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A novel detection method for *Escherichia coli* O157 by Peptide Nucleic Acid array

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Abstract: *E. coli* O157 is responsible for a number of human infections through the consumption of foods of animal origin, particularly those originating from cattle. Food poisoning by *Escherichia coli* O157 causes diarrhea, bloody stools and abdominal pain in humans, and may lead to severe kidney failure or brain injury. For these reasons, *E. coli* O157 has been the subject of increasingly stringent international regulations. A novel peptide nucleic acid (PNA) array was developed to identify *E. coli* O157 quickly and accurately. This PNA array exhibited high specificity, significantly detecting *E. coli* O157 strains without cross-reacting with other bacterial strains. The detection limit of the *E. coli* O157 PNA array was 100 CFU/ml. The *E. coli* O157 PNA array is indeed a better option for early detection and accurate diagnosis of the causative agent of a food-borne disease outbreak, which is crucial in planning and implementing strategic measures to prevent and control widespread outbreaks.

Keywords: *Escherichia coli* O157, Peptide Nucleic Acid Array, Food Poisoning

1. Introduction

Food poisoning with *Escherichia coli* O157 was first reported in 1982 [19]. From 1982 to 2002, a total of 8,598 cases of *E. coli* O157 infection were detected in the United States [17]. In England and Wales, 3,429 isolations of verocytotoxin-producing *E. coli* O157 from human sources were confirmed from 1995 to 1998 [26]. Infection with *E. coli* O157 is associated with the consumption of raw or undercooked meat and other foods or water contaminated with animal feces [6, 27]. Symptoms of *E. coli* O157 infection may be life-threatening, and include abdominal cramps, watery diarrhea, fever, vomiting, and hemorrhagic colitis [8].

Current schemes for the detection and identification of *E. coli* O157 rely on a combination of culture methods and biochemical and serological tests. However, established culture methods and biochemical tests for *E. coli* O157 identification are laborious and time consuming [10]. Moreover, serotyping requires skilled workers to carry out the tests [6, 25]. With recent advances in genetic technologies, molecular-based tests using PCR [5, 10, 12] and DNA microarrays have led to remarkable improvements in methods for the direct detection of *E. coli* O157 [16, 23]. The *E. coli* O157 O side chain contains four different sugars, including GDP-perosamine, which form the O side chain of the lipopolysaccharide (LPS) [20]. GDP-perosamine synthetase (*per*) is specific for *E. coli* O157, so it has been utilized for the identification of this bacterial strain [16].

Peptide nucleic acid (PNA), a novel oligonucleotide in which the sugar phosphate backbone is replaced by a pseudo-peptide skeleton, is notable for its exceptional biological and chemical stability as a nucleic acid analogue [13, 14]. PNA has been used to develop assays with improved detection limits, detection of sequence variations, and sensitivity [3, 15]. Moreover, PNA-DNA hybridization is more specific than the corresponding DNA-DNA duplex [2, 18, 24]. Due to these advantages, PNA has been widely used as a tool for molecular-based detection [1, 4, 9]. In the present study, we developed a PNA array-based method for the specific detection of *E. coli* O157 and compared its

detection limit and specificity with those of traditional culture-based methods.

2. Materials and Methods

2.1. Bacterial Strains and Serotyping

The bacterial strains containing *E. coli* O157:H7 are shown in Table 1. The four substrains of *E. coli* O157:H7 used were *E. coli* O157:H7 ATCC 43894 (Manassas, VA, USA), *E. coli* O157:H7 ATCC 43895 (Manassas, VA, USA), *E. coli* O157:H7 C163 (*E.coli* Reference center, PA, USA), and *E. coli* O157:H7 WSU 3110 (Washington State University, WA, USA). The other strains (Korea Veterinary Culture Collection, Animal, Plant Quarantine Agency, Anyang, South Korea) listed in Table 1 were used as controls. Serological analysis of *E. coli* was performed by slide and tube agglutination with 51 O and 22 H antisera, according to the manufacturer's instructions (Denka Seiken, Tokyo, Japan).

Table 1. *Evaluation of the specificity of the E. coli O157 PNA array using pure culture bacteria*

Bacteria	Strain no.	Intensity value	PNA results*
E. coli O157:H7	ATCC 43894	14203.3±49.7	+
E. coli O157:H7	ATCC 43895	18894.7±19.5	+
E. coli O157:H7	C 163	19726.7±19.7	+
E. coli O157:H7	WSU 3110	23151.7±27.9	+
E. coli O6:H-	KVCC-BA0000515	-34.0±9.0	-
E. coli O8:H19	KVCC-BA0000516	-34.8±7.3	-
E. coli O15:H25	KVCC-BA0001440	-14.3±7.9	-
E. coli O20:H-	KVCC-BA0000520	-22.8±7.9	-
E. coli O26:H-**		-4.3±7.5	-
E. coli O86:H25	KVCC-BA0002388	110.2±10.4	-
E. coli O111:H-	KVCC-BA0002395	1.3±10.3	-
E. coli O112:H8	KVCC-BA0002396	-5.8±8.9	-
E. coli O128:H2	KVCC-BA0002407	-26.2±7.1	-
E. coli O139:H1	KVCC-BA0000533	411.0±16.3	-
E. coli O141:H4	KVCC-BA0000534	12.8±7.2	-
E. coli O147:H19	KVCC-BA0002414	1.5±9.4	-
E. coli O148:H28	KVCC-BA0002415	-35.5±7.9	-
E. coli O149:H10	KVCC-BA0002416	-32.2±4.8	-
E. coli O153:H7	KVCC-BA0002417	446.5±4.1	-
E. coli O159:H20	KVCC-BA0002419	-5.2±15.8	-
Campylobacter lari	KVCC-BA0902581	-80.5±7.7	-
Campylobacter jejuni	KVCC-BA0902577	-81.5±4.2	-
Clostridium perfringens	KVCC-BA0000578	-25.7±2.5	-
Clostridium perfringens	KVCC-BA1100001	-92.5±9.1	-
Enterococcus faecalis	KVCC-BA0000404	7.2±3.5	-
Enterococcus faecium	KVCC-BA0000412	- 3.8±16.3	-
Enterococcus hirae	KVCC-BA0700653	4.5±7.9	-
Listeria grayi	KVCC-BA0000577	-14.5±8.1	-
Listeria innocua	KVCC-BA0000576	-0.7±5.7	-
Listeria monocytogenes	KVCC-BA0000575	4.7±10.4	-
Pasteurella multocida	KVCC-BA0000513	15.2±7.8	-
Salmonella berta	KVCC-BA0000581	-36.0±5.3	-
Salmonella derby	KVCC-BA0000582	-20.3±4.4	-
Salmonella dublin	KVCC-BA0000584	-22.7±7.2	-
Salmonella enteritidis	KVCC-BA0400601	-90.7±6.0	-
Salmonella gallinarum	KVCC-BA0700722	-5.8±4.6	-
Salmonella glustrup	KVCC-BA0000583	30.3±4.6	-
Salmonella illinois	KVCC-BA0000585	-20.3±2.6	-
Salmonella muenster	KVCC-BA0000586	5.8±6.2	-
Salmonella oranienberg	KVCC-BA0000587	28.2±5.1	-
Salmonella paratyphi B	KVCC-BA0000588	-10.2±8.4	-
Salmonella senftenberg	KVCC-BA0000590	-9.7±4.0	-
Salmonella tallahassee	KVCC-BA0000589	-17.7±8.7	-
Salmonella tennessee	KVCC-BA0000592	-33.0±7.8	-
Salmonella typhimurium	KVCC-BA0000591	-24.5±7.4	-
Salmonella uganda	KVCC-BA0000596	-25.8±5.5	-

Bacteria	Strain no.	Intensity value	PNA results*
Salmonella virchow	KVCC-BA0000595	-28.2±5.1	-
Salmonella typhimurium	KVCC-BA0400600	-102.2±8.2	-
Staphylococcus epidermidis	KVCC-BA0001452	6.5±16.3	-
Staphylococcus aureus	KVCC-BA0000098	-4.2±4.9	-
Streptococcus dysgalactiae	KVCC-BA0001419	2.3±3.8	-
Streptococcus equi	KVCC-BA0201420	1.7±3.5	-
Streptococcus iniae	KVCC-BA0000100	6.2±4.9	-
Streptococcus parauberis	KVCC-BA0000187	-0.8±7.5	-
Streptococcus suis	KVCC-BA0000579	-3.2±4.9	-
Vibrio parahaemolyticus	KVCC-BA0001825	-5.0±7.1	-
Yersinia enterocolitica	KVCC-BA0000716	-14.5±0.7	-

* Results of the PNA array were defined as negative (<3,000) or positive (>3,000) on the basis of the intensity value. ** *E. coli* O26:H- was kindly provided by Dr. Min-soo Kang of Animal, Plant Quarantine Agency.

2.2. Assay of CFU and Preparation of E. coli O157 DNA

Analysis of colony forming units (CFU) was based on varying lengths of incubation time (0, 1, 3, and 5 h) of *E. coli* O157 culture that was inoculated into modified *Escherichia coli* (mEC) broth. The culture broth was then inoculated into MacConkey agar and incubated for 24 h at 37 °C. After incubation, the colonies were counted, and then, for the PNA assay, bacterial DNA was extracted using a genomic DNA extraction kit (iNtRon Biotechnology Inc., Seongnam, South Korea), and stored at -20 °C until use.

2.3. Design and Synthesis of PNA Probe

The PNA probe (NH2-linker-CGATGCCAATGTACTCGG) was designed on the basis of the *E. coli* O157-specific *per* region. The spotting mixture buffer (Panagene, Daejeon, South Korea), including the probe, was printed onto epoxy-coated slides with a 2470 arrayer (Aushon Biosystems, Billerica, MA, USA). The printed slide was blocked with succinic anhydride. Nested PCR for the detection of *E. coli* O157 using the PNA array was performed twice with a biotin-conjugated primer set (forward, 5'-TTCACACTTATTGGATGGTCTCA-3', and reverse, 5'-TCGATAGGCTGGGGAAACT-3'). The first reaction mixture (25 μl) contained 1 U of *Taq* DNA polymerase (Solgent, Daejeon, South Korea) and PCR buffer with 1.5 mM $MgCl_2$, 150 μM deoxynucleoside triphosphate mixture, and 0.08 μM of each specific primer. The second reaction mixture was the same as the first PCR mixture, except that 0.04 μM forward primer and 2 μM reverse primer were used. The first PCR program was 5 min at 95 °C, 15 cycles of 30 sec at 95 °C, 30 sec at 50 °C, and 60 sec at 72 °C, and final incubation of 10 min at 72 °C. The second PCR program consisted of 45 cycles of the same temperature and time conditions.

2.4. Hybridization and Scanning of PNA Array

Five microliters of biotin-labeled target DNA were mixed with 70 μl of PNA hybridization buffer (Panagene, Daejeon, South Korea) containing Cy5-streptavidin (Amersham Pharmacia, Buckinghamshire, UK) and applied to the PNA array. The PNA array was incubated for 2 h at 40 °C. After hybridization, PNA array images were taken by a nonconfocal fluorescent scanner (Axon Instruments, Union City, CA, USA) with a typical laser power of 100% and photomultiplier tube gain of 700. Scanning to detect Cy5 was performed with GenPro Pix 6.0 software (Axon Instruments, Union City, CA, USA), at a wavelength of 635 nm. Fluorescence signal intensities represent the hybridization signals of the PNA probe-target duplexes.

3. Results and Discussion

3.1. Efficacy of the PNA Probe for E. coli O157

The performance of the PNA array as a detection assay for *E. coli* O157 is shown in Table 1. A positive signal for the PNA array was defined as an intensity value of ≥ 3,000. Among the 57 strains, only four *E. coli* O157 strains (ATCC 43894, ATCC 43895, C163, and WSU 3110) gave positive results, with intensity values from >14,203.0±49.7 to 23,151.7±27.9. The remaining 53 strains gave negative signals, with intensity values from -102.2±8.2 to 446.5±4.1 (Table 1). The *E. coli* O157-specific probes hybridized specifically to their corresponding targets and did not cross-hybridize with other strains.

3.2. CFU Change with Incubation Time and Detection Limit of the PNA Array

The CFU and PNA array results for *E. coli* O157 with incubation times of 0, 1, 3, and 5 h are shown in Fig. 1 and Fig. 2. Four *E. coli* O157 strains were inoculated into the mEC broth. CFU was calculated using the culture and colony counting method at different incubation time intervals. The CFU values for all four *E. coli* O157 strains were 10^2 after 1 h, 10^3 after 3 h, and 10^5 after 5 h of incubation. After 1 h of incubation, the PNA intensity values of the four *E. coli* O157 substrains (ATCC 43894, ATCC 43895, C163, and WSU 3110) were 3,052.2±5.1, 3,750.0±5.0, 3,543.8±8.6, and 7,415.0±7.2, respectively. Thus, all four of the substrains gave positive signals. The detection limit of the *E. coli* O157 PNA array was 100 CFU/ml. After 5 h of incubation, the PNA intensity values of the four substrains were 14,203.3±49.7, 18,894.7±19.5,

19,726.7±19.7, and 23,151.7±27.9, respectively (Fig. 2).

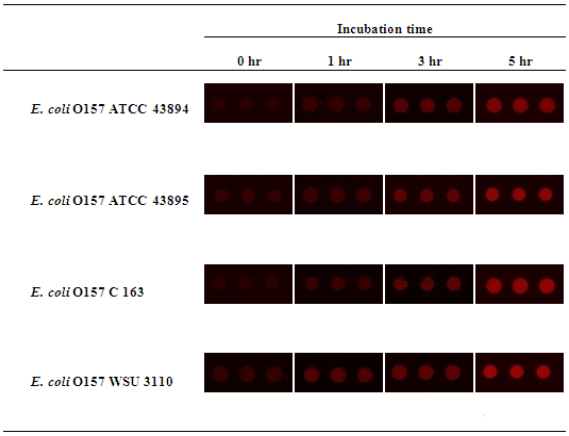
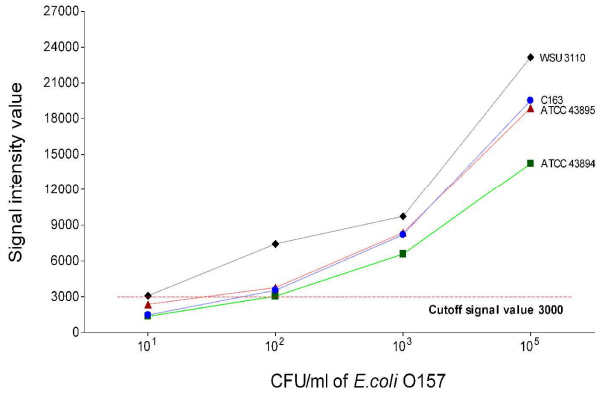


Figure 1. *Scanned images of the peptide nucleic acid (PNA) microarray, with E. coli O157 at different incubation time intervals.*



■, *E. coli* O157 ATCC 43894; ▲, *E. coli* O157 ATCC 43895; ●, *E. coli* O157 C163; ◆, *E. coli* O157 WSU 3110.

Figure 2. *Colony forming units (CFU) and sensitivity of four E. coli O157 strains detected with the peptide nucleic acid (PNA) microarray.*

Recent advances in nucleic acid recognition, including the introduction of PNA, provide exciting opportunities for DNA biosensors. PNA has several advantages as a probe molecule, such as superior hybridization characteristics, detection of single-base mismatches, and improved chemical and enzymatic stability compared with nucleic acids. In addition, its unique molecular structure enables new modes of label-free detection, contributing to the establishment of rapid, stable, and reliable analytical processes. PNA sensing can be used to detect pathogens of interest with high stability and increased affinity for target sequences [22]. Accordingly, PNA probes have been applied to fluorescence *in situ* hybridization and single nucleotide polymorphism detection [11, 21]. Using current culture methods, detection of *E. coli* O157 would take about 7 days, whereas identification by the PNA array required only 1–2 days. To improve the culture methods, molecular-detection methods (PCR, enzyme immunoassay, rapid kit methods) have been developed. However, these methods have low specificity and high detection limits. The pathogenicity of *E. coli* O157 is associated with numerous virulence factors, including verotoxin 1 (VT1) and verotoxin 2 (VT2). Although VT1 and VT2 detection methods have been used to detect *E. coli* O157 by PCR, this diagnostic method is not accurate because some *E. coli* O157 strains do not produce verotoxin. We detected VT1 in *E. coli* strains O157:H7 ATCC 43894, ATCC 43895 and C163, and VT2 in *E. coli* strains O157:H7 ATCC 43894, ATCC 43895 and WSU 3110 (data not shown). An enzyme immunoassay for *E. coli* O157 has been developed as a sandwich-type assay with polyclonal antibodies to the bacteria; however, this method can only detect *E. coli* O157 at concentrations $\geq 1.3 \times 10^5$ CFU/ml [7]. Recently, Real-time PCR methods have been used to detect low density of *E. coli* O157, but this diagnostic method can not be used on large-scale because of the reagent and instrumentation cost. Taken together, these results show that the PNA array is an effective method for detecting *E. coli* O157.

4. Conclusion

We have developed a novel PNA-based microarray for detecting *E. coli* O157. This method allows rapid and accurate high-throughput screening of bacteria that cause food-poisoning, from a large number of food animal samples. The *E. coli* O157 PNA array is indeed a better option for early detection and accurate diagnosis of the causative agent of a food-borne disease outbreak, which is crucial in planning and implementing strategic measures to prevent and control widespread outbreaks. Furthermore, this PNA array could also be an effective research and diagnostic protocol to gather epidemiological data useful for policy formulation in establishing food safety and ensuring national food security.

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