



Development of a Method for Detection of Shigatoxin-Producing *Escherichia coli* Belonging to Clinically Important Twelve O Serotypes Based on the Combination of PickPen-Assisted Immunomagnetic Separation and Loop-Mediated Isothermal Amplification

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Abstract

Not only O157 Shigatoxin-producing *Escherichia coli* (STEC) but some of non-O157 STECs are attracting attentions as clinically important STEC and a possible food-borne spread of these pathogens is becoming a worldwide concern. In this study, we developed a sensitive, specific, and simple method to detect STECs of clinical significance in retail beef so that it can be used even in resource-limited countries. We designed and fine-tuned a protocol characterized by PickPen devise-assisted immunomagnetic separation (IMS) for specific and simultaneous screening of O157 and eleven other O serotypes and quick and easy *stx* gene detection in STEC by the loop-mediated isothermal amplification (LAMP) assay. In vitro experiments showed 91CFU/ml sensitivity for IMS and 100% specificity for the IMS-LAMP-based method. We detected by the IMS-LAMP-based method the *stx* gene in 41 of 74 (55.4%) and none of 28 (0%) of beef purchased in, respectively, Thailand and Japan. Analyses of the STEC isolates from the Thai beef demonstrated high sensitivity and high (80-100 %) specificity of the detection method and regional difference in the STEC distribution in beef; and therefore the results support that this IMS-LAMP-based method is suitable for detection of clinically important STEC in beef.

Keywords

Shigatoxin-producing *Escherichia coli*, Retail beef, Immunomagnetic separation, PickPen, loop-mediated isothermal amplification

List of abbreviations

STEC: Shigatoxin-Producing *Escherichia coli*, EHEC: Enterohaemorrhagic *Escherichia coli*, *stx*: Shigatoxin gene, *stx*₁: Shigatoxin gene type 1, *stx*₂: Shigatoxin gene type 2, IMS: Immunomagnetic Separation, IMB: Immunomagnetic Beads, IMBV: Immunomagnetic Beads Volume, bIMBV: Best Immunomagnetic Beads Volume, LAMP: Loop-Mediated Isothermal Amplification, CE: Capture Efficiency

Introduction

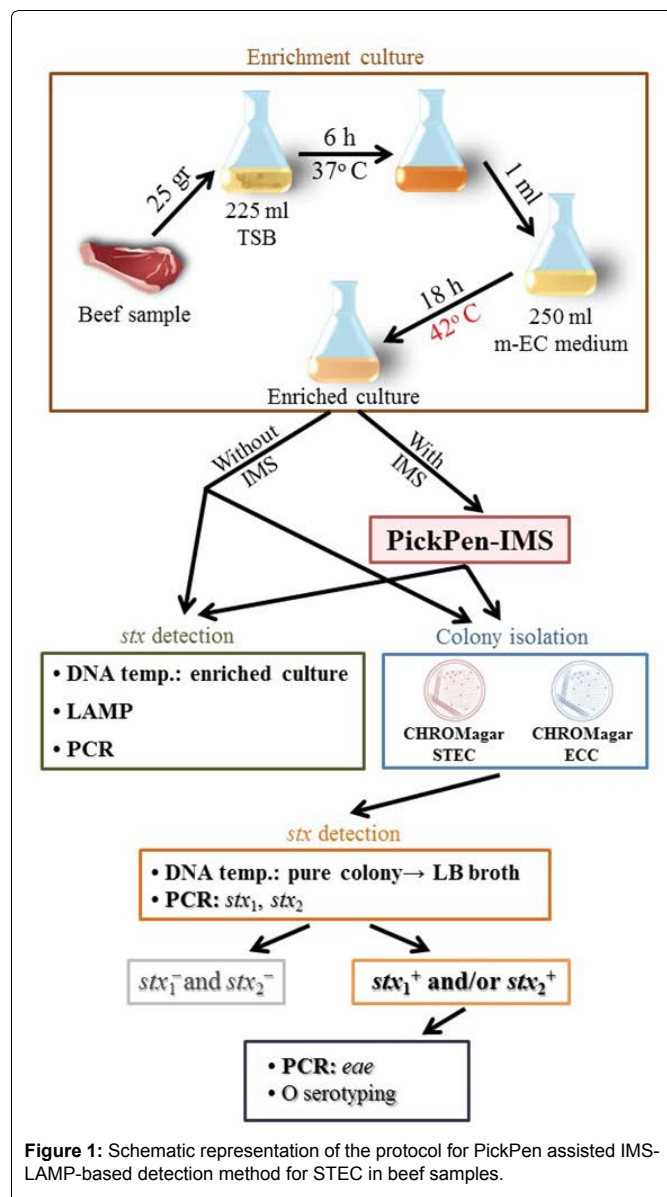
Shiga toxin (Stx)-producing *Escherichia coli* (STEC; also called verocytotoxin-producing *E. coli* or VTEC) can cause mild diarrhea in humans. Enterohaemorrhagic *E. coli* (EHEC) is a highly virulent subgroup of STEC and is one of major categories of pathogenic *E. coli* that cause important enteric infections in humans [1]. The common virulence factor of STEC and EHEC is the production of one or both subtypes of Stx (Stx1 or Stx2). EHEC is defined, based on the typical symptom of the patient, as STEC causing severe bloody diarrhea in humans. The EHEC may be confirmed experimentally by detection of additional virulence factors intimin and the large EHEC plasmid [2].

Strains of STEC/EHEC are differentiated by the O antigen on their outer membrane into over 180 serotypes [3]. Of all EHEC serotypes *E. coli* O157:H7 is the one which has caused most food borne outbreaks and has led to the highest number of hemolytic uremic syndrome cases, a life-threatening sequela of STEC infection; however, some of non-O157 serotypes are also becoming an important health concern because they have been isolated from the patients with the typical symptoms of EHEC infection with increasing frequency than before [4,5]. EHEC belonging to O157 and six other O serotypes (O26, O91, O103, O111, O121, and O145) are considered important in Japan [6-8]; and additional eight serotypes (O15, O45, O55, O104, O113, O118, O128, and O153) totaling to fifteen O serotypes for all countries in the world [9-15]. We therefore considered these fifteen O serotypes of EHEC are of particular importance for a global-scale detection method.

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In the inspection of environmental samples including food, the STEC expressing the known O antigens of EHEC are targeted as a potential public health hazard. Serogroup-specific method and virulence-specific method were combined for detection of STEC belonging to a clinically important O serogroup in some studies. Combinations of simple to sophisticated and high-cost techniques targeting a narrow spectrum of the important O serotypes of STEC/EHEC (a maximum number of eight of target O serotypes). These include multiplex PCR [16], PCR-ELISA and multiplex real-time PCR assays [17] and LAMP [18]; multiplex real-time PCR assays screening for virulence genes and O-antigens encoding genes [15]; immunomagnetic separation (IMS) targeting certain O serotypes of *E. coli* individually or simultaneously [19,20]; and a combination of multiplex real-time PCR and IMS assays [21-23].

Due to the rapid development of transportation, the food trade among various countries has become very active these days. Therefore, specific, sensitive and easy-to-perform methods are necessary for examination of food for a wider range of the important O-serotypes of STEC/EHEC even in resource-limited and tropical countries. In this study, we combined a unique IMS method, as a specific and easy-to-perform, covering clinically important O serotypes worldwide and a loop-mediated isothermal amplification (LAMP) method [24], as a simple, sensitive and low-cost technique, to achieve the goal described above.

Materials and methods

Immunomagnetic beads (IMB)

Magnetic beads were coated with antibodies against seven *E. coli* O antigens using antibodies partially purified from rabbit polyclonal antiserum specific to each of target O antigen (rabbit anti-*Escherichia coli* O157, O26, O91, O103, O111, O121 or O145 antiserum, Denka Seiken Co. Ltd., Tokyo, Japan). These resulted in bead group 1 suspension that targets the O serotypes of clinical importance in Japan. Similarly, bead group 2 suspension containing the beads coated with antibodies against anti- O15, O55, O104, O128 and O153 was prepared. Antibodies against anti- O45, O113 and O118 were unavailable for us and thus were not included. Equal volumes of bead group 1 suspension and bead group 2 suspension were mixed, resulting in the final bead suspension and designated as IMB in this study. This bead suspension (IMB) targets the O serotypes of clinical significance on a global scale. IMB was stored at 4° C and used whenever needed throughout this study.

Beef sample examination

Japanese beef was purchased from 4 local stores in Kyoto City, Japan, in 2013. Malaysian and Thai beef were purchased from a local market in Hat Yai City, southern Thailand, in 2013 and 2014. The protocol used to examine the beef samples is outlined in Figure 1. Twenty five grams of each sample was incubated at 37°C in 225ml of tryptic soy broth (Bacto™ Tryptic Soy Broth; Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA). After 6 h, 1 ml of the culture was added to 250ml of modified EC medium containing 25mg/l novobiotin (Nobobiotin added m-EC medium, Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and incubated at 42° C for 18 h. The broth culture with or without IMS treatment was examined in two steps. Step 1: the DNA template was prepared by a boiling method: boiling 1ml of the broth culture or bead suspension for 10 min, chilling on ice for 10 min, followed by centrifugation at 15,000 rpm for 5 min and collection of the supernatant. The DNA template was examined for the presence or absence of the *stx* genes by a conventional PCR method as previously described in [25] and the LAMP method according to the instruction by the manufacturer (Loopamp™ Verotoxin-producing *Escherichia coli* Detection Kit, Eiken Chemical Co., Ltd., Tokyo, Japan). Step 2: ten µl of the broth culture or bead suspension was streaked onto two selective agar media and incubated at 37°C for 18 h. Up to 5 suspected colonies from each triplicated agar plate were selected: mauve colonies growing on CHROMagar™ STEC (with 50 mg/l STEC supplement) and blue colonies growing on CHROMagar™ ECC (CHROMagar, Paris, France). The selected colonies were grown in 2 ml of Luria-Bertani broth [26] and the DNA template was prepared and examined by conventional PCR for *stx*₁ and *stx*₂ genes. The isolates that gave *stx*₁⁺ and/or *stx*₂⁺ result were examined for the *eae* gene as previously described in [27] and for O serotype by an agglutination test using *E. coli* O antisera (*E. coli* antisera “SEIKEN”, Set 1, Denka Seiken Co., Ltd.).

PickPen-Immunomagnetic separation (PickPen-IMS)

A 96-deep well (2-ml capacity) titer plate-based IMS procedure using an eight-channel PickPen devise (PickPen® 8-M, BIOCONTROL, Washington, USA) was performed as follows. IMB were placed at the room temperature on a shaker (140 rpm) for 30 min before use. Thirty µl of IMB were mixed with 1 ml of the broth culture in the first well and incubated at the room temperature on a shaker (140 rpm) for 30 min. The PickPen device was applied for 5 min and the captured IMB were transferred to the second well and washed in 1-ml washing buffer [phosphate-buffered saline consisting of 0.01M phosphate and 0.16M NaCl with pH 7.0 (PBS) with added 0.1 % Tween 20]. The washing step was repeated in the third well. Finally, the washed IMB were suspended in 1 ml of the washing buffer in the fourth well (designated as Yeild).

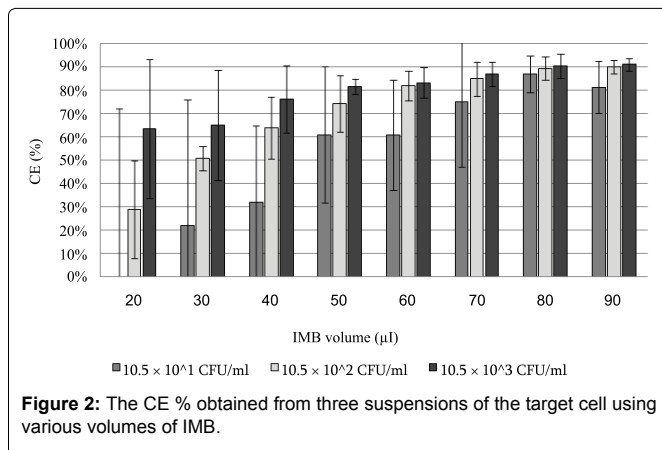
IMS sensitivity

The capture efficiency (CE) % was selected as indicator to evaluate four factors (IMB volume hereinafter referred to as IMBv, incubation

Table 1: Reference strains of *E. coli* used in IMS sensitivity and specificity experiments

Strain name	Origin	Year of isolation	O serotype	Presence of gene:		Literature/source
				<i>stx</i> ₁	<i>stx</i> ₂	
K-H-1	Human, Korea	1995	O143	—	—	[27]
K-H-2	Human, Korea	1995	O166 ¹	—	—	[27]
M47	Beef, Malaysia	1997	OUT ²	—	—	Laboratory stock
PE-7	Human, Brazil	1989	O126	—	—	[29]
KETE	Human, USA	Unknown	O6	—	—	[30]
KEIE	Human, Thailand	1991-1992	O124	—	—	[31]
EDL993	Human, USA	1982	O157	+	+	[32]
PV11-004	Human, Japan	2011	O91	+	—	Laboratory stock
PV11-006	Human, Japan	2011	O26	+	—	Laboratory stock
PV11-035	Human, Japan	2011	O145	—	+	Laboratory stock
PV10-104	Human, Japan	2010	O103	+	—	Laboratory stock
PV08-103	Human, Japan	2008	O111	+	+	Laboratory stock

¹Reported as OUT in the indicated literature, but subsequently determined to be O166 by the author (A.Y.K.).
²OUT: untypable for O serogroup.



time of the IMB-cell suspension mixture, PickPen application time, and washing step) in IMS sensitivity test. *E. coli* PV10-104 was used in this experiment (Table 1). The strain was grown in Luria-Bertani broth at 37°C for 18 h with shaking (160 rpm). Serial 10-fold dilutions of the culture were made in PBS without Tween 20.

IMBv: one milliliter of cell suspension of each of 10⁻⁵, 10⁻⁶ and 10⁻⁷ dilutions was mixed with (20 to 90μl) of IMBv. The best IMB volume (bIMBv) was determined.

IMB-target incubation time: four incubation times (5, 15, 30, and 45 min) were tested by incubating 10⁻⁵ cell suspension of the target cell with bIMBv.

PickPen application time: CE % at selected time points (0.5, 1.5, 3.5, 5, 7 min) of the PickPen application (duration of attracting the IMB by the PickPen) was determined as described below. The CE % relative to the increase in PickPen application time was calculated and the appropriate PickPen application time was determined.

Washing step: 10⁻⁵ target cell suspension was incubated with the bIMBv for 30 min. The IMB-target cell complex was washed twice and the CE % was determined and the loss of the target cell was calculated.

The CE was calculated as previously described, $CE\ (%) = (C_0 - C_a) / C_0 \times 100$, where C_0 is the total number of cells present in the sample (CFU/ml), and C_a is the number of cells not bound to IMB (CFU/ml) [28].

The IMS sensitivity (CFU/ml) was defined as the minimum concentration of target cells detectable by IMB at the bIMBv. This was calculated as follows, $IMS\ sensitivity\ (CFU/ml) = (C_L \times CE) / 100$, where C_L is the low concentration of the examined cell suspensions.

IMS specificity

To evaluate the specificity of the PickPen-IMS used in this study a mixture of *E. coli* strains consisting 6 strains each of targeted and non-targeted O serotypes was treated by this method. After the

Table 2: The result of examination of DNA from enriched culture of beef samples for the *stx* gene purchased in Thailand

Result of the <i>stx</i> gene detection					No. of beef samples
Pattern desig. ¹	Without IMS and by:		With IMS and by:		
	LAMP	PCR	LAMP	PCR	
A	—	—	—	—	26
B	+	—	—	—	7
C	+	—	+	—	28
D	—	—	+	—	11
E	+	+	+	+	1
F	+	+	+	—	1
Total	37	2	41	1	74

¹Based on the combination of IMS (with or without) and the kind of genetic method (LAMP or PCR) used for detection of the *stx* gene in the enriched culture of the beef sample.

PickPen-IMS treatment the O serotypes and *stx* genotypes of the *E. coli* remaining in the solution in each deep well of the 96-deep well titer plate were analyzed relative to those of the starting mixture. The details of the experiment are as follows. The twelve *E. coli* strains (Table 1) were grown in Luria-Bertani broth at 37°C for 18h with shaking (160rpm) and the cultures were diluted to 10⁻⁵CFU/ml. Equal volumes of each bacterial suspension were mixed in one tube (designated as the starting mix). 20μl of the starting mix was spread on CHROMagar™ ECC in triplicate to screen the starting composition of O serotypes without IMS (as a control). 1 ml of the starting mix was transferred to the first well and mixed with bIMBv, and then PickPen-IMS was performed as described above. The beads were washed in the second and the third wells and finally suspended in the fourth well. Appropriate volumes of the remaining solution in each well were spread on CHROMagar™ ECC in triplicates and incubated at 37°C for 18 h (20, 50, 50, and 20μl, from the first to the fourth well, respectively). Colonies were picked up randomly (up to 24 per plate) and the O serotypes were examined for the selected colonies. DNA templates were prepared from the starting mix and from the solutions remaining in the four wells by boiling and examined by LAMP as described above.

Statistical Analysis

All experiments were conducted in triplicate. The means and standard deviations of all collected data were calculated from independent group of replicates. A student's t-test was used for statistical analysis of the data between two groups. A p-value ≤0.1 was considered statistically significant.

Results and Discussion

Detection of STEC in the beef samples

In this study, we aimed at developing a specific, sensitive and easy-to-perform detection method for clinically important O serotypes of STEC that can be used in all countries in the world. We examined retail beef samples (not artificially contaminated) by the procedure outlined

Table 3: Characteristics and isolation procedure and origin of the STEC strains isolated in this study

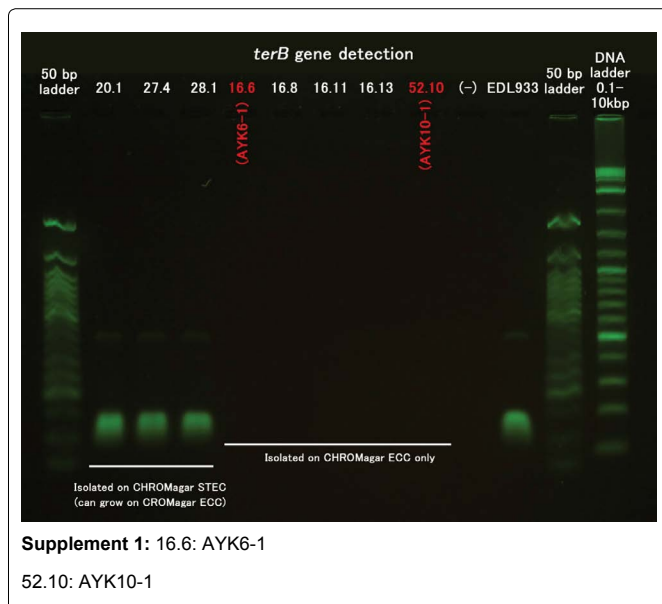
Strain no. ¹	Beef samples		Pattern ² desg.	Isolation of <i>stx</i> ⁺ strains						
				Isolation procedure ³			Characteristics of the isolated strains			
	O serotype	Presence of gene ⁴ :								
Medium		W/O IMS	W/IMS		<i>stx</i> ₁	<i>stx</i> ₂	<i>eae</i>			
AYK1-1	Malaysian	2013	C	S	—	+	O157	—	+	+
AYK2-1	≐	≐	E	S	—	+	O157	—	+	+
AYK3-1	≐	≐	C	S	—	+	O111	+	—	—
AYK4-1	≐	2014	D	S	+	+	O111	+	—	—
AYK4-2	≐	≐	D	S	+	—	O71	+	—	—
AYK4-3	≐	≐	D	S	+	+	O103	+	—	—
AYK5-1	≐	≐	D	S	—	+	O103	+	—	—
AYK6-1	≐	≐	F	E	+	—	O103	+	—	—
AYK7-1	≐	≐	D	S	+	+	O103	+	—	—
AYK8-1	≐	≐	D	S	—	+	O103	+	—	—
AYK9-1	≐	≐	C	S	+	—	O140	+	+	—
AYK10-1	Thai	≐	C	E	+	—	O144	+	+	—

¹Strains AYK4-1, AYK4-2 and AYK4-3 were isolated from the same beef sample; other strains from different beef samples.

²Corresponding to the pattern designation of Table 2 regarding the combination of examination methods for the *stx* gene in the enriched culture of the beef sample.

³W/O IMS: without IMS, W/IMS: with IMS, S: CHROMagar STEC, E: CHROM agar ECC, — : not isolated, + : isolated

⁴—: absent, +: present



in Figure 1 to evaluate our IMS-LAMP based detection method. We conducted our investigation in Japan and Thailand where distribution of the target bacterium is relatively scarce and prevalent respectively [25,33-35]. In our strategy, initial screening utilized detection of the *stx* gene. We examined not only the boiled supernatant of the enriched culture but also isolated colonies for the *stx* gene in order to check the possibility of false positive and false negative result of the LAMP assay. Twenty-eight Japanese beef samples were examined in 2013. And a total of 74 beef samples (22 and 52 samples, respectively, in 2013 and 2014) purchased in the market in southern Thailand were examined. All 28 Japanese beef samples examined were negative for the *stx* gene with or without IMS treatment. The Japanese result is largely reflecting the high quality and hygienic conditions of beef production in Japan.

The result of the examination for the presence of the *stx* gene in the enriched culture of the 74 beef samples purchased in Thailand was classified into patterns from A to F. This classification is based on the difference in the result obtained by the two genetic detection methods (LAMP and PCR) of the enriched culture treated without or with IMS. Forty-eight (64.9 %) of the 74 samples purchased in southern Thailand gave *stx*⁺ in at least one result of the four categories when enriched beef cultures treated with or without IMS were examined by LAMP and PCR (Table 2). The total number of *stx*⁺ DNA samples obtained by LAMP assay was 26 times (78/3 DNA samples) higher than that obtained by conventional PCR assay.

The 74 beef samples consisted of 35 Thai beef samples and 39 Malay beef samples. 57.1% of the Thai beef and 71.8% of the Malay beef samples gave *stx*⁺ result (data not shown). This result supports the previous report that Malay beef is more frequently contaminated by STEC than Thai beef [33].

In the study in Thailand, we observed two false negative cases of LAMP assay; where LAMP assays gave negative but some isolated colonies gave *stx*⁺ result (Tables 2,3, Pattern D, AYK4-1, AYK4-2, AYK4-3 and AYK7-1, LAMP without IMS). The result may suggest the need for a different DNA template preparation method for the LAMP assay other than the boiling method.

The beef samples classified to Patterns C and D amounted to 39 (52.7 %) beef samples that gave positive result only by LAMP but not by PCR regardless of IMS although *stx*⁺ result could be confirmed for 4 (14.3 %) and 4 (36.4 %) beef samples of Patterns C and D, respectively, by obtaining *stx*⁺ isolates. This result demonstrated how LAMP is more sensitive than PCR and that PCR failed in detecting the *stx* gene in the 8 (Patterns C and D combined) beef samples confirmed by isolation of the *stx*⁺ strains (false negative PCR result). The Pattern D is composed of 11 (14.9 %) samples that gave *stx*⁺ result by LAMP only when IMS treatment was applied, indicating the improvement of the sensitivity by IMS treatment prior to LAMP assay.

However, the Pattern B consists of 7 samples that were positive by LAMP without IMS and turned into negative by LAMP with IMS and we could not isolate any STEC strain which can be explained by a possibility that these 7 samples were contaminated with non-target *E. coli* O serotypes or non-*E. coli* bacteria possessing the *stx* gene [36]. We suspect that the LAMP positive result in Pattern B is most likely to be false positive.

To our surprise, one sample showed Pattern F where STEC O103, one of our target O serotypes, was isolated without IMS but not with IMS; and this particular strain was isolated on CHROMagar ECC only but not on CHROMagar STEC (Tables 2 and 3, AYK6-1). A possible explanation for the first unexpected observation that no STEC was isolated after IMS treatment is that the sample contained a large number of competing populations (*E. coli* expressing target O serotypes but lacking the *stx* gene). Because of abundance of competing population, the total number of STEC O103 after IMS treatment probably became below the detection limit of the PCR assay but was still detectable by the LAMP assay. To explain the second unexpected observation that the strain isolated on CHROMagar ECC was not isolated on CHROMagar STEC (contains potassium tellurite), we investigated the possibility that the isolated strain may lack the tellurite resistance gene (*terB*) [15]. We confirmed this was the case by the PCR assay (Supplement 1).

The selective isolation of STEC through CHROMagar ECC but not CHROMagar STEC was also observed in another case (Table 3, AYK10-1) which was also confirmed to be *terB*⁻ (Supplement 1). Our result summarized in Table 3 showed that 10 of 12 (83.3%) STEC strains were isolated on CHROMagar STEC but that 2 of 12 (16.7%) STEC strains could be isolated only on CHROMagar ECC. This result is similar to the report by Tzschoppe et al. [15] where 13.6% of EHEC and EHEC-like strains did not grow on CHROMagar STEC. We therefore propose simultaneous use of CHROMagar ECC for the isolation of STEC/EHEC.

In general, we could isolate *stx*⁺ *E. coli* strains more often from CHROMagar STEC (83.3 %, medium S in Table 3) compared to CHROMagar ECC (16.7 %, medium E in Table 3). Twelve strains belonging to six O serotypes were isolated from 10 beef samples. Nine and one of the ten beef samples were imports from Malaysia into Thailand and domestic product of Thailand, respectively. Of the six O serotypes three (O157, O111 and O103) belonged to the O serotypes of IMS target; two strains of O157 were obtained only with IMS and two strains of O111 and five strains of O103 were obtained with or without IMS. The remaining three O serotypes (O71, O140 and O144) were IMS non-target O serotypes; one strain each of these serotypes was obtained exclusively without IMS. The isolated strains included two O157 strains carrying the *stx*₂ and *eae* genes; and the other non-O157 strains sharing the *stx*₁ gene and lacking the *eae* gene (Table 3).

There are the studies reporting isolation of STEC/EHEC belonging to O157 serotype, possessing the *stx*₂ gene, but producing no or low-level Stx2 from the beef marketed in Hat Yai, southern Thailand [25,33,35]. Sukhumungoon et al. [35] also reported a non-O157 strain carrying the *stx*₁ gene from the beef purchased in the same mentioned city in Thailand. Their *stx*₁⁺ non-O157 strain produced a large amount of Stx1. In our study carried out in the same city, we isolated 10 strains of non-O157 STEC and all carried the *stx*₁ gene. Since this type of non-O157 STEC seems to be prevalent in the beef marketed in this area they may be of public health significance.

Improving IMS sensitivity

The result of the analysis of retail beef samples showed that we could isolate the strains carrying the *stx* gene from 20.8 % of the *stx*⁺

samples. We explored by in-vitro experiments the possibilities that IMS-LAMP performance can be further improved by fine tuning the factors involved in the IMS. The following four factors were evaluated in details using CE % as the indicator.

IMBv: incubation time of IMB-target cell mixture (30 min) and PickPen application time (5 min) were fixed. The effect of the combination of various concentrations of the target cells (10.5×10^1 , 10.5×10^2 , and 10.5×10^3 CFU/ml) and the various IMBv (20, 30, 40, 50, 60, 70, 80, and 90 μ l) were compared (Figure 2). The average of the CE % was gradually increased and its standard error was reduced with increase in the IMBv. For comprehensive evaluation of IMBv effect, when the result of CE % of the three concentrations of the target cells were combined, the average $\pm$ standard error increased from 25.0 ± 47.3 CE % at 20 μ l IMBv to 88.4 ± 5.71 CE % at 80 μ l IMBv. However, increase in the IMBv from 80 to 90 μ l had no significant effect on the increase of CE % whereas IMBv increase from 70 to 80 μ l showed significantly increase CE % for the middle and high concentrations of target cell suspension ($p=0.1$). Therefore, the 80 μ l of IMBv was judged to be the bIMBv. We found, when IMBv was increased to 80 μ l or above, the CE value reached a plateau regardless of the cell concentration. Since the ability of IMB to capture the maximum amount of the target cells is limited by the amount of antibodies coating the beads we judged the IMBv of 80 μ l (bIMBv) is the most suitable for the studies where the concentration of the target cells is unpredictable. The 80 μ l of IMBv is much more than the volume reported by other workers (20 μ l) [21,37] and close to the maximum volume that can be handled by the PickPen devise in our hands.

Incubation time of IMB-target cell: 2.7×10^3 CFU/ml of the target cell incubated with 80 μ l of IMB were employed. The result (5 min, 13.3 ± 3.5 CE %; 15 min, 70.9 ± 6.9 CE %; 30 min, 90.2 ± 0.4 CE %; 45 min, 86.1 ± 5.1 CE %) showed that the CE % was increased significantly when the incubation time was increased from 5 to 15 min and from 15 to 30 min, but not from 30 to 45 min ($p = 0.1$). Thus, 30 min incubation time was judged as the required time for the best CE % result.

PickPen application time (duration of attracting the IMB by the PickPen) : 1.1×10^3 CFU/ml of the target cell incubated with 80 μ l (bIMBv) of IMB were employed. The results (time - CE) were as follows: 0.5 min - 62.1 ± 30.6 CE %; 1.5 min - 57.6 ± 21.7 CE %; 3.5

Table 4: O serotype-based grouping and the number of the colonies randomly selected from the agar plates inoculated with the starting mix (without IMS) and the solution or the suspension remaining in each of the four wells after PickPen-IMS treatment (with IMS).

O serotype-based grouping		Number of the colonies selected ¹ :				
Group	O serotype	Without IMS	With IMS ²			
		(Starting mix)	No wash (First well)	First wash (Second well)	Second wash (Third well)	Yield (Fourth well)
Non-target	O143	—	++++	—	—	—
	O166	—	—	—	—	—
	OUT	+	+++	+	+	—
	O126	++	+++	+	—	—
	O6	++	++	+	—	—
	O124	++	++	—	—	—
	Subtotal	24 (33.3 %)	61 (84.7 %)	4 (26.7 %)	4 (19.0 %)	0 (0 %)
Target	O157	+	+	—	—	+++
	O91	++	—	—	+	++++
	O26	+++	+	—	+	+++
	O145	—	—	+	—	+
	O103	+++	+	+	—	+++
	O111	++	++	++	+++	++
	Subtotal	48 (66.7 %)	11 (15.3 %)	11 (73.3 %)	17 (81.0 %)	72 (100 %)
Total		72	72	15	21	72

¹Designations for the number of the colonies: —, 0; +, 1 – 5; ++, 6 – 10; +++, 11 – 15; +++, 16 – 23.

²Designation of the sample solution or suspension is explained in Materials and Methods

min - 65.8 ± 11.6 CE %; 5 min - 83.0 ± 4.73 CE %; 7 min - 61.5 ± 14.2 CE %. P values between the neighboring two time points were: $p = 0.44$, 0.5 - 1.5 min; $p = 0.24$, 0.5 - 3.5 min; $p = 0.08$, 3.5 - 5 min; $p = 0.08$, 5 - 7 min. The p value was gradually reduced as the time increased, but it appeared to have reached a plateau at 5 min. We therefore judged 5 min is the appropriate application time of PickPen.

Washing step on the loss of IMB-target cell complex in the buffer: the first and the second washing had insignificant effect ($p = 0.1$) (<1 CE % and <2 CE %, respectively).

After fine tuning of the IMS factors were fixed to as follows, the IMBv to 80 μ l, incubation time to 30 min, PickPen application time to 5 min and washing step to be repeated twice. These conditions determined the sensitivity of the IMS as 91 CFU/ml at the low concentration of the target cell (Figure 2).

IMS specificity

To evaluate the specificity of the IMS method in vitro, 6 strains of *E. coli* carrying the *stx*₁ and/or the *stx*₂ genes, each expressing different target O antigens, were mixed with 6 strains of *E. coli* lacking the *stx* gene and each expressing non-target O antigens. After completion of the IMS treatment, distributions of non-target and target O serotypes in the starting mix (not treated with IMS) and solutions or the cell suspension remaining in the four wells after IMS treatment were examined (Table 4). The result showed that IMS helped to separate and concentrate all target O serotypes (Table 4, Yield). The transition of the change in percentages of target vs. non-target groups showed how and to what extent each step of the PickPen-IMS treatment could contribute to change in the composition of the starting mix. The examination of DNA templates of the 5 suspensions by the commercially available loopamp VT *E. coli* detection kit reflected how LAMP can perform with the help of IMS in this particular experiment as follows: without IMS, no wash, first wash, second wash, and yield, showed respectively, positive, positive, negative, negative, and positive result for the *stx* gene. This result suggests that the remaining target cells in the solutions, first wash and second wash, were below the detection limit of the loopamp kit (60 CFU/test).

We have evaluated the specificity of the IMS by an in vivo assay (Table 3). In the in vivo study of the beef purchased in Thailand, STEC strains belonging to the target and non-target O serotypes were isolated from 8 and 2 LAMP-positive beef samples, respectively. These non-target O serotypes were O140 and O144 (strains AYK9-1 and AYK10-1, respectively, in Table 3), and they were isolated from two different beef samples only without IMS treatment (Table 2, Pattern C). The LAMP positive result with IMS treatment of these samples may or may not be due to these non-target O serotypes. Based on this result, the specificity of the IMS-LAMP detection method can be judged to be very good ranging from 80 % (due to non-target O serotypes) to 100 % (due to target O serotypes). In addition, the IMS was shown to be 100 % specific by an in vitro assay where only target O serotypes were isolated from the Yield (Table 4).

Conclusion

In our current study we designed an IMS-LAMP method targeting as many as twelve O serotypes and to the best of our knowledge, this is the first time to report this number of O serotypes of STEC targeted at once. We have evaluated the specificity of this method by in vivo and in vitro assays and it showed to be 80 to 100 % specific. We regret that we could not include three more O serotypes as the targets of the PickPen-IMS because the antibodies were unavailable when we started this study. However, we have found the antibodies are available recently. The system can therefore be expanded to include the three additional antibodies so that the targets will cover the 15 important O serotypes in the future. We think addition of the antibodies rose to the three or more O antigens, if needed, are possible although the optimal conditions (i.e. bIMBV) and results of the detection method may change. Using a similar PickPen-IMS, our colleagues successfully prepared a set of IMB targeting 69 different K antigens at once and demonstrated its utility by detecting the target organisms from

seafood [38]. Therefore, this method may be expanded to include three more O serotypes as a targets totaling to the fifteen O serotypes proposed in the introduction to cover important O157 and non-O157 serotypes in different parts of the world.

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References

- Farrokh C, Jordan K, Auvray F, Glass K, Oppegaard H, et al. (2013) Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. *Int J Food Microbiol* 162: 190-212.
- Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11: 142-201.
- Ballmer K, Korczak BM, Kuhnert P, Slickers P, Ehrlich R, et al. (2007) Fast DNA serotyping of *Escherichia coli* by use of an oligonucleotide microarray. *J Clin Microbiol* 45: 370-379.
- Stigi KA, macdonald KJ, Tellez-Marfin AA, Lofy KH (2012) Laboratory practices and incidence of non-O157 shiga toxin-producing *Escherichia coli* infections. *Emerg Infect Dis* 18: 447-449.
- Gould LH, Bopp C, Strockbine N, Atkinson R, Baselski V, et al. (2009) Recommendations for diagnosis of Shiga toxin-producing *Escherichia coli* infections by clinical laboratories. *MMWR Recomm Rep* 58: 1-14.
- NIID (National Institute of Infectious Diseases) and Tuberculosis and Infectious Diseases control division, Ministry of Health, labour and welfare, Japan (2012) Symptoms associated with EHEC infection, by serotype. *Infectious Agents Surveillance Report (IASR)* 33:117.
- NIID (National Institute of Infectious Diseases) and Tuberculosis and Infectious Diseases control division, Ministry of Health, labour and welfare, Japan (2013) Symptoms associated with EHEC infection, by serotype. *Infectious Agents Surveillance Report (IASR)* 34: 125.
- NIID (National Institute of Infectious Diseases) and Tuberculosis and Infectious Diseases control division, Ministry of Health, labour and welfare, Japan (2014) Symptoms associated with EHEC infection, by serotype. *Infectious Agents Surveillance Report (IASR)* 35: 119.
- Mathusa EC, Chen Y, Enache E, Hontz L (2010) Non-O157 Shiga toxin-producing *Escherichia coli* in foods. *J Food Prot* 73: 1721-1736.
- EFSA (European Food Safety Authority) (2009) Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food) on request of EFSA. *The EFSA Journal* 7: 1-43.
- Bettelheim KA (2007) The non-O157 shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. *Crit Rev Microbiol* 33: 67-87.
- Johnson KE, Thorpe CM, Sears CL (2006) The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. *Clin Infect Dis* 43: 1587-1595.
- Garcia A, Fox JG (2003) The rabbit as a new reservoir host of enterohemorrhagic *Escherichia coli*. *Emerg Infect Dis* 9: 1592-1597.
- De Moura C, Ludovico M, Valadares GF, Gatti MSV, Leite DS (2012) Detection of virulence genes in *Escherichia coli* strains isolated from diarrheic and healthy feces of dairy calves in Brazil. *Arquivos do Instituto Biológico, São Paulo* 79: 273-276.
- Tzschoppe M, Martin A, Beutin L (2012) A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. *Int J Food Microbiol* 152: 19-30.
- DebRoy C, Roberts E, Valadez AM, Dudley EG, Cutter CN (2011) Detection of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the *wzx* gene of the O-antigen gene cluster. *Foodborne Pathog Dis* 8: 651-652.
- Perelle S, Dilasser F, Grout J, Fach P (2007) Screening food raw materials for the presence of the world's most frequent clinical cases of Shiga toxin-encoding *Escherichia coli* O26, O103, O111, O145 and O157. *Int J Food Microbiol* 113: 284-288.
- Wang F, Jiang L, Ge B (2012) Loop-mediated isothermal amplification assays for detecting shiga toxin-producing *Escherichia coli* in ground beef and human stools. *J Clin Microbiol* 50: 91-97.

19. Safariková M, Safarik I (2001) Immunomagnetic separation of *Escherichia coli* O26, O111 and O157 from vegetables. *Lett Appl Microbiol* 33: 36-39.
20. Kanki M, Seto K, Kumeda Y (2014) Simultaneous Immunomagnetic Separation Method for the Detection of *Escherichia coli* O26, O111, and O157 from Food Samples. *J Food Prot* 77: 15-22.
21. Fratalico PM, Bagi LK, Cray WC Jr, Narang N, Yan X, et al. (2011) Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in ground beef. *Foodborne Pathog Dis* 8: 601-607.
22. Fratalico PM, Bagi LK (2012) Detection of Shiga toxin-producing *Escherichia coli* in ground beef using the GeneDisc real-time PCR system. *Front Cell Infect Microbiol* 2: 152.
23. Madic J, Vingadassalon N, de Garam CP, Marault M, Scheutz F, et al. (2011) Detection of Shiga Toxin-Producing *Escherichia coli* Serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in Raw-Milk Cheeses by Using Multiplex Real-Time PCR. *Applied and Environmental Microbiology* 77: 2035–2041.
24. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, et al. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28: E63.
25. Koitabashi T, Vuddhakul V, Radu S, Morigaki T, Asai N, et al. (2006) Genetic characterization of *Escherichia coli* O157: H7/- strains carrying the stx2 gene but not producing Shiga toxin 2. *Microbiol Immunol* 50: 135-148.
26. Griffith KL, Wolf RE Jr (2002) Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. *Biochem Biophys Res Commun* 290: 397-402.
27. Kim YB, Okuda J, Matsumoto C, Morigaki T, Asai N (1998) Isolation of an *Escherichia coli* O157:H7 strain producing Shiga toxin 1 but not Shiga toxin 2 from a patient with hemolytic uremic syndrome in Korea. *FEMS Microbiology Letters* 166: 43–48.
28. Zeng J, Wei H, Zhang L, Liu X, Zhang H, et al. (2014) Rapid detection of *Vibrio parahaemolyticus* in raw oysters using immunomagnetic separation combined with loop-mediated isothermal amplification. *Int J Food Microbiol* 174: 123-128.
29. Tsukamoto T (1996) PCR methods for detection of enteropathogenic *Escherichia coli* (localized adherence) and enteroaggregative *Escherichia coli*. *Kansenshogaku Zasshi* 70: 569–573.
30. Stacy-Phipps S, Mecca JJ, Weiss JB (1995) Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. *J Clin Microbiol* 33: 1054-1059.
31. Sethabutr O, Venkatesan M, Murphy GS, Eampokalap B, Hoge CW, et al. (1993) Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *J Infect Dis* 167: 458-461.
32. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, et al. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308: 681-685.
33. Sukhumungoon P, Nakaguchi Y, Ingviya N, Pradutkanchana J, Iwade Y, Seto K, Radu R, Nishibuchi M, Vuddhakul V (2011a) Investigation of stx2+ eae+ *Escherichia coli* O157:H7 in beef imported from Malaysia to Thailand. *International Food Research Journal* 18: 381–386.
34. Ikeda T, Morimoto Y, Tamate N, Shimizu S, Kumada H, Komagome R, Kubo A, Yamaguchi K (2007) Surveillance of foodborne in food. Report of Hokkaido Institute of Public Health 57: 73–75 (article in Japanese).
35. Sukhumungoon P, Mittraparp-arthorn P, Pomwised R, Charemjiratragul W, Vuddhakul V (2011b) High concentration of Shiga toxin 1-producing *Escherichia coli* isolated from Southern Thailand. *International Food Research Journal* 18:683–688.
36. Mauro SA, Koudelka GB (2011) Shiga toxin: expression, distribution, and its role in the environment. *Toxins (Basel)* 3: 608-625.
37. Nou X, Arthur TM, Bosilevac JM, Brichta-Harhay DM, Guerini MN, et al. (2006) Improvement of immunomagnetic separation for *Escherichia coli* O157:H7 detection by the PickPen magnetic particle separation device. *J Food Prot* 69: 2870-2874.
38. Tanaka N, Iwade Y, Yamazaki W, Gondaira F, Vuddhakul V, et al. (2014) Most-probable-number loop-mediated isothermal amplification-based procedure enhanced with K antigen-specific immunomagnetic separation for quantifying tdh+ *Vibrio parahaemolyticus* in molluscan shellfish. *Journal of Food Protection* 77:1078-1085.