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

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독소원성 대장균과 *Clostridium perfringens* A형이 생산하는 장독소의 검색을 위한 RPLA법과 PCR기법의 감도 비교

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요 약

독소원성 대장균(enterotoxigenic *Escherichia coli*, ETEC, EC81, serotype O148:H28)이 생산하는 heat-labile enterotoxin(LT)를 검색해 본 결과, reversed passive latex agglutination(RPLA)법에 있어서는 2배로 희석한 용액(50 ng)으로부터 64배로 희석한 용액(1.56 ng)에서까지 양성반응을 보였으며 polymerase chain reaction (PCR)기법에 있어서는 10 ng으로부터 1 pg희석용액에서까지 417-base pair(bp)의 LT DNA fragment가 확인되었다. *Clostridium perfringens* A형(NCTC8238, Hobbs serotype 2)이 생산하는 장독소를 검색해 본 결과, RPLA법에 있어서는 2배로 희석한 용액(50 ng)으로부터 64배로 희석한 용액(1.56 ng)에서 까지 양성반응을 보여 독소원성대장균이 생산하는 LT와 일치하였으나, PCR기법에 있어서는 10 ng로부터 10 pg 희석용액에서 까지 364-bp의 DNA fragment가 확인되어 독소원성대장균이 생산하는 LT보다 1/10의 낮은 감도를 보였다. PCR기법은 RPLA법에 비하여 훨씬 신속하고 소량의 sample로 장독소를 확인할 수 있었다.

Key words: *Escherichia coli*, *Clostridium perfringens*, RPLA, PCR.

Ⅰ. Introduction

Enterotoxigenic *Escherichia coli* is a pathogen that has a major role in diarrheic diseases of humans and animals (Moon, 1974; Sack, 1975). Two distinct toxins have been implicated in the diarrheal process, LT(Moon, 1974; Smith and Gyles, 1970) and 2 families of heat-stable toxins(ST: ST_a and ST_b). The ST_a toxins consist of methanol-soluble peptides with similar core sequences that are active in the suckling mouse bioassay (Burgess, Bywater, and Cowley, 1978; Chan and Giannella, 1981; Giannella, 1976; Ronnberg, Wadstrom, and Jernvall,

1983): ST_b is a methanol-insoluble peptide active in the ligated pig jejunal loop test.

It is less well characterized than ST_a (Burgess, Bywater, and Cowley, 1978: Chan and Giannella, 1981: Giannella, 1976: Ronnberg, Wadstrom, and Jernvall, 1983: Greenberg and Guerrant, 1980: Jung, 1999a). The LT is of high molecular weight, consists of 2 subunits, is functionally, structurally, and genetically related to the enterotoxin of *Vibrio cholerae*, and is highly homologous among various strains of LT-production *E. coli* (Dallas, Gill, and Falkow, 1979: Jung, 1999b).

The enterotoxin from *Clostridium 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 food contaminated with large numbers of *C. perfringens* vegetative cells, and it is characterized by diarrhea and abdominal pain (Uemura, Akai, and Sakaguchi, 1990: Jung, 1997). Human stool specimens frequently contain *C. perfringens* and some foodstuffs contain a large amount of this organism (Kokubo, Jinbo, Murakami, and Murakami, 1982: Uemura, 1977). 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 (Uemura, 1977: Tsukamoto et al., 1981: Itoh, 1979). It is often difficult to obtain adequate stool specimens and sensitivity of assayable methods to detect enterotoxin. Assuming the suckling mouse bioassay is the accepted criterion referenced standard, the gene probe was more specific and the ELISA was more sensitive of the assay. Also, the gene probe assay had specificity of 99% and sensitivity of 90.4%, compared with the infant mouse method (Cryan, 1990a). The DNA-based method, such as the PCR assay, are not affected by health or expression of the bacteria. Phenotypic methods such as the RPLA and the ELISA, have problems with reproducibility in determining the existence of biologically active LT and ST_a toxins of ETEC (Cryan, 1990a: Cryan, 1990b).

The objective of this study was to define sensitivity between LT of ETEC (EC81) and *C. perfringens* enterotoxin(CPE, NCTC8238) using the RPLA and the PCR.

Ⅱ. Materials and Methods

1. Bacterial Strains

One of *E. coli* (EC81, serotype O148:H28, ST_a^+, LT^+) and the control group of one toxin-negative strain (EC84, serotype O1:H7, ST_a^-, LT^-) were used in this study. These strains isolated from human beings were donated by Cho, M. J. from the Department of Microbiology, Kyungsang University, Medical College, Korea. Also, one of *C. perfringens* strain NCTC8238 (Hobbs serotype 2, ET^+) and the control group of one toxin-negative strain (serotype ATCC3629, ET^-) were used in this study. These strains isolated from human beings were donated by Tsukamoto, Osaka Prefectural Institute of Public Health, Japan.

2. Detection of Enterotoxin of Strains by Use of the RPLA

Tryptic soy broth(DIFCO, Detroit, MI)was prepared, and LT of ETEC by use of the RPLA with the VET-RPLA kit (Denka Seiken, Tokyo, 1990), and from 2-fold (50 ng) dilutions were carried out up to the second-to-last wells. The

microtiter plate was shaken well with a microtiter plate mixer. *C. perfringens* were cultured in the thioglycollate medium(DIFCO, Detroit, MI) at 37℃ for 18 to 20 hours and deactivated by heating at 75℃ for 20 minutes. If the spore-formation of *C. perfringens* cells were poor, the cells were cultured repeatedly in the thioglycollate medium (DIFCO, Detroit, MI) and heating 2 to 3 times. Then, *C. perfringens* were inoculated in the Duncan and Strong medium (DS medium: Duncan and Strong, 1968) and Gifu anaerobic medium broth (GAM, Nissui, Tokyo) prepared for promoting enterotoxin production, and cultured at 37℃ for 18 to 48 hours. After cultivation, the culture fluid was cold-centrifuged at 3,000 rpm for 20 minutes and the supernatants were assayed for CPE by use of the RPLA with the PET-RPLA kit (Denka Seiken, Tokyo, 1990).

3. Purification of Chromosomal DNA from the Strains

For *E. coli*, chromosomal DNA was extracted by use of Murry and Thompson procedure(1980). And, for *C. perfringens*, chromosomal DNA was purified by Saito et al. procedure(1992).

4. Extension Primers

For *E. coli*, from the base sequence of LT gene of ETEC (Murray and Thompson, 1980), two oligonucleotides were synthesized as primers. For LT, primer PT-1, was synthesized from the nucleotide sequence between base pair (bp) 713 and 733, also primer PT-2, was synthesized from the sequence between bp 351 and 368(Table 1, Bioneer, Korea). These two primers were designed to generate for a 417-bp fragment for LT gene of ETEC.

For *C. perfringens*, from the base sequence of CPE gene (Damme-Jongsten et al., 1989), two oligonucleotides were synthesized as primers. Primer PT-1, was commercially synthesized from the nucleotide sequence between bp 426 and 446 also primer PT-2, was synthesized from the sequence between bp 770 and 789 (Table 2, Takara Biotech., Kyoto). These two primers were designed to generate for a 364-bp fragment for CPE gene.

Table 1. Nucleotide sequences of PCR primers for the amplification of *Escherichia coli* enterotoxin gene

Primers	Nucleotide sequences	Location
PT-1	5'-CAGACTATCAGTCAGAGGTTG-3'	713-733
PT-2	5'-TTCATACTGATTGCCGCA-3'	351-368

Table 2. Nucleotide sequences of PCR primers for the amplification of *Clostridium perfringens* type A enterotoxin gene

Primers	Nucleotide sequences	Location
PT-1	5'-TGTAGAATATGGATTTGGAAT-3'	426-446
PT-2	5'-AGCTGGGTTTGAGTTTAATG-3'	770-789

5. Detection of Enterotoxin by Use of the PCR Analysis

The PCR analysis was performed, using a 50-μl reaction mixture. The reagents for the PCR analysis were prepared in the following manner.

For *E. coli* LT, preincubation was 5 minutes at 94℃. The DNA underwent denaturation by incubation for 1 minute at 94℃. The primers were allowed to anneal for 1 minute at 55℃, then were extended by incubation with DNA polymerase for 1 minute at 72℃ in the DNA thermal cycler (Pharmacia, Alameda, Calif.) that automatically changed the temperature for 30 cycles. After the reaction, the final extension was performed by keeping the tube for 7 minutes at 72℃ then cooling it to 4℃.

For *C. perfringens*, incubation for DNA denaturation was 1.5 minutes at 94℃, and primers were allowed to anneal for 2.3 minutes at 55℃. Incubat-

ion for primer extension by the DNA polymerase for 1.3 minutes at 72℃, using the DNA thermal cycler (Pharmacia, Alameda, Calif.) that changed the temperature automatically 25 to 30 times. After the reaction, the final extension was performed by keeping a tube for 5 minutes at 72℃, then cooling the tube to 4℃. Sensitivity of the PCR analysis in detecting enterotoxin gene extract (contained with 1 μg DNA extract 1 μl using the DNA calculator, Pharmacia, Alameda, Calif.) was performed as 10-fold dilutions from 10 ng to 100 ag in *E. coli* 81 (LT^+) and *C. perfringens* NCTC 8238 (Hobbs serotype 2, ET^+).

Ⅲ. Results and Discussion

Use of gene probe for detection of LT and ST in human and animal *E. coli* has become an established laboratory technique (Woodward et al., 1990: Patamoroj et al., 1983: Jung, 1999). The DNA sequence of LT from the chicken, ETEC strain was identified by direct dideoxy sequencing of the PCR amplified DNA and was compared with those of LT from porcine and human ETEC strains EWD 299 and H10407 (Inoue et al., 1993). Clones in *E. coli* carrying parts of the CPE gene were detected with the synthetic DNA probe. For deducing the hypothetical DNA sequence from the amino acid sequence of CPE, those took into account the high AT-contents of Clostridial DNA and the codon usage of the *C. tetanus* neurotoxin gene (Marmur et al., 1963: Eisel et al., 1986).

Detection for LT of ETEC by use of the RPLA was positive reaction from 2-fold (50 ng) to 64-fold dilutions(1.56 ng) in EC81 (serotype O148: H28) (Table 3), also detection for CPE by the RPLA was positive from 2-fold (50 ng) to 64-fold dilutions(1.56 ng) in NCTC8238 (Hobbs serotype 2, ET^+) (Table 4), which was in agreement with the findings of Jung (1997) and Jung (1999b).

Table 3. Dectection of *Escherichia coli* enterotoxin by use of passive latex agglutination

Strains	Dilution							
	×2	×4	×8	×16	×32	×64	×128	×256
EC81 (O148:H28, LT^+:100 ng)	+++	+++	++	++	++	+	±	−
Control: EC84(O1:H7, LT^-)	−	−	−	−	−	−	−	−

Remark: −: reaction of enterotoxin non-producing; ±: reaction of enterotoxin doutable producing; +, ++, +++: reaction of enterotoxin producing.
LT: heat-labile enterotoxin of *E. coli*.

Table 4. Detection of *Clostridium perfringens* enterotoxin by use of the reversed passive latex agglutination

Strains	Dilution							
	×2	×4	×8	×16	×32	×64	×128	×256
NCTC8238 (Hobbs serotype 2, ET^+ 100 ng)	+++	++	++	++	++	+	±	−
Control: ATCC3629(ET^-)	−	−	−	−	−	−	−	−

Remark: −: reaction of enterotoxin non-producing; ±: reaction of enterotoxin doubtable producing; +, ++, +++: reaction of enterotoxin producing.
ET: *C. perfringens* enterotoxin.

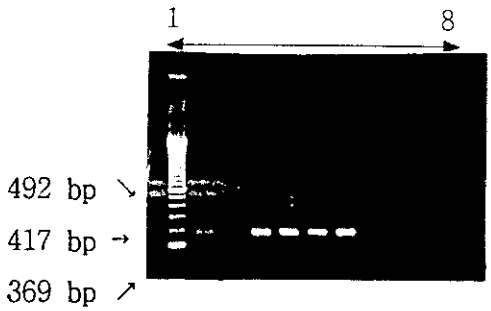

Fig. 1. Electrophoretic analysis of the PCR-amplified of *E. coli* enterotoxin gene. Lanes 1: 100-bp ladder DNA marker; 2: *E. coli* 81(LT^+); 3: *E. coli* 84 (LT^-); 4~8: 10-fold dilutions of a DNA extract of *E. coli* 81 (LT^+) from 10 ng to 100 ag (anneal temperature: 55℃).

The PCR using LT gene-specific primers of ETEC with the detection could be from 10 ng to 1 pg of a DNA fragment of 417-bp in EC81 (Fig. 1), but the PCR which was using *C. perfringens* enterotoxin gene-specific primers with a detection could be from 10 ng to 10 pg of a DNA fragment of 364-bp in NCTC8238 (Hobbs serotype 2, ET^+) (Fig. 2), which was in agreement with the findings of Jung (1997) and Jung (1999b).

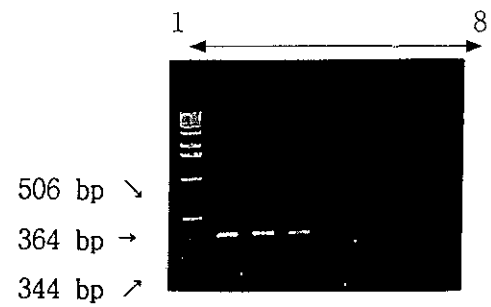

Fig. 2. Electrophoretic analysis of the PCR-amplified of *C. perfringens* enterotoxin gene. Lanes 1: 1-Kb ladder DNA marker; 2~7: 10-fold dilutions of a DNA extract of *C. perfringens* NCTC8238 (Hobbs serotype 2, ET^+); 8: ATCC3629 (ET^-) from 10 ng to 100 ag(anneal temperature: 55℃).

Concordance of test results between antigen -antibody reaction, based on the RPLA, and detection of a LT gene of ETEC and a CPE gene, based on the PCR analysis, was 100% in this study, and detection of a LT gene of ETEC appeared 10-fold sensitivity than a CPE gene, also the PCR assay was more rapid and required use of a few sample than the RPLA for detection of LT of ETEC and CPE. Analysis of these results suggested that additional diagnostic tests, such as amplified fragment length polymorphism analyses should be developed, using the gene-based PCR assay, to provide greater specificity and sensitivity for the detection of LT of ETEC and CPE.

Ⅳ. Abstracts

Detection for heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC, EC81, serotype O148 : H28) by use of the reversed passive latex agglutination (RPLA) was positive reaction from 2-fold (50 ng) to 64-fold dilutions(1.56 ng) in EC81 and polymerase chain reaction (PCR) using LT gene-specific primers of ETEC could detect from 10 ng to 1 pg of a DNA fragment of 417-bp in EC81. And, detection of *Clostridium perfringens* enterotoxin (CPE, NCTC 8238, Hobbs serotype 2) by use of the RPLA was also positive from 2-fold (50 ng) to 64-fold dilutions(1.56 ng) in NCTC8238 but the PCR using CPE gene-specific primers could detect be from 10 ng to 10 pg of a DNA fragment of 364-bp in NCTC8238. Detection of a LT of ETEC gene appeared 10-fold sensitive than a CPE gene, and the PCR assay was more rapid and required smaller sample than the RPLA for detection of LT of ETEC and CPE.

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