DETECTION OF *ESCHERICHIA COLI* O157: H7 *VT* AND $_{RFB_{O157}}$ BY MULTIPLEX POLYMERASE CHAIN REACTION

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Abstract. A rapid method for detection of *Escherichia coli* O157: H7 using multiplex PCR was developed. Two oligonucleotide primer pairs were used for simultaneously detection of *vt* encoding verotoxin genes for virulence factor and rfb_{O157} encoding the O-antigen specific for *E. coli* O157: H7. Multiplex PCR generated two products of 215 bp and 420 bp for *vt* and rfb_{O157}, respectively. Multiplex PCR detected reference strain O157: H7 (NF-7777) with a sensitivity of 10^5 CFU per ml with no amplification of other 15 pathogenic bacteria. After incubation of 10^2 CFU/25 gram raw meat in tryptic soy broth at 37ºC for 8 hours, multiplex PCR conducted with the addition of 100 mg bovine serum albumin produced the two specific PCR products for *E. coli* O157: H7. This modified multiplex PCR is a rapid, sensitive, and specific technique for detecting and differentiating *E. coli* O157: H7 and has the potential to be used as an alternative to conventional methods for the screening of O157: H7 strains isolated from raw meat.

INTRODUCTION

Examination of foods for the presence of pathogenic bacteria has been increasing in recent years because food service operations and consumers use food frequently (Tortorello, 2000). *Escherichia coli* O157: H7, an organism in the family of Enterobacteriaceae, grow well and sometimes found in the intestine of human and other mammals, is of an important pathogen contaminating food. It is grouped as Enterohemorrhagic *Escherichia coli* (EHEC) causing symptoms of diarrhea, hemorrhage and acute kidney failure.

E. coli O157: H7 synthesizes 2 types of verotoxin, namely, Verotoxin 1 (vt_1) and Verotoxin 2 (vt_2) (Paton and Paton, 1998). During outbreaks of food poisoning, a number of cases were reported with the organism as the etiological agent (Tortorello, 2000). The first report of *E. coli* O157: H7 outbreak was in 1982 in USA, Canada and England where this organism was found contaminating undercooked beef (Riley *et al*, 1983). There have been many outbreaks linked to ingestion of not only beef but also vegetables and fruits, including lettuce, cantaloupe, cabbage, alfalfa, spruots, radish sprouts and apple juice (Paton and Paton, 1998). *E. coli* O157: H7 possesses characteristics that are different from other *E.coli*. The strain O157: H7 is a slow or non sorbitol fermenter, beta-glucuronidase negative and fails to grow at temperature of 44-45ºC (Tortorello, 2000). Culture media including Celfixime and Tellurite Sorbitol McConkey agar (CT-SMAC) or Chrom agar O157 have been developed to suit its growth requirement. The Most Probable Number (MPN) method is used to quantify the number of organisms in liquid culture and is suitable even for small

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numbers (Tortorella, 2000).

As biochemical tests have low sensitivity and specificity and are time consuming, a number of alternative tests with enhanced sensitivity, specificity and accuracy have been developed. Using the nucleotide sequences of vt_1 and vt_2, Calderwood *et al* (1987) employed standard PCR for the diagnosis of verotoxigenic *E. coli* (VTEC). Karch and Myer (1989) designed a set of primers specific to both types of *vt* for VTEC diagnosis. Pollard *et al* (1990) used 2 pairs of primers, each specific for a particular *vt*, in PCR base test that efficiently identified verotoxigenic *E. coli* in food samples. Gannon *et al* (1992) designed a set of primers specific to both *vt* genes that was effective in identifying verotoxigenic *E. coli* in fecal samples. Despite these available techniques, none of them has been able to specifically identify *Escherichia coli* O157: H7 since they are a number of other *E. coli* that encoded verotoxin, such as *E. coli* O26, O111 and O113 (Goldwater and Betteiheim, 1994). After the nucleotide sequence of rfb_{O157} encoding O-Ag of *E. coli* O157 was determined by Bilge *et al* (1996), test to detect *E. coli* O157 in milk was then developed using primers specific to rfb_{O157} in PCR reaction (Maurer *et al*, 1999). Multiplex PCR, a more specific and more rapid method employing multiple sets of primers specific for the target genes, has been employed by Fratamico *et al*, (1995) who used four pairs of primers specific for vt_1, vt_2, *eaeA* and EHEC-*hly*A. Paton and Paton (1998), using the same set of primers, were able to detect VTEC in stool samples. However, detection via the use of 3 primer sets or more to indicate the presence of *E. coli* O157:H7 is not attractive due to the complexity of the reaction and length of time required.

In the present study, we have developed a PCR system using 2 primers specific for *vt* and rfb_{O157} and applied it for the detection of spiked *E. coli* O157: H7 in meat samples, which produced a clear result. Comparative studies between PCR, immunomagnetic seperation (IMS) and direct plating technique on selective media for detecting *E. coli* O157: H7 were also conducted.

MATERIALS AND METHODS

Bacterial cultures

The following *E. coli* O157 reference strains were used: NF-1709, NF-14577, NF-22895, and NF-23379 are verotoxin vt_1 producing strains; NF-9492 and NF-23380 are verotoxin vt_2 producing strains; NF-7777 and NF-9879 are both verotoxin vt_1 and vt_2 producing strains. *E. coli* wild type strain used was strain K12. All *E. coli* reference strains and tested bacteria species were obtained from Department of Microbiology, Faculty of Public Health, Mahidol University; Department of Microbiology, Faculty of Science, Chulalongkorn University; and The National Institute of Health, Thailand (Table 1). All strains were grown in Luria and Bertani (LB) broth at 37ºC overnight before being subjected to DNA isolation. For preservation, bacteria were grown in LB broth for 20 hours and then 3 ml of cell cultures were mixed with 7 ml of glycerol and aliquots were kept at -20ºC and -70ºC. Verotoxin production of all tested *E. coli* O157: H7 strains were confirmed by using PCR verotoxin kit (Takara Shuzo, Tokyo, Japan).

Multiplex polymerase chain reaction for detection of verotoxin genes and rfb_{O157}

Two pairs of oligonucleotide primers were synthesized according to Paton *et al* (1993) and Maurer *et al* (1999). The primer sequences are listed in Table 2. Template DNA from *E. coli* O157: H7 strain was prepared by the boiling method according to *Chen et al* (1998). Conditions for PCR are shown in Table 3 and amplifications were conducted using DNA Thermal Cycles (Perkin Elmer, USA). PCR products were detected by 2% agarose gel-electrophoresis at 100 V for 1 hour and staining with 10 µg/ml ethidium bromide. Positive result was indicated by band at 215 and 420

Table 1
Reference and tested bacteria used.

Bacteria
vt_1 producing reference strains
Escherichia coli O157: H7 (NF-1709) [a]
Escherichia coli O157: H7 (NF-14577) [a]
Escherichia coli O157: H7 (NF-22895) [a]
Escherichia coli O157: H7 (NF-23379) [a]
vt_2 producing reference strains
Escherichia coli O157:H7 (NF-9492) [a]
Escherichia coli O157: H7 (NF-23380) [a]
vt_{1+2} producing reference strains
Escherichia coli O157: H7 (NF-7777) [a]
Escherichia coli O157: H7 (NF-9879) [a]
Tested
Aeromonas sobria[a]
Campylobactor jejuni[a]
Enterobacter aerogenes[a]
Klebsiella pneumoniae[a]
Salmonella enteritidis[a]
Salmonella typhimurium[a]
Escherichia coli K12
Listeria monocytogenes
Proteus vulgaris
Pseudomonas aeruginosa
Serratia marcescens
Citrobacter freundii DMST 1959
Shigella dysenteriae DMST 15110 (Type I)
Shigella dysenteriae DMST 15111 (Type II)
Vibrio cholerae DMST 2873
Yersinia enterocolitica DMST 8012

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bp for verotoxin and *E. coli* O157, respectively.

Sequencing of PCR product

DNA templates from reference strains *E. coli* NF-1709 (vt_1), NF-9492 (vt_2) and NF-7777 (vt_{1+2}), were purified using QIA quick Gel Extraction kit (Qiagen, Germany), and sequenced by Bioservice Unit, Biotec, NSTDA, Thailand.

Determination of specificity and sensitivity of multiplex PCR

DNA of *E. coli* NF-7777 was used as the positive control for specificity determination. Sensitivity of multiplex PCR was determined using a ten-fold dilution of overnight growth *E. coli* NF-7777 in 0.85% normal saline solution. Genomic DNA of the each dilution of reference strain was extracted by boiling method (*Chen et al*, 1998). The minimum number of cells required to give a positive signal with the primers was recorded.

Detection by multiplex PCR of *E. coli* O157: H7 spiked in ground beef

E. coli O157: H7 NF-7777 was grown in LB broth in a shaking incubator at 37ºC overnight. Solutions of 1, 10, 10^2, and 10^3 CFU/ml were prepared in 0.85% normal saline. One ml aliquot of each cell suspension was added to 25 g of ground beef sample in 225 ml of Tryptic Soy Broth (TSB ; Difco, USA) or modified *E. coli* broth (mEc+novobiocin). The meat samples were incubated in a shaking incubator (110 rpm) at 37ºC for 6, 8, and 24 hours. Then, 1 ml aliquot of supernatant was analyzed for presence of verotoxin and O-antigen genes by a modified multiplex PCR protocol

Table 2
Oligonucleotide primers used for multiplex PCR.

Name of primer	Primer sequences (T_m)	Target gene	Basepair (bp)
VT-F[a]	5′- ATACAGAG(GA)G(GA)ATTTCGT -3′ (50ºC)	vt_1 and vt_2	215
VT-R[a]	5′- TGATGATG(AG)CAATTCAGTTA -3′ (54ºC)		
O157PF-8[b]	5′- CGTGATGATGTTGAGTTG -3′ (52ºC)	rfb_{O157}	420
O157PR-8[b]	5′- AGATTGGTTGGCATTACTG -3′ (54ºC)		

[a]Paton *et al* (1993); [b]Maurer *et al* (1999)

Table 3
Condition of PCR assays.

	PCR assay		
	VT[a]	O157 *rfb*[b]	Multiplex[c]
1X PCR buffer	20 mM Tris, pH 8.0	20 mM Tris, pH 8.0	20 mM Tris, pH 8.0
dNTP	0.2 mM	0.2 mM	0.2 mM
$MgCl_2$	3.0 mM	3.0 mM	3.0 mM
VT-F	50 pmol	-	50 pmol
VT-R	50 pmol	-	50 pmol
O157 PF8	-	50 pmol	50 pmol
O157 PR8	-	50 pmol	50 pmol
Taq DNA polymerase	1.0 U	1.0 U	1.0 U
Template DNA	(1 µg) 10 µl	(1 µg) 10 µl	(1 µg) 10 µl
PCR-Programs	94ºC: 1 min	94ºC: 0 sec	94ºC: 1 min
	55ºC: 1 min	55ºC: 0 sec	55ºC: 1 min
	72ºC: 1 min	72ºC: 15 sec	72ºC: 1 min
	35 cycles	30 cycles	35 cycles

[a]Paton *et al* (1993); [b]Maurer *et al* (1999); [c]This study

in which 100 µg of bovine serum albumin were included in the reaction and 25 µl of template DNA were employed.

Detection of *E. coli* O157:H7 in ground beef sample by IMS and culture method

After pre-enrichment of spiked ground beef samples, aliquots were taken for further analysis by IMS and direct plating method. Direct plating of pre-enrichment samples were assayed on Sorbitol MacConKey agar with added Potassium Tellurite and Cefixime (CT-SMAC, Oxoid, England) and Cellobiose-Lactose-Indole-beta-D-Glucuronidase agar (CLIG, Kyokuto, Japan). For immunological test, O157: H7 strains were confirmed by serological test with antisera O157 and H7 (S&A Reagent Lab, Thailand).

For IMS method, target bacteria from the pre-enriched samples were specifically caught onto magnetic beads coated with anti *E. coli* O157: H7 antibodies. One ml of pre-enriched samples was mixed with 20 µl of Dynal beads (Dynal, Norway). The suspension of bacteria and beads was separated using a magnet and the beads were washed several times to remove food debris and other microorganisms according to the Dynal Manual (Dynal, Norway). *E. coli* O157:H7 cell numbers were determining by suspending in 100 µl of phosphate buffer-Tween solution and then plating on CT-SMAC.

RESULTS

In this study, a rapid method for detection of O157 VTEC was developed. Two oligonucleotide primer pairs were used in multiplex PCR assays for simultaneous detection of *vt* and O157 *rfb* gene. All O157 strains containing either vt_1 or vt_2 or both genes could be identified by the presence of amplified products of 420 (*rfb* O157) and 215 bp (*vt*) (Fig 1). No PCR products were generated from *E. coli* K12 strain (data not shown).

The identification of *E. coli* O157 strains was confirmed by determining the presence of *vt* gene using O157 typing set. This kit amplifies vt_1 and vt_2 using primer set EVT-1/EVT-2 and EVS-1/EVS-2, generating a fragment of 349 bp and 404 bp, respectively (data not shown).

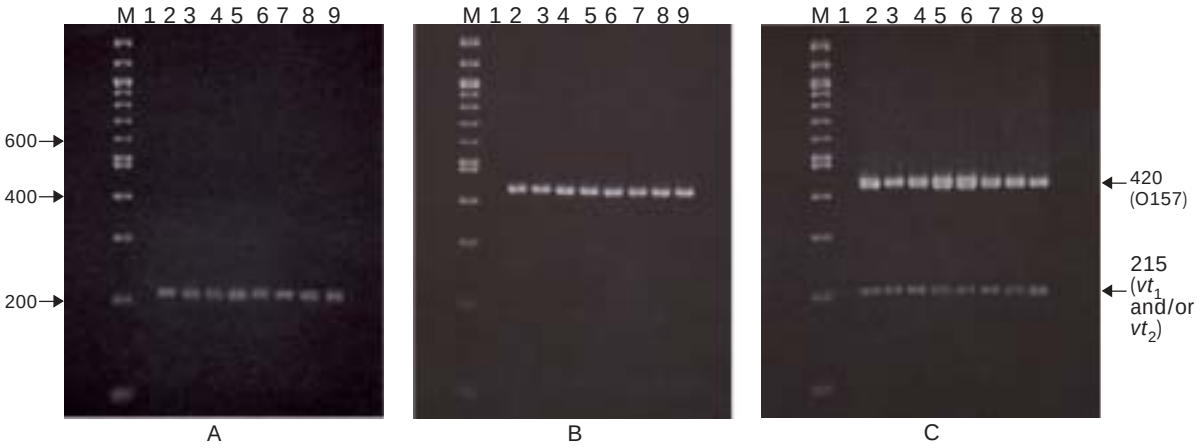

Fig 1–Agarose gel-electrophoresis of PCR and multiplex PCR products of reference strains of *Escherichia coli* O157. (A) PCR products of *vt* gene. (B) PCR products of O157 *rfb* gene. (C) multiplex PCR products. Lane M, 100 bp DNA ladder; 1, strain K-12; 2, strain NF-14577; 3, strain NF-22895; 4, strain NF-23379; 5, strain NF-1709; 6, strain NF-23380; 7, strain NF-9492; 8, strain NF-9879; 9, strain NF-7777.

Homology study of PCR products of verotoxin genes and rfb_{O157} with sequences in GenBank

The nucleotide sequences were compared with those in GenBank, using BlastN version 2.2.7. Gene vt_1 from *E. coli* O157:H7 NF-1709 shared 86% nucleotide sequence identity with vt_1 of *E. coli* O157:H7 RIMD O509952 and vt_2 from *E. coli* O157:H7 NF-9492 shared 89% nucleotide sequence identity with vt_2 of *E. coli* O157:H7 RIMD O509952. Gene *rfb* O157 from *E. coli* O157: H7 NF-7777 shared 97% nucleotide identity with *rfb*B of *E.coli* O157: H7 C664-1992.

Verotoxin 1 from *E. coli* O157:H7 NF-1709 shared 60% amino acid sequence identity with verotoxin 1 (subunit A) of *E. coli* O157:H7 RIMD O509952 and verotoxin 2 from *E. coli* O157:H7 NF-9492 shared 69% amino acid sequence identity with verotoxin 2 (subunit A) of *E.coli* O157:H7 RIMD O509952. The O-antigen from *E. coli* O157: H7 NF-7777 shared 86% amino acid sequence identity with O-antigen from *rfb*B of *E. coli* O157: H7 ATCC 35150.

Specificity determination of multiplex PCR detection

To evaluate the speciticity of the primers, 16 strains of enteric bacteria, namely, *Aeromonas sorbia*, *Campylobactor jejuni*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Citrobacter freundii*, *Shigella dysenteriae*, *Shigella dysenteriae*, *Vibrio cholerae* and *Yersinia enterocolitica*, were tested. *E. coli* O157: H7 NF-7777 was used as positive control. All strains failed to produce any band, except *S. dysenteriae* type 1 that produced the vt_{1+2} amplicon (215 bp) (Fig 2).

Sensitivity determination of multiplex PCR detection

To evaluate the sensitivity of the primers, ten-fold dilutions in 0.85% normal saline of an overnight growth *E. coli* O157:H7 NF-7777 were prepared and DNA extracted. Multiplex PCR reaction revealed that both 215 and 420 bp could be detected at the minimum cell concentration of 10^5 CFU/ml (Fig 3).

Detection of *E. coli* O157:H7 spiked in ground beef by multiplex PCR

Following spiking with *E. coli* O157:H7, 25 grams of raw meat were incubated in TSB at 37ºC for of 6, 8 and 24 hours. Multiplex PCR with the addition of 100 µg bovine se-

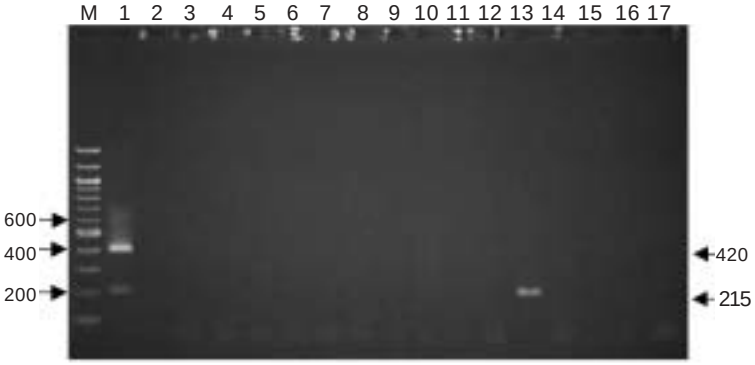

Fig 2–Agarose gel-electrophoresis of multiplex PCR products from different bacteria strains. Lane M, 100 bp DNA ladder; 1, *E. coli* NF-7777; 2, *Aeromonas sorbia*; 3, *Campylobactor jejuni*; 4, *Citrobacter freundii* DMST 1959; 5, *Enterobacter aerogenes*; 6, *E. coli*; 7, *Klebsiella pneumoniae*; 8, *Listeria monocytogenes*, 9; *Proteus vulgaris*; 10, *Pseudomonas aeruginosa*; 11, *Salmonella enteritidis*; 12, *Salmonella typhimurium*; 13, *Shigella dysenteriae* DMST 15110 (Type I); 14, *Shigella sysenteriae* DMST 15111 (Type II); 15, *Serratia marcescens*; 16, *Vibrio cholerae* DMST 2873; 17, *Yersinia enterocolitica* DMST 8012.

rum albumin gave two PCR products specific for *E. coli* O157: H7 at cell concentration of 1CFU only after 24 hours incubation (Fig 4A, panel C). However, incubation of 25-gram raw meat containing *E. coli* O157:H7 in the same medium allowed detection of 10^2 CFU after 8-hour incubation (Fig 4A, panel b).

Detection of *E. coli* O157: H7 by culture method and IMS in spiked ground beef samples

Cell cultures from spiked meat samples were incubated in two different enrichment broths for different times and then plated on CT-SMAC medium. TSB pre-enrichment broth showed better growth results than mEC+n broth (Table 4).

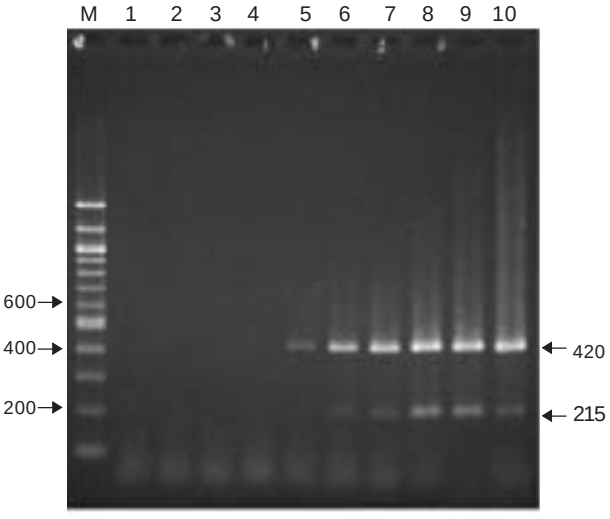

Fig 3–Agarose gel-electrophoresis of PCR products from different cell concentrations of *E. coli* O157: H7 NF-7777. Lane M, 100 bp DNA ladder; 1, 1 (CFU/ml); 2, 10 (CFU/ml); 3, 10^2 (CFU/ml); 4, 10^3 (CFU/ml); 5, 10^4 (CFU/ml); 6, 10^5 (CFU/ml); 7, 10^6 (CFU/ml); 8, 10^7 (CFU/ml); 9, 10^8 (CFU/ml) and 10, 10^9 (CFU/ml).

Standard protocol of IMS using anti-*E. coli* O157: H7 Dynabeads was followed. Samples were cultivated in pre-enrichment broth at 37°C and incubated for various times, and then were diluted and plated on to CT-SMAC plates at 37°C for 24 hours. The minimum cell concentration of 1 cell/ml could be detected after pre-enrichment in TSB for 6 hours, indicating that IMS is a more sensitive method for detecting *E. coli* O157: H7 than the culture standard plating procedure (Table 4).

DISCUSSION

Numerous researchers have studied and diagnosed *E. coli* O157: H7 using PCR on various samples, such as fecal samples from infected persons or contaminated food samples (raw milk or meat) suspected as causative of outbreak. The use of a single pair of primers specific to target genes that are characteristics of *E. coli* O157:H7, *eg*, *vt eaeA* EHEC-*hlyA* or *uidA*, is unable to give an unambigu-

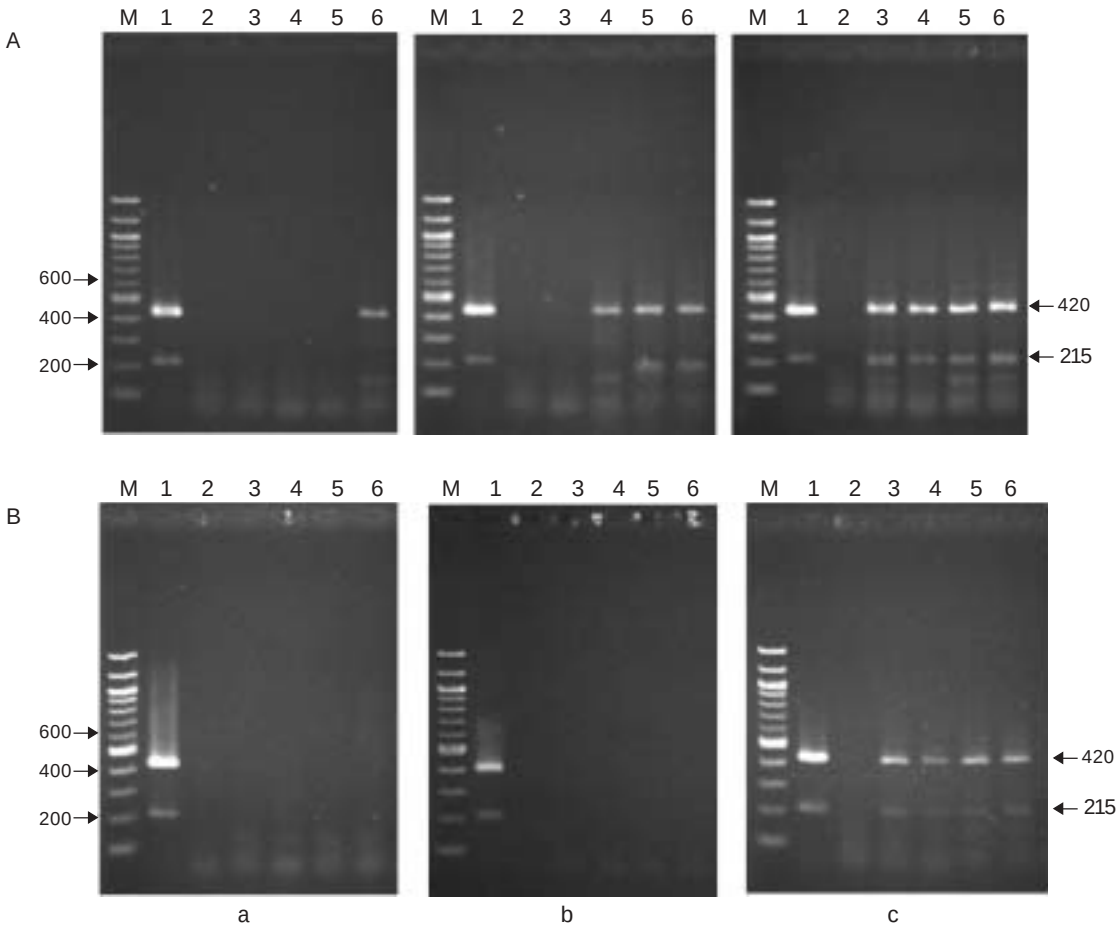

Fig 4–Agarose gel-electrophoresis of multiplex PCR products of *E. coli* O157: H7 NF-7777 spiked in 25 g raw meat. Cells were incubated in TSB broth (A) and mEC + n broth (B) for 6 hours (a) 8 hours (b) and 24 hours (c). Lane M, 100 bp DNA ladder; 1, positive control: NF-7777 DNA; 2, negative control: without *E. coli* O157:H7 DNA; 3, *E. coli* O157:H7 at cell concentration 1 CFU/25 g; 4, 10 CFU/ 25 g; 5, 10^2 CFU/25 g and 6, 10^3 CFU/25 g.

ous positive result as there are a variety of other gram-negative organisms possessing the same target genes of *E. coli* O157:H7 (Paton and Paton, 1998). Even when using multiplex PCR or multisets of primers as means to increase specificity, the same ambiguity is still encountered. When this is coupled to the complexity of the procedure and time required, this approach has not been attractive. The present study employed 2 sets of primers and tested the modified multiplex PCR to detect the presence of *E. coli* O157:H7 spiked in raw meat. One primer set is specific for *vt* that can be used for either vt_1 or vt_2 and the other primer set is specific for rfb_{O157} which is unique to *E. coli* O157:H7.

Optimal conditions for the detection of *E. coli* O157: H7 using our multiplex PCR were determined. When the PCR conditions reported by Paton *et al* (1993) for primer VT-F and VT-R (annealing temperature of 47ºC) amounts of PCR products obtained were relatively low. Furthermore, concentrations of $MgCl_2$ used were also varied in order to find optimal condition for PCR using primers for rfb_{O157}. We found that the annealing tempera-

Table 4
Comparison of detection of *E. coli* O157: H7 by cell culture method (pre-enrichment TSB and mEC + n broth) and IMS in raw beef samples.

Inoculum cell concentration	CFU								
	6 hours			8 hours			24 hours		
	TSB	mEC + n	IMS	TSB	mEC + n	IMS	TSB	mEC + n	IMS
100	2.8×10^2	<10	3.1×10^2	3.8×10^4	<10	4.3×10^4	$> 10^9$	$>10^9$	$>10^9$
101	2.1×10^3	<10	4.3×10^3	4.1×10^5	6.3×10^2	5.2×10^5	$> 10^9$	$>10^9$	$>10^9$
102	8.2×10^3	2.2×10^3	2.4×10^4	1.7×10^6	6.7×10^3	2.3×10^6	$>10^9$	$>10^9$	$>10^9$
103	2.7×10^5	7.5×10^3	4.6×10^5	8.6×10^6	1.2×10^4	1.3×10^7	$>10^9$	$>10^9$	$>10^9$

Cells were spiked in 25 g raw beef samples.

ture of 55ºC and $MgCl_2$ concentration of 3.0 mM are optimal for multiplex PCR employed for the detection of *E. coli* O157: H7.

Strains of *E. coli* that produced a verotoxin were first decribed in 1977 (Konowalchuk *et al,* 1997). Subsequently this toxin was shown to have immunological cross-reactivity, identical biological activity and similar structure to Shiga toxin produce by *S.dysenteriae* type 1 (Stephen *et al*, 1987; Karch and Mayer, 1989). Because of these similarities, a PCR product specific for *vt* was detected in *S. dysenteriae* type 1. However, use of two primer sets allowed detection of *E. coli* O157: H7 with high specificity.

The multiplex PCR could detect reference strain O157: H7 (NF-7777) with a sensitivity of 10^5 CFU/ml. Multiplex PCR was also applied for the detection of *E. coli* O157: H7 spiked in raw meat. After incubation of 25-gram raw meat in tryptic soy broth at 37ºC for 8 hours, 100 CFU of *E.coli* O157:H7 could be detected, using multiplex PCR containing the addition of 100 mg bovine serum albumin. This modified multiplex PCR protocol has a potential to be used for rapid, sensitive and specific method in the specific detection of *E. coli* O157: H7 and in the screening of O157: H7 strains isolated from raw meat as an alternative to conventional methods.

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REFERENCES

Bilge SS, Vary JC, Dowell SF, Tarr PI. Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus. *Infect Immun* 1996; 64: 4795-801.

Calderwood SB, Auclair F, Donohue-Rolfe A, Keusch GT, Mekalanos JJ. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc Natl Acad Sci USA* 1987 ; 84: 4364-68.

Chen J, Johnson R, Griffiths M. Detection of verotoxigenic *Escherichia coli* by magnetic capture-hybridization PCR. *Appl Environ Microbiol* 1998 ; 64: 147-52.

Fratamico PM, Sackitey SK, Wiedmann M, Deng MY. Detection of *Escherichia coli* O157:H7 by multiplex PCR. *J Clin Microbiol* 1995; 33: 2188-91.

Gannon VP J, King RK, Kim JY, Thomas EJ. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef by using the polymerase chain reaction. *Appl Environ Microbiol* 1992; 58: 3809-15.

Goldwater PN, Bettelheim KA. The role of

enterohaemorrhagic *E. coli* serotypes other than O157:H7 as causes of disease, In*:* Karmali MA, Goglio AG, eds. Recent advances in verocytotoxin producing *Escherichia coli* infections. Amsterdam: Elsevier Science BV, 1994: 57-60.

Karch H, Meyer T. Single primer pair for amplifying segments of distinct Shiga-like toxin genes by polymerase chain reaction. *J Clin Microbiol* 1989; 27: 2751-57.

Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli. Infect Immun* 1977; 18: 775-9.

Maurer JJ, Schmid D, Peterosko P, Sanchez S, Bolton L, Lee MD. Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. *Appl Environ Microbiol* 1999; 65: 2954-60.

Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev* 1998; 11: 450-79.

Paton AW, Paton JC, Goldwater PN, Manning PA. Direct detection of *Escherichia coli* Shiga-like toxin genes in primary fecal cultures by polymerase chain reaction. *J Clin Microbiol* 1993; 31: 3063-67.

Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J Clin Microbiol* 1990; 28: 540-5.

Riley LW, Remis RS, Helgerson SD, *et al*. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 1983; 308: 681-5.

Stephens PJ, Johnson JA. Direct inoculation into media containing bile salts and antibiotics is unsuitable for the detection of acid/salt stressed *Escherichia coli* O157:H7*. Lett Appl Microbiol* 1998; 27: 147-51.

Tortorello ML. *Escherichia coli* O157. In: Robinson RK, Batt CA, Patel PD, eds. Encyclopedia of food microbiology. London: Academic Press, 2000; 1: 646-52.

Wang L, Reeves PR. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infect Immun* 1998; 66: 3545-51.

Yokoyama K, Makino K, Kubota Y, *et al*. Complete nucleotide sequence of the prophage VT1-Sakai carrying the Shiga toxin 1 genes of the enterohemorrhagic *Escherichia coli* O157:H7 strain derived from the Sakai outbreak. *Gene* 2000; 258: 127-39.