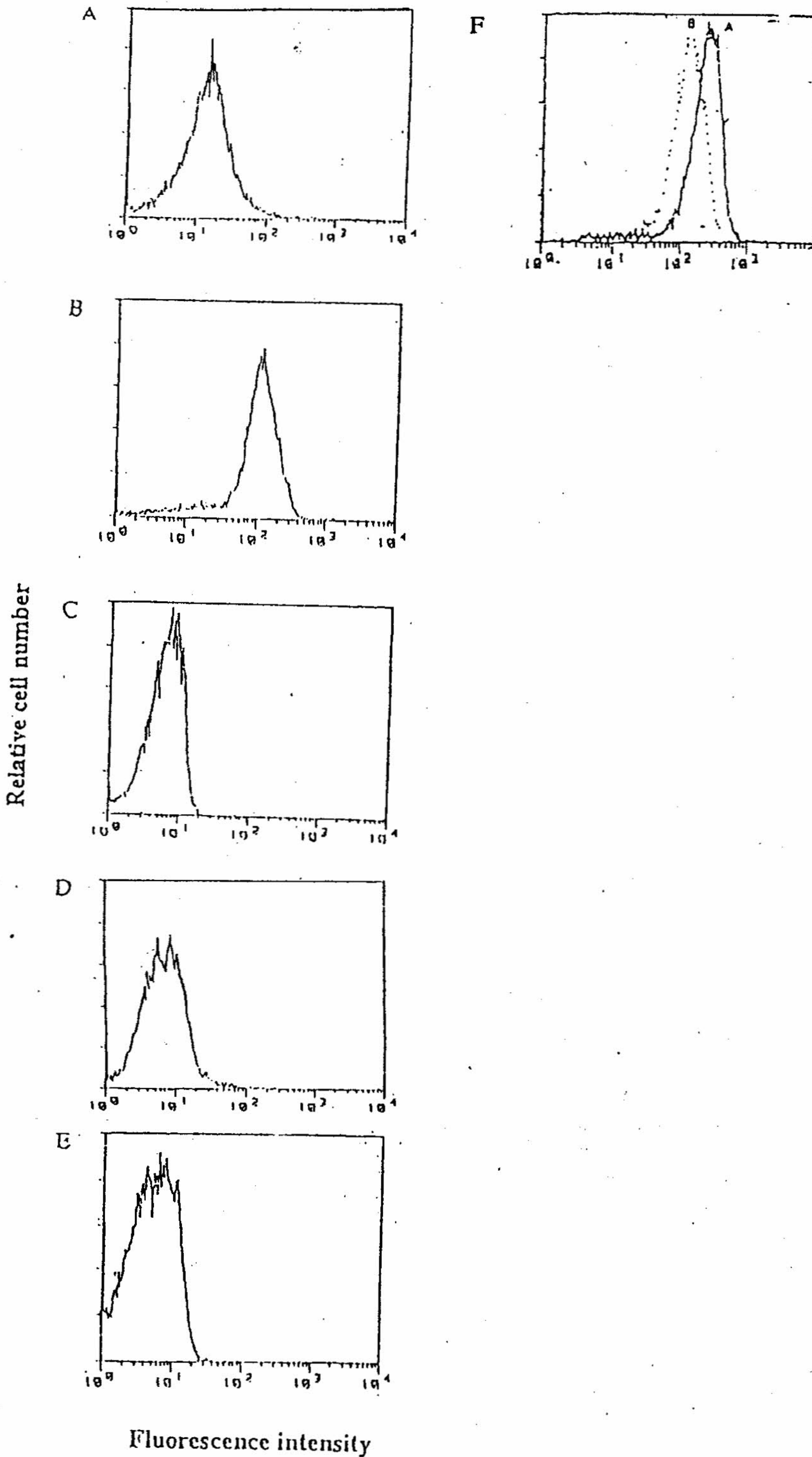

Fig 2. Flow cytometric analysis of *C. perfringens* NCTC 8239 by the use of MAb 3C3 specific for CPE, panel A; The vegetative cells, B; The sporangia incubated with biotin labeled MAb 3C3, C; The sporangia incubated with PBS D; The heat treated sporangia E; The sporangia incubated with negative control Mab.

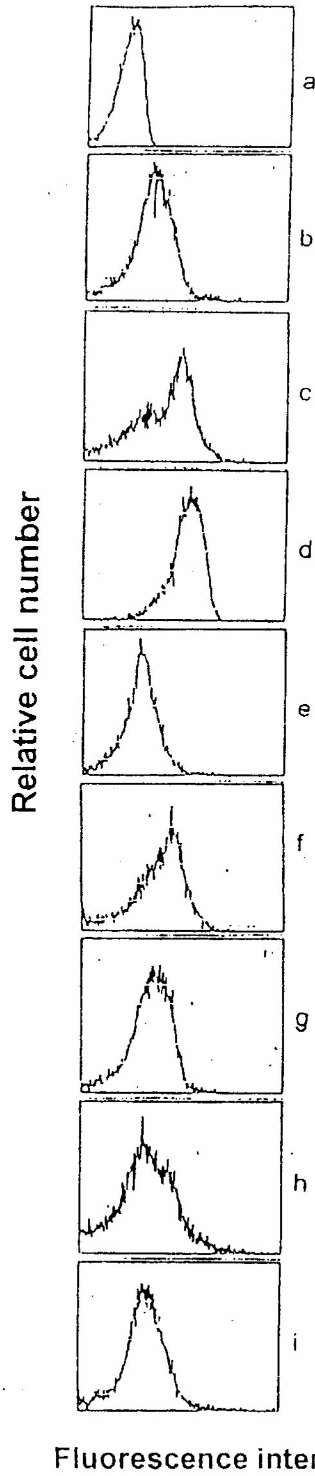

Fig 4. Flow cytometric analysis of FITC fluorescence produced by *C. perfringens* strains a) negative control b) NCTC 8797 c) NCTC 8238 d) NCTC 8239 e) NCTC 8679 f) NCTC 8235 g) NCTC 8798 h) NCTC 10240 i) NCTC 10611.

molecule is very important. However, determination of free toxin is limited due to poor CPE producing strains and difficulties in obtaining satisfactory level of sporulation under laboratory conditions.

Thus, the FCM method, which is specific, rapid, accurate and reproducible, can be used successfully in the detection of enterotoxigenic *C. perfringens*. Furthermore, the FCM method was also found to be more superior to the existing methods as the determination of CPE in thousands of sporangia is possible with in seconds. In the future, this technique might be of immense importance in *C. perfringens* outbreak surveillance programs.

REFERENCES

1. Bean, N. H. and ,P. M. Griffen1990. Food borne disease outbreaks in the United States, 1973-1987; pathogens, vehicles and trends. *Journal of Food Protection*, **53**, 804-817.

2. Duncan, C. L., Strong, H. and , M. Sebald 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens. Journal of Bacteriology*, **110,** 378-391.

3. Granum, P. E. and , R Skjelkvale. 1977. Chemical modification and characterization of enterotoxin from *Clostridium perfringens* type A. *Acta Pathol. Microbiol. Scand.* Sect, B, **85**, 89-94.

4. Granum, P. E., Telle, W., Olsvik, O. and A. Stavn 1984. Enterotoxin formation by *Clostridium perfringens* during sporulation and vegetative growth. International *Journal of Food Microbiology*, **1**, 43-49.

5. Itoh, T., Inaba, M., Saito, K., Sakai, S., Uemura, T. and G. Sakaguchi 1979. Assay for enterotoxin in fecal specimens of *Clostridium perfringens* food poisoning. *J. Jpn. Assoc. Infect. Dis.*, **53**, 409-416.

6. Labbe, R. 1989. *Clostridium perfringens*. In: M. P. Doyle(Ed.), *Foodborne bacterial pathogens*. Marcel Decker, New York. 192-234.

7. Labbe, R. G. 1991. *Clostridium perfringens. J. Assoc. Off. Anal. Chem.*, **74**, 711-714.b.

8. McClane, B. A. 1996. An overview of *Clostridium perfringens* enterotoxin. *Toxicon,* **34,** 1335-1343.

9. McClane, B. A. 1997. *Clostridium perfringens* In: M. Doyle, L., Beuchat, L., T. Montville (Eds) *Food microbiology: fundamentals and frontiers*. ASM press, Washington DC. 305-326.

10. McClane, B. A. 1998. New insights into the genetics and regulation of expression of *Clostridium perfringens* enterotoxin. *Curr. Top. Microbiol. Immunol.*, **225**, 37-55.

11. Nillo, L. 1977. Enterotoxin formation by *Clostridium perfringens* type A studied by the use of fluorescent antibody. *Can. J. Microbiol.*, **23**, 908-915.

12. Piyankarage, R. H., Tajima, T., Sugii, S. and T. Uemura 1999. Sandwich Enzyme Linked Immunosorbent Assay by using monoclonal antibody for the detection of *Clostridium perfringens* enterotoxin. *Journal of Veterinary Medical Science*, **61**, 45-47.

13. Roper, G., Short, J. A. and D.A. Walker 1976. *The ultra structure of Clostridium perfringens spores*. In: A. M. Barker, J. Wold, D. J. Ellar, G. H. Dring and G. W. Gould (Ed.), Spore Research. Academic Press Ltd., London, England, 279-296.

14. Saito, M. 1990. Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods and the natural environment in Japan. *Journal of Food Protection*, **53**, 115-118.

15. Saito, M., Matsumoto, M. and M. Funabashi 1992. Detection of *Clostridium perfringens* enterotoxin gene by polymerase chain reaction amplification procedure. *International Journal of Food Microbiology*, **17**, 47-55.

16. Stelma, G. N., Johnson, C. H. and D.B. Shah 1985. Detection of enterotoxin in colonies of *Clostridium perfringens* by a solid phase enzyme linked immunosorbent assay. *Journal of Food Protection*, **48**, 227-231.

17. Tsukamoto, T., Ishibashi, M., Asao, T., Ohtsu, K., Shinagawa, K., Kunito, D. and T. Uemura 1981. Detection of strain and enterotoxin in fecal specimens of *Clostridium perfringens* food poisoning. *Jpn. J. Publ. Health*, **28**, 487-491.