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Comparison of Sensitivity of Detection for *Clostridium perfringens* Type A Enterotoxin by the Reversed Passive Latex Agglutination and the Polymerase Chain Reaction

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Clostridium perfringens A형이 생산하는 장독소의 검색을 위한 RPLA법과 PCR기법의 감도비교

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국문요약

Clostridium perfringens A형이 생산하는 장독소를 검색해본 결과, RPLA법에 있어서는 2배로 희석한 용액으로부터 64배로 희석한 용액(NCTC 8239; Hobbs serotype 3; CPE$^+$)에서까지 양성반응을 보였으며 PCR 기법에 있어서는 10 pg 희석 용액까지 364 bp의 장독소 DNA fragment(NCTC 8238; Hobbs serotype 2; CPE$^+$)를 확인 할수 있었다. 그러므로 장독소를 검색하기 위해서는 PCR 기법이 RPLA법에 비하여 훨씬 감도가 높음을 확인할 수 있었다.

Keywords : *C. perfringens* Type A Enterotoxin, RPLA, PCR.

I. Introduction

C. perfringens type A food poisoning is widely reported.[1-6] The enterotoxin from *C. perfringens* type A is responsible for many cases of a mild type of food poisoning. It is produced in the intestinal tract during sporulation, after ingestion of 10^8-10^9 cells.[7] An enterotoxin produced by *C. perfringens* is responsible for a common form of bacterial food poisoning.[8] Food poisoning by *C. perfringens* occurs after ingestion of food contaminated with large numbers of *C. perfringens* vegetative cells, and it is characterized by diarrhea and abdominal pain.[9]

Human stool specimens frequently contain *C. perfringens* and some foodstuffs contain a large amount of this organism.[10,11] Since such organisms found in human and animal feces are mostly non-enterotoxigenic, it is necessary to distinguish the enterotoxigenic organisms from the non-enterotoxigenic ones to identify the true agents of food poisoning.

Enterotoxigenic *C. perfringens* causes food poisoning, which can be diagnosed by detecting enterotoxin in patient's stool specimens or by confirming the enterotoxigenicity of isolates from the incriminated strain or patient's stools. Excretion of enterotoxin in the stool, however, is limited only to the diarrheal stage.[12-14] It is often difficult to obtain adequate stool specimens and sensitivity of assayable methods to detect enterotoxin.

This study describes the comparison of sensitivity of detection for *C. perfringens* type A enterotoxin by the RPLA and the PCR.

II. Materials and Methods

1. Bacterial strains

Four well-toxin typed reference strains of *C.*

perfringens were used. These strains included: toxin type A strains NCTC 8791(Hobbs serotype 1), NCTC 8238(Hobbs serotype 2), NCTC 8239(Hobbs serotype 3) and NCTC 8235(Hobbs serotype 8), (received from Dr. Teizo Tsukamoto, Osaka Prefectural Institute of Public Health, Japan). Besides, one non-toxin typed reference strain was *C. perfringens* ATCC 3629 (received from Veterinary Research Institute, Rural Development Administration, Korea).

Twenty four *C. perfringens* strains (22 types-A toxins and 2 types-C toxins) were isolated from necrotic enteritis of chickens (7 strains), enterotoxemic piglets (14 strains) and enterotoxemic cattle (3 strains) in Korea.

2. Detection of enterotoxin of strains by the RPLA

C. perfringens were cultured in the thioglycollate medium at 37℃ for 18-20 hrs and deactivated by heating at 75℃ for 20 min. If the spore-formation of the *C. perfringens* cells were poor, the cells were repeated with thioglycollate medium and heating 2-3 times. Then *C. perfringens* were inoculated in the Duncan and Strong medium[15] prepared for promoting enterotoxin production, and cultured at 37℃ for 18-48 hrs.

After cultivation, the culture fluid was cold-centrifuged at 3000 rpm for 20 min and the supernatants were assayed for *C. perfringens* enterotoxin by the RPLA with a PET-RPLA kit (Denka Seiken, Tokyo).[16]

3. Purification of chromosomal DNA from the strains

Chromosomal DNA was purified by the following procedure.[16] Each strain was seeded on a cooked meat medium (DIFCO) and in the Gifu anaerobic medium broth (GAM, Nissui), then incubated for 24 hrs at 37℃. From a 1.5 ml portion of the culture, chromosomal DNA was purified by use of ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) and achromopeptidase (Wako Pure Chemical Industries, Osaka). The cultured cells were centrifuged and resuspended in 50 mM EDTA and washed with a 5 mM EDTA solution (pH 8.0). Washing was repeated with resuspension in 200 μl of 5 mM EDTA, to which 100 μl of achromopeptidase (10 mg/ml of 50 mM EDTA) was added. The mixture was then stirred gently and incubated for 90 min at 37℃, and 50 μl of 10% sodium dodecyl sulphate (SDS, Wako Pure Chemical Industries, Osaka) was added to the mixture, which was stirred gently and incubated for 10 min at 60℃. Freezing and thawing were repeated 3 times and 500 μl of phenol-chloroform was added to the mixture, which was stirred vigorously and centrifuged at 14000 xg for 10 min. 200 μl of ethyl and 1 ml of cold ethanol were added to the supernatants to allow chromosomal DNA to precipitate. The purified nucleic acids were diluted with 500 μl of distilled water, a 5 μl portion of which was added to the PCR system.

4. Extension primers

From the base sequence of the *C. perfringens* enterotoxin gene[17], two oligonucleotides were synthesized as primers.

One, PT-1, was synthesized from the nucleotide sequence from the 426 to 446 bp (5'-TGTAGAATATGGATTTGGAAT-3') and the other, PT-2, from the 770 to 789 bp (5'-AGCTGGGTTTGAGTTTAATG-3'). These two primers were designed to generate for a 364 bp (Takara Biotech. Co., Kyoto).

5. Detection for enterotoxin by the PCR

PCR was performed in a 50 μl reaction mixture. The reagents for the PCR were prepared in the following way. To a 500 μl sample tube, 33.7 μl of distilled water, 5 μl of the reaction buffer of 10-fold strength (Promega Corp., Madison, WI), 4.0 μl of dNTP mixture (2.5 mM), 0.3 μl of Taq DNA polymerase (Promega), 1.0 μl of PT-1 (20 μM), 1.0 μl of PT-2 (20 mM), and 5 μl of a DNA solution were added. The mixture was overlaid with sterilized mineral oil (Sigma, St. Louis, MD) to prevent evaporation during incubation. Incubation for DNA denaturation was 1.5 min at 94℃, that for annealing the primers for 2.3 min at 55℃. Incubation for primer extension by DNA polymerase for 1.3 min at 72℃, using a DNA thermal cycler (Pharmacia Co., Alameda, Calif.) that changed the temperature automatically 25-30 times.

After the reaction, the final extension was performed by holding the tube for 5 min at 72℃, then cooling the tube to 4℃. In the amplification of the DNA, 0.5 μl of the reaction mixture was subjected to electrophoresis in 1.5% agarose gel with a miniature electrophoresis system from mupid (Cosmobio Corp.,Tokyo).

The electrophoresis gel was stained with a 0.003 % ethidium bromide solution for 1-2 min and read at a wave length of 312 nm with a transilluminator (Vilber Lourmat, Vallee, France). Detection of the 364 bp DNA fragment was regarded as a positive enterotoxin gene.

III. Results

The detection of CPE by the RPLA was applied to specimens from NCTC 8239 (Hobbs serotype 3; ET$^+$) and ATCC 3629 (ET$^-$), as well as 7 isolates from necrotic enteritis of chickens, 14 isolates from enterotoxemic piglets and three isolates from enterotoxemic cattle as shown in Table 1. As this table also shows, detection of CPE by the RPLA was positive from 2-fold to 64 -fold dilution in NCTC 8239 (Hobbs serotype 3; ET$^+$), although it showed negative results for 24 samples.

Detected results for enterotoxin genes from strains of *C. perfringens* using the PCR are shown in Fig. 1 and Fig. 2 respectively in electrophoretic pattern of the PCR products obtained with purified nucleic acids from the template.

The sensitivity of the PCR in detecting enterotoxin gene extracts from *C. perfringens* was performed as 10-fold dilution from 100 ng to 100

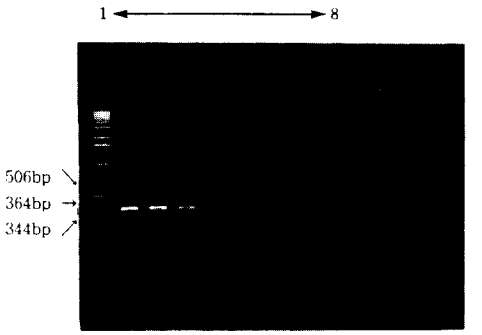

Fig. 1. Sensitivity of the PCR assay in detecting the *C. perfringens* enterotoxin gene. Lane 1: 1Kb ladder DNA marker; lanes 2-8: 10-fold dilution of DNA extract of *C. perfringens* NCTC 8238(Hobbs serotype 2; ET$^+$) from 100 ng to 100 ag (annealing temperature: 55℃).

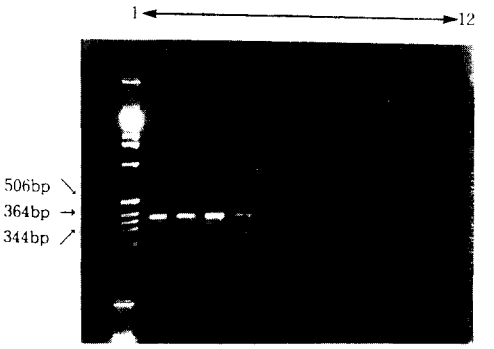

Fig. 2. Electrophoretic analysis of the PCR amplified *C. perfringens* enterotoxin gene. Lane 1: 1Kb ladder DNA marker; lane 2: *C. perfringens* NCTC 8791 (Hobbs serotype 1; ET$^-$); lane 3: *C. perfringens* NCTC 8238 (Hobbs serotype 2; ET$^+$); lane 4: *C. perfringens* NCTC 8239 (Hobbs serotype 3; ET$^+$); lane 5: *C. perfringens* NCTC 8235 (Hobbs serotype 8; ET$^-$); lane 6: *C. perfringens* ATCC 3629 (ET$^-$), and lanes 7-12: samples isolated from animal cases.

Table 1. Detection of *Clostridium perfringens* enterotoxin by the reversed passive latex agglutination

Strains	Dilution							
	×2	×4	×8	×16	×32	×64	×128	×256
NCTC 8239(Hobbs serotype 3; ET$^+$)*	+++	++	++	++	++	+	±	-
ATCC 3629 (ET$^-$)*	-	-	-	-	-	-	-	-
Necrotic enteritis of chickens (n=7)**	±	-	-	-	-	-	-	-
Enterotoxemic piglets (n=14)**	±	-	-	-	-	-	-	-
Enterotoxemic cattle (n=3)**	±	-	-	-	-	-	-	-

NOTE : -; reaction of enterotoxin non-producing, ±; reaction of enterotoxin doubtable producing, +, ++, +++; reaction of enterotoxin producing.
*ET : *C. perfringens* enterotoxin., **n : Strain numbers of isolated from cases.

ag for annealing temperature at 55℃ (364 bp) in NCTC 8238 (Hobbs serotype 2; ET$^+$), (Fig. 1).

Moreover, the PCR using *C. perfringens* enterotoxin gene-specific primers detected up to 10 pg (a DNA fragment of 364 bp) for annealing temperature at 55℃ in NCTC 8238 (Hobbs serotype 2; ET$^+$), (Fig. 1). But, specific clear band (364 bp) of *C. perfringens* enterotoxin gene was proved of negative results in 24 samples isolated from specimens of necrotic enteritis of chicken (n=7 strains), enterotoxemic piglets (n=14 strains) and enterotoxemic cattle (n=3 strains) in Korea. The results of detection of CPE by the RPLA are similar with those of the PCR but the PCR was more sensitive of the assays than the RPLA.

IV. Discussion

C. perfringens is widespread in the soil and sewage, and is commonly found in the intestinal tract of human and various animals. It is an opportunistic organism and causes great economic losses by death due to necrotic enteritis of chickens, enterotoxemic piglets and enterotoxemic cattle.[18-20] Type-A *C. perfringens* food poisoning is also widely reported.[1,2,4,6] The purpose of the study reported here were to the comparison of sensitivity of detection for *C. perfringens* type A enterotoxin by the RPLA and the PCR.

The detection of CPE by the RPLA was positive from 2-fold to 64-fold dilution in NCTC 8239 (Hobbs serotype 3; ET$^+$), although it gave negative results for 24 samples (Table 1).

The sensitivity of the PCR in detecting enterotoxin gene extracts from *C. perfringens* was performed as 10-fold dilution from 100 ng to 100 ag for annealing temperature at 55℃ (a DNA fragment of 364 bp) in NCTC 8238 (Hobbs serotype 2; ET$^+$), which is in agreement with Saito *et al.*[16] (Fig. 1). Moreover, the PCR using *C. perfringens* enterotoxin gene-specific primers detected up to 10 pg (a DNA fragment of 364 bp) for annealing temperature at 55℃ in NCTC 8238 (Hobbs serotype 2; ET$^+$) (Fig. 1).

The detection of *Escherichia coli* enterotoxin is compared with the enzyme-linked immunosorbent assay (ELISA) and the suckling mouse bioassay. Taking the suckling mouse assay as the "gold standard", the gene probe was the more specific and the ELISA was more sensitive of the assays[21], also the gene probe assay had a specificity of 99% and a sensitivity of 90.4% compared to the infant mouse method.[22]

Basically, the detection of *C. perfringens* enterotoxin by the RPLA and the PCR in these results, which is in similar with the findings of Cryan.[21,22]

These results suggest that to be rapid and acurate diagnostic tests for *C. perfringens* type A enterotoxin into their epidemiological investigations of animal infectious diseases and human food poisoning.

V. Conclusion

Detection for *Clostridium perfringens* enterotoxin (CPE) by the reversed passive latex agglutination (RPLA) was positive from 2-fold to 64-fold dilutions in NCTC 8239(Hobbs serotype 3; ET$^+$). The polymerase chain reaction (PCR) which was using *C. perfringens* enterotoxin gene-specific primers with a detection limit equivalent to 10 pg of a DNA fragment of 364 bp in NCTC 8238(Hobbs serotype 2; ET$^+$). The PCR provided a rapid and more sensitivity tool for the detection of CPE compare to the RPLA

References

1) Hobbs, BC. : *Clostridium welchii* as a food poisoning organism. J. App. Bacteriol., 28, 74-82, 1965.
2) Loewenstein, MS. : Epidemiology of *Clostridium perfringens* food poisoning. N. Engl. J. Med., 286, 1026-1028, 1972.
3) Itoh, T. : Incidence of heat-resistant *Clostridium perfringens* in faeces of healthy subjects, serotyping of isolates and food poisoning caused by new serotype organisms. Ann. Rep. Tokyo Metr. Res., Lab. Publ. Hlth., 24, 7-39, 1973.
4) Raevuori, M. : The occurrence of food poisoning outbreaks in Finland. Suomen Elainlaakaril, 82, 501-508, 1976.
5) Todd, ECD. : Food borne disease in six countries-a comparison. J. Food Prot., 41, 559-565, 1978.
6) Choudhary, SP. & Narayan KG. : Outbreak of

Clostridium perfringens type A food poisoning. 30th Ann. Carif. IPHA, All India Inst. of Hyg. Publ. Hlth. Jan., 16-18, 1985.

7) Richardson, M. & Granum, PE. : The amino acid sequence of the enterotoxin from *Clostridium perfringens* type A. Fed. Eur. Biochem. Soic., 182, 479-484, 1985.

8) Walker, HW. : Food-borne illness from *Clostridium perfringens*. Criti. Rev Food Sci. Nutr., 7, 71-104, 1975.

9) Uemura, T, Akai, T, & Sakaguchi, G. : Structure and function of *Clostridium perfringens* enterotoxin. microbial toxins in foods and feeds. Plenum Press, 203-212, 1990.

10) Yasukawa, A, Okada, Y, Kitase, T, & Miyamoto, S. : Distribution of enterotoxin-producing strains of *Clostridium perfringens* type A in human beings, foods and soils. J. Food Hyg. Soc. Jpn., 16, 313-317, 1975.

11) Kokubo, Y, Jinbo, K, Murakami, F, & Murakami, H. : Prevalence of spore-forming bacteria in sugar, starch, spices and vegetable protein. Ann. Rep. Tokyo Metr. Res. Lab. Publ. Hlth., 33, 155-160, 1982.

12) Uemura, T. : Incidence of enterotoxigenic *Clostridium perfringens* in healthy humans in relation to the enhancement of enterotoxin production by heat-treatment. J. App. Bacteriol., 44, 411-419, 1977.

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

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

15) Duncan, CL. & Strong, DH. : Improved medium for sporulation of *Clostridium perfringens*. App. Microbiol., 16, 82-89, 1968.

16) Saito, M, Matsumoto, M, & Funabashi, M. : Detection of *Clostridium perfringens* enterotoxin gene by the polymerase chain reaction amplification procedure. Int. J. Food Microbiol., 17, 47-55, 1992.

17) Damme-Jongsten, MV, Wernars, K, & Notermans S. : Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. Antonie Van Leeuwenhock, 56, 181-190, 1989.

18) Cho, SK, Kim, & JY, Park, JM. : Studies on enterotoxemia of calves caused by *Clostridium perfringens*. Kor. J. Vet. Publ. Hlth., 14, 255-263, 1990.

19) Kim, HJ. : Studies on necrotic enteritis of chickens caused by *Clostridium perfringens*. Thesis of PhD., Dept. Vet. Med., Graduate School, Chonnam National Univ., Korea, 1994.

20) Park, KY, Jung, SD, Yeh, JG, & Seo, IB. : Studies on the necrotic enteritis of chicken in Korea. Kor. J. Vet. Med., 34, 593-599, 1994.

21) Cryan, B. : Comparison of three assay system for detection of enterotoxigenic *Escherichia coli* heat-stable enterotoxin. J. Clin. Microbiol., 28, 792-794, 1990a.

22) Cryan, B. : Comparison of the synthetic oligonucleotide gene probe and infant mouse bioassay for detection of enterotoxigenic *Escherichia coli*. Eur. J. Clin. Microbiol. Inf. Dis., 9, 229-232, 1990b.