Sandwich Enzyme-Linked Immunosorbent Assay by Using Monoclonal Antibody for Detection of Clostridium Perfringens Enterotoxin

Ramani H. PIYANKARAGE, Tomoko TAJIMA1), Shunji SUGII1) and Takashi UEMURA*

Laboratories of Veterinary Public Health and 1)Veterinary Microbiology, Department of Veterinary, Science College of Agriculture, Osaka Prefecture University, 1–1 Gakuen-cho, Sakai, Osaka 599–8531, Japan

(Received 5 February 1998/Accepted 17 August 1998)

ABSTRACT. Sandwich enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative estimation of Clostridium perfringens enterotoxin (CPE) with monoclonal and polyclonal antibodies as capturing and detecting antibodies, respectively. The dose-dependent relationship between absorbance at 405 nm and concentration of purified CPE was obtained over the range of 0.64–400 ng/ml. The sandwich ELISA was found to detect crude CPE in culture and CPE in 10% fecal extracts. This method is convenient, rapid and sensitive for specific detection of CPE.—KEY WORDS: CPE, ELISA, monoclonal antibody.


Clostridium perfringens is a common food bacterium distributed widely in human foods, especially meat and poultry products [12]. Most strains of C. perfringens type A produce an intestinally active polypeptide known as enterotoxin (CPE), a causative factor of human food poisoning [11]. CPE is synthesized during sporulation of C. perfringens vegetative cells and the lysis of sporulated cells liberates CPE into the intestinal tract [3]. Various immunoassay methods such as immunoelectrophoresis, hemagglutination, and other methods have been developed during the past few decades for detection of CPE [2, 8, 10, 14]. At the present time, enzyme-linked immunosorbent assay (ELISA) is one of the common methods to detect CPE. The most sensitive ELISA for CPE by the use of polystyrene beads detects CPE in picogram quantities [15], whereas several plate ELISA methods in nanogram levels [1, 6, 7, 9, 16]. Even though monoclonal antibodies (MAb) have been developed against CPE [5, 17], MAb-based ELISA has not been reported. The purpose of this study was to develop a rapid, convenient, quantitative ELISA by using MAb for specific detection of CPE.

C. perfringens strains NCTC 8239 (Hobb’s type 3) and NCTC 8798 (Hobb’s type 9) were used in this study. Crude and purified CPE were prepared according to the methods described previously [3, 11]. Rabbit antiserum against purified CPE was produced by injecting 15–40 μg of purified CPE in Freund’s complete adjuvant according to the methods described previously [14].

For the preparation of MAb, purified CPE detoxified with formalin was used as the antigen. Balb/c CrSlc mice were immunized in the same manner as described previously [4, 5]. Mouse spleen cells were fused with mouse myeloma cells P3X63Ag8.653 by using polyethylene glycol 1,500 (Boehringer Mannheim GmbH, Germany). Cell fusion, screening, cloning, and production of ascitic fluid were performed as described previously [4, 5]. MAb isotypes were determined by ELISA with a Mouse Typerr Subisotyping Panel (Bio-Rad Laboratories, Richmond, CA, U.S.A.) according to the instructions given by the manufacturer. Specificity of MAb was determined by Western blotting [13] with crude CPE produced by C. perfringens strain NCTC 8239 as the antigen.

For sandwich ELISA to detect CPE, each well of a 96-well microplate (Iwaki Glass, Tokyo, Japan) was coated with 100 μl of ascitic fluid produced by MAb 3C3 (diluted 1:4,000) in 0.05 M carbonate buffer, pH 9.6 by incubating for 1 hr at 37°C in a humid chamber. In order to block non-specific binding, 200 μl of Block Ace (diluted to 1:4 with distilled water) (Dainippon Pharmaceutical Co., Osaka, Japan) was added to each well and the plates were incubated for 1 hr at 37°C. At the end of incubation, plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST). Then, 5-fold serial dilutions of purified CPE starting from 1:50 in 0.01 M phosphate buffer, pH 6.8, were applied in 100 μl amounts into appropriate wells and the plates incubated for 1 hr at 37°C. After incubation, the plates were washed in the same way as earlier and 100 μl of rabbit anti-CPE serum (diluted to 1:5,000 with Block Ace) was added to each well and the plates were incubated for further 1 hr at 37°C. This incubation was followed by four washing steps with PBST, and 100 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG (Biosource International, diluted to 1:500) was added to each well. Then, the plates were incubated at 37°C for 30 min. After 4 washings, 100 μl of 0.05 M citrate phosphate buffer (pH 4.0) containing 0.2 mM 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.) and 0.04% H2O2, was added to each well and the plates were left standing at room temperature for 20 min for color development. The absorbance at 405 nm was measured with a microplate reader (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Each sample was tested in duplicate with appropriate controls.

For detection of crude CPE produced by C. perfringens strains NCTC 8239 and NCTC 8798, 5-fold serial dilutions

* Correspondence to: Dr. UEMURA, T., Laboratory of Veterinary Public Health, Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599–8531, Japan.
were prepared in 0.01 M phosphate buffer, pH 6.8. Sandwich ELISA was carried out in the same manner as described above.

To determine CPE in fecal materials, 1 ml of purified CPE was added to a fecal suspension prepared by adding 1 g of CPE-free goat feces to 9 ml of diluted Block Ace. The suspension was vortexed well, incubated for 30 min at room temperature, and centrifuged at 10,000 rpm for 20 min. With the supernatant, sandwich ELISA was performed as described above. All procedures adopted were the same except that the blocking buffer was replaced by 10% fetal calf serum.

Eight hybridomas secreting anti-CPE antibody were finally obtained. Among them, MAb 3C3 was found to contain the highest antibody titer in the titration assay and used for subsequent studies. MAb 3C3 was found by Western blotting to produce IgG1(κ) and to react with CPE (34 kDa).

Figure 1 shows the standard curve for detection of purified CPE in the sandwich ELISA. The dose-dependent relationship between absorbance at 405 nm and concentration of purified CPE was obtained over the range of 0.64–400 ng/ml. Dose-response curve was also observed with crude CPE produced by C. perfringens strain NCTC 8239 over the range of dilutions of culture fluid from 1:5 to 1:3,125 (Fig. 2a). By the latex agglutination test, the culture fluid at these dilutions contained approximately 1.2 μg/ml–1.7 ng/ml of CPE. Similar results were obtained by using CPE produced by C. perfringens NCTC 8798 over the range of dilution from 1:125 to 1:3,125 and it contained approximately 104–4.16 ng of CPE/ml (Fig. 2b). These results indicate that this ELISA was applied successfully in the detection and quantitative assay of both purified and crude CPE. The most sensitive ELISA method reported previously [15] could detect 1.0 pg of purified CPE. However, this method is laborious and it takes more than 2 days for the completion. In the present study, we were able to complete the assay within 5.5 hr. By comparison, the present ELISA method is simple and quick. This is an important achievement as the rapidity is combined with high sensitivity and specificity.

With fecal samples, more than 10 ng of CPE per gram of feces (>1 ng/ml) was specifically detected by this sandwich ELISA method (Fig. 3). As reported previously [6], nonspecific high background reactions were observed during analysis of CPE in fecal extracts. This was minimized successfully by using 10% fetal calf serum (FCS) as the blocking buffer. According to Skjelkvale and Uemura [10], the detectable amount of CPE in fecal samples of food poisoning patients is 0.5–16 μg/g. On the other hand, another report [1] has shown that 38% of stool samples in outbreaks contained less than 0.5 μg/g of CPE. It has also shown a much wider range of CPE concentrations ranging from 12 ng/g to 140 μg/g in stool specimens. The lowest detectable level of fecal CPE was found to be 10 ng/g (1 ng/ml) in the sandwich ELISA system described in this study. Hence, it could be adopted successfully in analyzing fecal samples of infected patients.

The sandwich ELISA described here was found to be a rapid and quantitative method with a high specificity for the detection of CPE. It could be adopted successfully in analyzing CPE in not only spent cultures but also in fecal samples.

ACKNOWLEDGMENT. This study was supported in part by a grant-in-Aid for Scientific Research (No. 09460146) from the Ministry of Education, Science, Culture, and Sport of Japan.

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**Figure 1.** Standard curve for ELISA determinations of purified *Clostridium perfringens* enterotoxin. \( y = 0.41x + 0.30, r = 0.97 \)
Fig. 2. ELISA determinations of crude CPE produced by
a) *Clostridium perfringens* NCTC 8239 (Hobb’s type 3),
b) *Clostridium perfringens* NCTC 8798 (Hobb’s type 9).

Fig. 3. ELISA determinations of CPE in fecal samples spiked with purified CPE.

REFERENCES