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Development of A Modified Enrichment Method for the Rapid Immunoassay of *Escherichia coli* O157 Strains in Fresh Cut Vegetables

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ABSTRACT

Escherichia coli O157:H7 is a pathogen that colonizes human intestinal epithelial membrane and causes a characteristic lesion and severe syndromes, including diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. It is difficult to isolate and identify this pathogen. The purpose of this research was to establish a two-stage enrichment method for the detection of *E. coli* O157:H7 in fresh cut vegetables. Fresh cut vegetable samples were first enriched in modified EC broth with novobiocin at 42˚C for 6 hr, and then transferred to sorbitol MacConkey broth containing cefixime and tellurite at 35˚C for 18 hr. Finally, the enriched broth was verified for the existence of *E. coli* O157 by using an automated enzyme-linked fluorescence immunoassay (mini-VIDAS) or an immunostrip test. This two-stage enrichment method would decrease the false-positive *E. coli* O157 tested by immunostrip or mini-VIDAS. The pathogen in food sample could be as low as 10 CFU/g and selectively enriched to 10^5 or 10^7 CFU/mL required for the detection by mini-VIDAS or immunostrip. The presence of *E. coli* O157:H7 in enriched broth was confirmed by multiplex polymerase chain reaction (PCR). Thus, the two-stage enrichment method can be used to rapidly screen food samples for *E. coli* O157.

Key words: immunostrip, enzyme-linked fluorescence immunoassay (mini-VIDAS), multiplex polymerase chain reaction, *Escherichia coli* O157:H7, fresh cut vegetables, rapid detection

INTRODUCTION

Since first isolated from the victims of bleeding diarrhea in 1975, *Escherichia coli* O157:H7 has become an object of increasing alarm in 1982 as implicated in several outbreaks of food poisoning linking to a variety of food, such as hamburger, roasted meat, drinking water, apple juice, raw milk, etc in Europe and America(1). From 1993 to 1996, episodes of foodborne disease associated with *E. coli* O157:H7 were reported in Americans who consumed fresh vegetable salad(2,3). In 1996, an outbreak of foodborne disease affecting over ten thousands people claiming in Japan was caused by food products contaminated by *E. coli* O157:H7(4).

Isolation of *E. coli* O157:H7 residing in food is more difficult when the cell number of *E. coli* O157:H7 is low(5). In the past decade, however, the techniques for detecting *E. coli* O157:H7 have achieved a great advance in the development of either new methods or rapid detection kits, especially immunoassays such as immunostrip test and enzyme-linked fluorescence immunoassay (ELFA)(6). In addition to rapid determination, the advantages of immunoassay include easiness to perform and greater detection sensitivity by incubating specimens to increase bacterial numbers. The principle of immunostrip is specific antibodies immobilized onto colored-microbeads. If a specimen solution contains *E. coli* O157:H7 antigen, the mixture will react with *E. coli* O157:H7-specific antibodies and yield a color band(7). ELFA is most commonly conducted by using a mini-VIDAS automated immunoassay analyzer(8). *E. coli* O157 antigens present in the specimen will bind to specific antibodies immobilized on the inner wall of solid phase receptacle, while unbound antigens are removed away by washing. The enzyme substrate added is then converted into a fluorescent product. The intensity of emitted fluorescence is automatically measured by a VIDAS optical scanner within 45 min(9). A specimen determined negative by the VIDAS method would suggest that the time-consuming standard culture methods and biochemical tests can be suspended to spare time required for isolation and incubation of specimens(10,11).

According to a previous study by Huang *et al.*(12), a total of 116 specimens of fresh cut vegetables were added to modified EC (mEC) broth containing novobiocin and shaken at 37˚C for 24 hr for *E. coli* O157:H7 analysis. Ten milliliter of specimen were heated at 100˚C for 15 min and applied to a mini-VIDAS analyzer. The result showed that 31 of the 116 specimens were positive for *E. coli* O157. However, only one bacterial strain isolated from vegetable mixture was confirmed to be *E. coli* O157:H7 by PCR amplification for DNA fragments of *eae*, *stx* and *hly*. This indicated that this one-stage enrichment method was not

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specific for *E. coli* O157:H7 and it accounted for false-positive results of mini-VIDAS method. In order to avoid false-positive results and increase rapidity and sensitivity of the detection of *E. coli* O157:H7 in food products, the present study exploited a two-stage enrichment method.

MATERIALS AND METHODS

I. *Fresh Cut Vegetables*

Lettuce, cabbage, cucumber and alfalfa sprouts produced by the Tao-Yuang Agricultural Improvement Station were packaged aseptically in plastic bags and transported to laboratory within 1 hr at low temperature. Prior to use, the vegetables were cut into shreds of 2 × 1 cm, soaked in 1:10 (w/w) 60 ppm chlorine dioxide solution for 10 min, and then cleaned with sterilized distilled water.

II. *Bacterial Strains and Culture Media*

E. coli O157:H7 BCRC 13084, 13085, 13086, 13089, *Salmonella typhimurium* BCRC 12947 and *Klebsiella pneumoniae* BCRC 10692 strains were obtained from the Bioresources Collection and Research Center (BCRC) of Food Industry Research and Development Institute.

III. *Immunostrip Products and Mini-VIDAS*

The immunostrip products are Singlepath *E. coli* O157:H7 (Merck, Darmstadt, Germany), Pathstik *E. coli* O157:H7 (Lumac, Landgraaf, Netherlands), and Revel *E. coli* O157:H7 (Neogen, Lansing, MI, USA).

The mini-VIDAS (Vitek Immuno Diagnostic Assay System, bioMerieux Vitek, Inc., France) instrument performs reactions and takes readings automatically.

IV. *Detection of E. coli O157:H7 and Selective Enrichment of Broth Culture*

E. coli O157:H7 was spread on SMAC agar plates (sorbitol MacConkey agar), incubated at 37˚C for 18 hr, and then observed for the presence of typical colonies[13].

The flowchart of selective enrichment of broth culture for the assay of *E. coli* O157:H7 is shown in Figure 1.

(I) *One-stage enrichment*

According to Okrend *et al.*[13], 25 g of fresh cut vegetables was added to 225 mL of sterilized mEC broth containing novobiocin (20 µg/mg). The mixture was inoculated with 10 and 100 CFU/g of mixed strains of BCRC 13084, 13085, 13086, 13089 and homogenized by a stomacher for 2 min before incubation at 37˚C for 24 hr, as procedure (1) of Figure 1. After first enrichment, the broth was examined by immunostrip of Pathstik for the presence of *E. coli* O157:H7, In addition, another 10 mL of sample was taken, heated at 100˚C for 15 min and examined by mini-VIDAS for *E. coli* O157. The broth was also directly confirmed by multiplex PCR.

(II) *Two-stage enrichment*

In procedure (2-1) and (2-2) as shown in Figure 1, fresh cut vegetables were added to mEC broth containing novobiocin, inoculated with BCRC 13084, 13085, 13086 and 13089, and then incubated at temperature up to 42˚C for 6 or 24 hr. After the first enrichment, 1 mL of the sample broth was transferred to 9 mL of sorbitol MacConkey (TC-MAC) broth containing potassium tellurite (2.5 µg/mg) and cefixime (0.05 µg/mg). Afterwards, the broth was incubated at 37˚C for 18 hr. The second selectively enriched broth was then examined by immunostrip or mini-VIDAS, as well as tested by multiplex PCR.

In procedure (3-1) and (3-2) of Figure 1, fresh cut vegetables were added to novobiocin-containing mEC broth containing 1% of novobiocin-tellurite-cefixime, inoculated with BCRC 13084, 13085, 13086 and 13089, and incubated at 37˚C or 42˚C for 24 hr, respectively. For the second enrichment at 37˚C for 18 hr, 1 mL of the first enriched broth culture was transferred to 9 mL of TC-MAC broth containing 1% tellurite-cefixime,. The second enriched broth was examined by immunostrip or mini-VIDAS and confirmed by multiplex PCR.

V. *Sensitivity of Mini -VIDAS and Isolation of False Positive Strains*

The strains of BCRC 13085 was cultured overnight in nutrient broth at 37˚C for 24 hr. After dilution to 10^6, 10^5 and 10^4 CFU/mL, respectively, 10 mL of the selectively enriched sample was heated at 100˚C for 15 min. An aliquot of 500 µL was transferred to a mini-VIDAS ECO strip, and the intensity of fluorescence was detected by mini-VIDAS. Samples of reading ≧0.1 RFV (relative fluorescence value) were regarded as positive for *E. coli* O157, and those of reading <0.1 RFV were negative[6]. The lowest detection limit was sensitivity.

Vegetable samples not inoculated with *E. coli* O157:H7 but gave false positive results were concentrated by mini-VIDAS ICE. An aliquot of 0.1 mL was spread onto the selective Sorbitol MacConkey agar (SMAC agar) with 1% tellurite-cefixime. After an 18-hr incubation at 37˚C, colorless bacterial colonies which displayed no fluorescence under UV light were taken and re-suspended in 0.85% NaCl solution[13]. The bacterial solution was then mixed well with reagents of *E. coli* O157 Latex Test Kit (Oxoid, Hampshire, UK). The bacterial strain was preserved if agglutination was observed within 1 min, otherwise discarded[14]. After determined as Gram-negative bacilli, the bacterial strain was identified with API 20E test (BioMerieux Co. Vercieu, France).

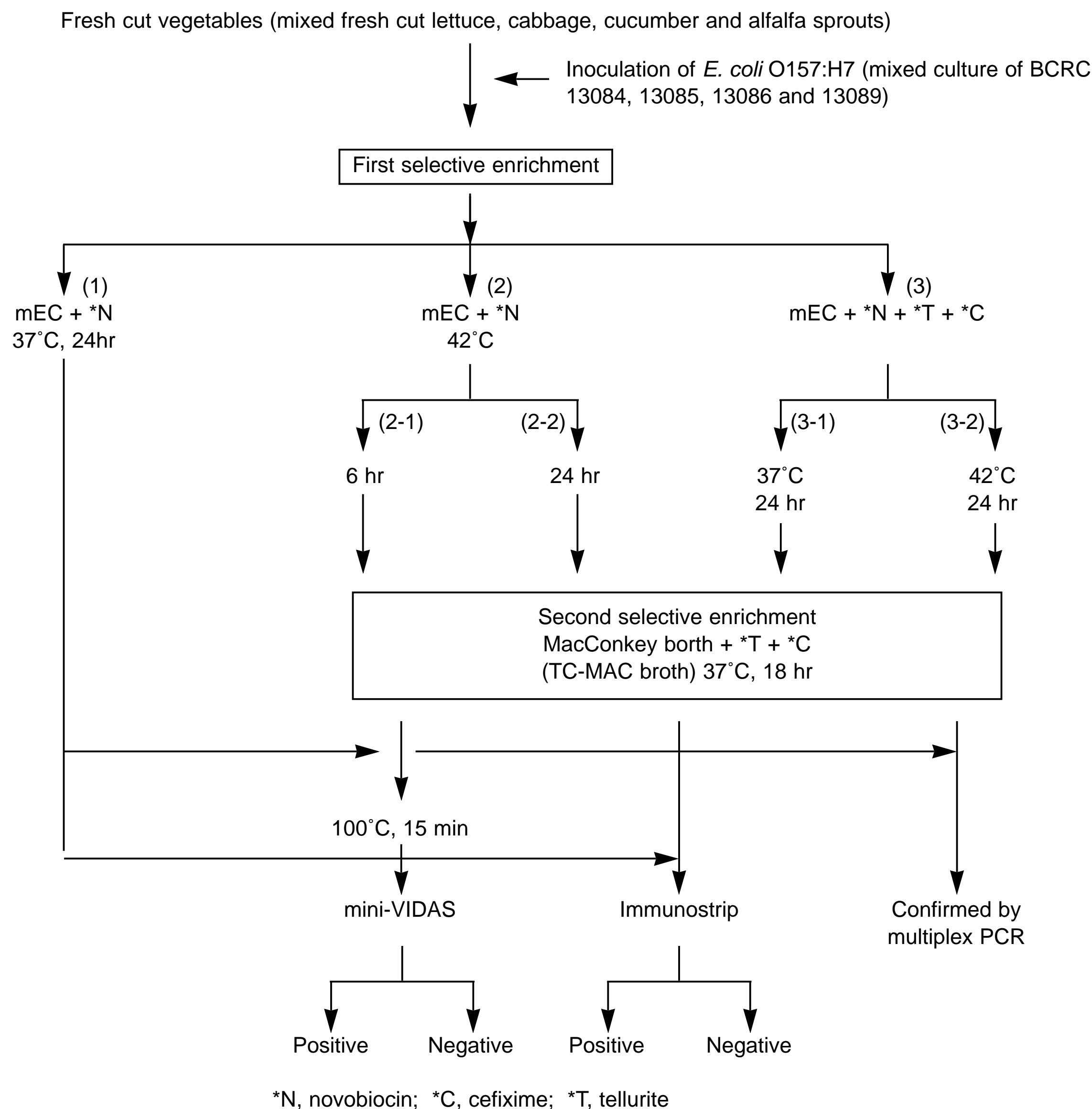

Figure 1. Flowchart of first and second enrichment of broth culture for the immunoassay of *E. coli* O157.

VI. *Sensitivity of Immunostrip Assay*

The BCRC 13084, 13085 and 13086 strains were cultured overnight in nutrient broth at 37˚C for 24 hr, and then diluted to 10^8, 10^7 and 10^6 CFU/mL, respectively. Afterwards, the strains were transferred to immunostrip products provided by Singlepath *E. coli* O157:H7, Pathstik *E. coli* O157:H7, and Revel *E. coli* O157:H7. Disposable pipette was used to dispense three drops of diluted sample into circular sample port on immunostrip (Pathstik *E. coli* O157:H7, the strip end was immersed into the enriched broth), and results were observed after 20 min. The sensitivities of different immunostrips were also compared with *E. coli* O157:H7 or mixed with bacterial strains (*Mogella morganii* RV-1 and *Enterobacter cloacae* RV-2) isolated from raw vegetables and giving false positive results by mini-VIDAS assay. The lowest detection limit was sensitivity.

VII. *Confirmation by Multiplex Polymerase Chain Reaction*

The second enriched broth was transferred to 1.5 mL Eppendorf tubes and centrifuged at 4,000 rpm for 20 min. Bacterial chromosomes and plasmids in cell pellets were extracted with the Nucleobond Nucleic Acid Purification Tools (Clontech, California, USA). An aliquot of DNA extract was added to Ready-To-Go PCR beads (Pharmacia, Piscataway, NJ, USA). The sample was heated at 95˚C for 5 min using a Hybaid Thermal Cycler (Hybaid Limited, Teddington, UK).

Table 1 shows of the number, sequence, location and PCR product size of the three pairs of gene-specific primers. Genotype was confirmed by three *E. coli* O157:H7-specific genes, *stx*, *hly* and *eae*. The *stx* and *eae*

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genes are responsible for the production of shiga toxin and intimin, respectively[15,16,17,18]. On the other hand, the *hly* gene is localized in the plasmid pO157, and encodes the sequence of enterohemolysin[17,18,19]. The PCR reaction mixture was consisted of 10 mM Tris-HCl (pH8.0), 50 mM KCl, 3.0 mM $MgCl_2$, 0.1% Triton x-100, 200 μM (each) of the four deoxynucleoside triphosphates (dNTPs), 1.25 u Taq DNA polymerase (Gene Amp PCR Reagent kit, Perkin Elmer, Branchburg, NJ, USA). 0.50 μM of primers AE20-2, AE22, MK1 and MK2, and 0.25 μM of primers MFSIF and MFSIR. The reaction mixtures were heated at 95˚C for 5 min and subjected to 35 cycles of denaturation at 95˚C for 30 s, annealing at 55˚C for 1 min, extension at 72˚C for 1 min, and an additional 10 min extension at 72˚C. Products of the multiplex PCR were analyzed by agarose gel electrophoresis (1.5%) followed by ethidium bromide staining of the separated DNA.

RESULTS AND DISCUSSION

I. *One-stage Enrichment Culture*

As shown in procedure 1 of Table 2, 20 and 40% of the control samples were shown to be *E. coli* O157 positive by immunostrip and mini-VIDAS assays, while no positive result was found by multiplex PCR. There were two possible explanations. Firstly, a small number of *E. coli* O157:H7 might be already present in fresh cut vegetables samples. Such a low bacterial level reflected the fact that mini-VIDAS had the highest sensitivity, followed by immunostrip and then multiplex PCR. Secondly, *E. coli* O157:H7 is not present in fresh cut vegetable samples, but both immunostrip and mini-VIDAS yielded false positive results. The two positive samples in the control group were subjected to *E. coli* O157 isolation with TC-SMAC agar for several times. No colorless colony capable of emitting fluorescence under UV light was obtained. The species were identified by API 20E test to be mainly *M. morganii* RV-1 and *E. cloacae* RV-2 (result not shown). It was possible that some bacterial strains with similar structure of "O" antigens might covalently bound to *M. morganii* RV-1 and *E. cloacae* RV-2, resulting in false positive results.

According to Melvina[20], *Citrobacter freundii, Salmonella typhimurium,* and *Salmonella tennessee* would interfere with the outcomes of mini-VIDAS by yielding false positive results due to close similarity of antigen. Among the samples inoculated with 10 CFU/g of *E. coli* O157, only 40% and 60% samples were determined to be positive by immunostrip and min-VIDAS assays, respectively. Among those inoculated with 100 CFU/g of *E. coli* O157, there were 60% and 80% samples were determined to be positive, respectively (procedure 1 of Table 2). However, PCR could detect all of these inoculated samples positively. Since the sensitivity of PCR is approximately 1,000 CFU/mL, *E. coli* O157:H7 might not replicate to a

Table 1. Sequence of oligonucleotides used as PCR primers

Primer	Gene primer sequence	Gene	Product size (bp)	References
AE20-2	5'TCAGCGTGGTTGGATCAACCT3'	*eae*	397	15,16,17
AE22	5'ATTACCATCCACACAGACGGT3'			
MK1	5'TTTACGATAGACTTCTCGAC3'	stx_1/stx_2	228/225	16,17,18
MK2	5'CACATATAAATTATTTCGCTC3'			
MFS1F	5'ACGATGTGGTTTATTCTGGA3'	*hly*	166	17,18,19
MFS1R	5'CTTCACGTCACCATACATAT3'			

Table 2. Effects of selective enrichment method on the detection results of immunoassay and PCR

Procedure*	Inoculated level of *E. coli* O157:H7 (CFU/g samples)	No. of positive samples/No. of samples tested		
		Immunostrip	Mini-VIDAS	Multiplex PCR
1	0	1/5	2/5	0/5
	10	2/5	3/5	5/5
	100	3/5	4/5	5/5
2-1	0	0/5	0/5	0/5
	10	5/5	5/5	5/5
	100	5/5	5/5	5/5
2-2	0	0/5	0/5	0/5
	10	5/5	5/5	5/5
	100	5/5	5/5	5/5
3-1	0	0/5	0/5	0/5
	10	0/5	0/5	3/5
	100	0/5	2/5	4/5
3-2	0	0/5	0/5	0/5
	10	0/5	0/5	2/5
	100	0/5	1/5	3/5

*Refer to Figure 1.

level over the detection limit of mini-VIDAS, otherwise interference might occur by other contaminating bacteria proliferating during selective enrichment. Therefore, in the first stage of enrichment, mEC broth was used to promote the growth of *E. coli* O157:H7 in samples, and novobiocin was added to inhibit some Gram-negative bacteria (e.g., *Neisseria* spp. and *Haemophilus influenzae*) and Gram-positive bacteria (e.g., *Staphylococcus* spp. and *Streptococcus* spp.)(21). However, a positive result of sample detected by mini-VIDAS did not necessarily indicate the presence of *E. coli* O157. It might be just a false positive result(12,22,23). Thus, the enrichment efficacy of mEC broth directly affected the results of immunostrip and mini-VIDAS assays.

II. *Two-stage Enrichment Culture*

The two-stage enrichment of culture mainly exploited mEC broth containing tellurite and cefixime. Incubation temperature was raised to 42˚C to inhibit the growth of non-*E. coli* O157 (procedure 2-1 and 2-2 in Figure 1). The results in Table 2 reveal that all control samples were negative and samples inoculated with 10 and 100 CFU/mL were determined to be positive by immunostrip, min-VIDAS or multiplex PCR. This indicates that sample broth could be subjected to second enrichment after 6 hr or 24 hr of the first enrichment. For such a two-stage enrichment method, detection results were significantly affected by temperature, incubation time and the presence of cefixime and tellurite. However, when both cefixime and tellurite were added to mEC containing 20 ppm of novobiocin, enriched at either 37˚C (procedure 3-1 of Figure 1) or 42˚C (procedure 3-2 of Figure 1), no positive results of samples later incubated with cefixime- and tellurite-containing SMAC broth for 18 hr in the second stage of enrichment were reported by immunostrip. Only 40% (inoculated with 100 CFU/g at 37˚C) and 20% (inoculated with 100 CFU/g at 42˚C) samples were positively detected by mini-VIDAS. It was likely that *E. coli* O157:H7 could not grow to levels above 10^5 CFU/mL during two enrichment procedures in two days with enrichment broth containing three kinds of antibiotics. According to the ratio of positive results by multiplex PCR (2/5~4/5), the cell number of *E. coli* O157:H7 were estimated to be 10^2~10^3 CFU/mL.

Therefore, higher incubation temperature (42˚C) could reduce false positive results, and additional antibiotics of cefixime and tellurite adversely affected the growth rate of *E. coli* O157:H7. Burtscher and Wuertz(24) developed a two-stage enrichment procedure and PCR-based method to detect potential pathogens in aerobic and anaerobic organic waste. They found that their design was effective especially when bacterial count was low, and that no false positive results were caused by DNA of dead cells.

Whether an immunoassay can accurately detect indicator bacteria is determined by correct methods of cultivating indicator bacteria. Sewell *et al.*(25) employed a two-stage enrichment method with enzyme immunofluorescence assay to detect *Listeria* rapidly and accurately in food products. However, it is concerned whether their two-stage enrichment procedure would reduce the sensitivity of mini-VIDAS or not. Therefore, in the present study, diverse levels of *E. coli* O157:H7 were established to determine the sensitivities of different two-stage enrichment procedures and interference caused by contaminating bacteria. Figure 2 shows the electrophoresis result of multiplex PCR amplification with bacterial DNA directly extracted from enriched broth and primers specific to these three genes. Samples inoculated with BCRC 13089 as low as 10 CFU/25 g could be detected by multiplex PCR after two-stage enrichment procedures (Figure 2, lane 2). Although contaminating bacteria (*S. typhimurium* and *K. pheumoniae*) were even at levels of 1,000 fold of indicator bacterium in cell count, PCR could accurately detect *E. coli* O157:H7 present in samples (Figure 2, lane 5 and 6). No *E. coli* O157:H7 was detected by PCR in samples not inoculated with *E. coli* O157:H7 after two-stage enrichment procedures (Figure 2, lane 1, 3 and 4).

III. *Sensitivity of Mini-VIDAS and Interference from Contaminating Bacteria*

Table 3 reveals whether the detection of *E. coli* O157:H7 was affected by false-positive bacteria *M. morganii* RV-1 and *E. cloacae* RV-2, which are the main bacterial species causing false-positive results in procedure 1 of Table 2. The result of mini-VIDAS assay remained negative when either *M. morganii* RV-1 or *E. cloacae* RV-2 alone was present at <log 5.0 CFU/mL. Table 3 also shows that the detection limit of mini-VIDAS for *E. coli* O157:H7 CCRC 13085 alone was 10^5 CFU/mL, when one contaminating bacterial strain was present at 10^5 CFU/mL, *E. coli* O157:H7 remained detectable. On the contrary, when both

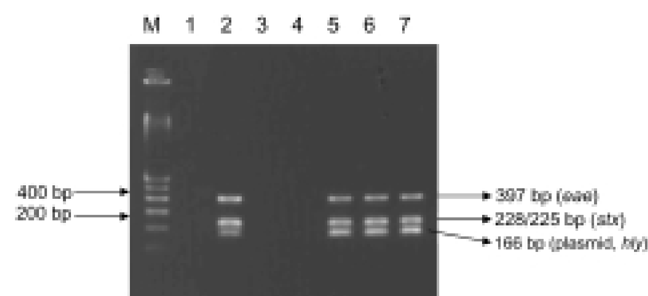

Figure 2. Amplification of specific target gene in bacterial DNA directly extracted from the enriched broth. DNA fragments of *eae*, *stx* and *hly* were amplified by multiplex PCR. Lane M: 100 bp ladder as molecular weight marker; Lane 1, Negative control (without any bacterial inoculation); Lane 2, *E. coli* O157:H7 BCRC 13089 (10 CFU/25 g); Lane 3, *Salmonella typhimurium* BCRC 12947 (10^4 CFU/25 g); Lane 4, *Klebsiella pneumoniae* BCRC 10692 (10^4 CFU/25 g); Lane 5, *E. coli* O157:H7 BCRC 13089 (10 CFU/25 g) + *S. typhimurium* BCRC 12947 (10^4 CFU/25 g); Lane 6, *E. coli* O157:H7 BCRC 13089 (10 CFU/25 g) + *K. pneumoniae* BCRC 10692 (10^4 CFU/25 g); Lane 7, Positive control, *E. coli* O157:H7 BCRC 13089 (10^4 CFU/25 g).

Table 3. Detection sensitivity of mini-VIDAS for *E. coli* O157:H7 BCRC 13085 mixed with or without unspecific bacterial culture

Detection levels of pure and mixed cultures (CFU/mL)			Result by
E. coli O157:H7 BCRC 13085	*M. morganii* RV-1	*E. cloacae* RV-2	mini-VIDAS
1×10^4	0	0	—
1×10^5	0	0	+
1×10^6	0	0	+
0	1×10^4	0	—
0	1×10^5	0	—
0	1×10^6	0	—
0	0	1×10^4	—
0	0	1×10^5	—
0	0	1×10^6	—
1×10^5	0	1×10^5	+
1×10^5	1×10^5	0	+
1×10^5	1×10^5	1×10^5	—

—, negative; +, positive.

Table 4. Detection sensitivity of immunostrips for different strains of *E. coli* O157:H7 and cell density

Tested strains of *E. coli* O157:H7	Inoculated levels (CFU/mL)	Result by immunostrip		
		Singlepath	Pathstik	Revel
BCRC 13084	10^6	—	±	—
	10^7	±	+	—
	10^8	+	++	±
BCRC 13085	10^6	—	±	±
	10^7	—	+	±
	10^8	+	++	+
BCRC 13086	10^6	—	+	—
	10^7	±	+	±
	10^8	+	++	+

—, negative; ±, slightly positive; +, positive; ++, strongly positive.

contaminating bacteria strains were present at 5×10^5 CFU/mL, the results of mini-VIDAS became negative. Therefore, the sensitivity of mini-VIDAS was affected by the presence of both contaminating bacteria at the same time. Keith(26) also estimated the sensitivity of mini-VIDAS assay for *Salmonella* in food products. His study revealed that *Salmonella* could be positively detected at $\geqq 10^4$ CFU/mL after enrichment procedures.

IV. *Sensitivity of Immunostrip and Interference from Contaminating Bacteria*

Table 4 shows the sensitivity of immunostrip of different brands for the detection of *E. coli* O157:H7 BCRC 13084, 13085 and 13086 at diverse levels. The immunostrip manufactured by Pathstik had the highest sensitivity, and could positively detect 10^6 CFU/mL of these three strains. This indicates that, in addition to bacterial count and strain, factors affecting the sensitivity of immunostirp also included the account of coating antibody specific to *E. coli* O157:H7 on the immunostrips manufactured by different brands and in different batches of a certain brand. Table 5 shows the detection sensitivity of immunostrips for pure BCRC 13086 or mixed with other bacteria. *E. coli* O157:H7 BCRC 13086 at 3×10^6 CFU/mL could be positively detected by immunostrip assay when mixed with equal amount of unspecific bacteria *M. morganii* RV-1 and *E. cloacae* RV-2. When the indicator bacteria was mixed with one unspecific bacteria 20~27 fold of indicator bacteria in cell count, the reaction became unclear. When mixed with two unspecific bacteria up to 47 fold of indicator bacteria, the indicator bacteria became undetectable. When the indicator bacteria was at 3×10^7 CFU/mL, the reaction was not interfered by two unspecific bacteria up to 50 fold of indicator bacteria.

Table 5. Detection sensitivity of Pathstik immunostrips for pure *E. coli* O157:H7 BCRC13086 mixed with or without unspecific bacterial culture

Detection levels by pure and mix cultures (CFU/mL)			Result by immunostrips
E. coli O157:H7 BCRC 13086	*M. morganii* RV-1	*E. cloacae* RV-2	
0	8×10^8	0	—
0	0	6×10^8	—
3×10^6	0	0	+
3×10^6	8×10^6	6×10^6	—
3×10^6	8×10^7	0	±
3×10^6	0	6×10^7	±
3×10^6	8×10^7	6×10^7	±
3×10^7	8×10^7	0	+
3×10^7	0	6×10^8	+
3×10^7	8×10^7	6×10^7	+
3×10^7	8×10^8	6×10^7	+
3×10^8	8×10^8	6×10^9	+

—: negative; +: positive; ±: slightly positive.

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