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Identification of *Escherichia coli* O157:H7 with Oligonucleotide Arrays

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The bacterium *Escherichia coli O157:H7*, which causes major, often prolonged outbreaks of gastroenteritis with hemolytic-uremic syndrome (HUS) is a worldwide threat to public health and has been implicated in many outbreaks of haemorrhagic colitis, some of which included fatalities caused by haemolytic uraemic syndrome (Riley, L. W. et al. 1983; Slutsker et al. 1998). Most human *E.coli O157:H7* infections are caused by consumption of contaminated food and water, and cattle are generally considered the major reservoir for this organism (Dorn and Angrick 1991). Close to 75,000 cases of *E.coli O157:H7* infection were estimated to occur annually only in the United States (Mead, P. S. et al. 1999). Lack of rapid and sensitive detection methods has contributed to our poor understanding of the ecology of this organism in the animal reservoir, and consequently hindered the design of intervention strategies to control the prevalence of *E.coli O157:H7*. At the same time, lack of effective treatment and the potential for large-scale outbreaks from contaminated food supplies have propelled intensive research on the pathogenesis and detection of *E.coli O157:H7*. As sporadic manifestations can rapidly turn into major outbreaks, the development of rapid, automated, yet sensitive and specific diagnostic assays for the detection of *E.coli O157:H7* has become a major concern worldwide. Conventional method for the detection of *E.coli O157:H7* involves colony isolation and isolate confirmation by biochemical and serological tests. These methods are time-consuming and laborious (Meng, J., et al.1994). A number of PCR-based assays have been developed for detection of *E.coli O157:H7*, and most of these assays have been carried out to establish a multiplex PCR method, which could simultaneously detect the specific genes of *E. coli O157:H7* (Gannon et al. 1997; Paton and Paton 1998). Although these gene sequences are associated with *E.coli O157:H7*, they are also present in other pathogenic *E.coli* strains, and are not unique for serotype *O157:H7*. Moreover, many assays using DNA probes have also been developed to detect *E.coli O157:H7* and it is not specific for serotype *O157:H7*. Tyagi and Kramwr (1998) introduced a new type of fluorescent probe, called molecular beacon, for simple and accurate DNA sequence analysis, but it was limited by the type of fluorophore.

In recent years, the use of oligonucleotide microarrays has be demonstrated to be a promising approach for nucleic acid analysis (Pease, A. C., et al. 1994; Parinov, S., et al. 1996), microorganism determination (Bavykin,S.G., et al. 2001) and diagnostics of diseases (Yershov, G., et al. 1996; Drobyshev, A., et al. 1997). The standard bioassays used for identification of pathogenic *E.coli* are difficult to adapt for the screening of large numbers of *E.coli* isolates. The array format has great potential for providing

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strong tools to overcome the shortcomings described as above owing to its advantage of being scalable, flexible and easy to fabricate and perform. The highly specific recognition properties of hybridization between the probes and the PCR products make these hybridization assays attractive for use in the fields of biodiagnostics as sensitive probes for multiple PCR products simultaneous detection, and the array format has been used in systematical analysis of microbial virulence factors (Chizhikov,V., et al. 2001; Small, J., et al. 2001) and microbial identification (Bavykin,S.G., et al. 2001) using fluorescent labeled targets. However, there are few reports for microbial identification using biotionylated target DNA. As a model system, we chose to analyze the *E.coli* strains, especially *E.coli O157:H7*. Here we describe a novel method, based on streptavidin-Cy3 interaction with biotin, to detect *E.coli* O157:H7 in a reproducible, accurate and sensitive manner with oligonucleotide arrays.

MATERIALS AND METHODS

Normal *E.coli* ATCC 25922 strain, *E.coli O1:H7*, *E.coli O157:H19* and *E.coli O157:H7* (882364) strain was obtained from Center of Disease Prevention and Control of Jiangsu Province. Genomic DNA was extracted from the bacterium cells by conventional phenol-chloroform method. PCR amplification was performed in a total volume of 25 μl, containing 1 U *Taq* polymerase (TaKaRa, Takara Shuzo, Japan), 2.5 μl 10×PCR buffer, 200 μM dNTP, 1 μl DNA template sample, and approximately 0.25 μM each pairs of primers. The synthesis of biotin-labeled multiple PCR products were also amplified by the same method above expect that substitute 200 μM dTTP with 190 μM dTTP and 10μM biotin-16-dUTP (Roche Inc.). The amplification procedure was as follows. The reaction mixture was incubated at 94℃ for 5 min. Then, it was subjected to 35 cycles of incubation at 94℃ for 30 s to denature the DNA, at 60℃ for 60 s to anneal the primers, and at 72℃ for 60 s to extend the annealed primers. Following amplification, the sample was subjected to electrophoresis on a 2 % (w/v) agarose gel and the products were visualized by 0.5ug/ml ethidium bromide staining. A 100 base pair DNA ladder (DL2000, Takara Shuzo, Japan) was used as the marker. Seven probes were designed with 20~30 nucleotides length corresponding to an internal segment of the each gene fragment to be amplified in the *E.coli* strains (Table 1). An alignment of these nucleotides sequences was carried out with the OMIGA software, version 2.0. All the oligonucleotides were synthesized using a DNA synthesizer and purified by Shanghai Shengyou Ltd., China. Spotting solutions were obtained by dissolving the probes in sodium carbonate buffer (0.1 M, pH=9.0) at the concentration of 10 μM. Pin-based spotting robot PixSys5500 (Cartesian Tech. Inc.) with CMP3 pin was used to perform the array spotting. About 500 pl spotting solution was spotted on the slide with 120 μm diameter and 300 μm spacer. After spotting, the slides were incubated at room temperature for 2 hours and at 37°C for 2 hours. Then the slides were washed thoroughly with 0.1% Tween in deionized distilled water and dried. After PCR reaction, the PCR products were directly heated for 5 minutes at 96℃ to denature the PCR products, and then snap chilled on ice for 3 minutes before hybridization. The hybridization procedure was described everywhere. Simply, The arrays were then hybridized with the denatured sample at 37℃ for 15 min and washed in 2×SSC/0.1% SDS for 2 min at room temperature and in 0.1×SSC/0.1% SDS for 3 min at room temperature. Finally, slides were stained by streptavidin-Cy3 (Zymed Inc.) for 8 min at room temperature and rinsed with 0.01 M PBS buffer (pH=7.4) and then dried. After staining with the streptavidin-Cy3, fluorescent images of the microarrays were generated by scanning the slides by using a ScanArray 4000 (GSI Lumonics,

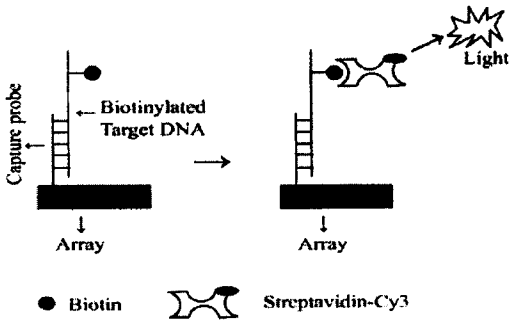

Figure 1. Schematic representation of the fluorescent detection of hybridized target DNA on a microarray based on the use of streptavidin-Cy3.

Table 1. Oligonucleotide probes and primers for virulence factor targets

Target gene	Primer sequence(5' to 3')	Oligonucleotide targets to microchip
Stx 1	F, ataaatcgccattcgttgactac	H_2N-taacatcgctcttgccacagactgc
	R, agaacgcccactgagatcatc	gtcag
Stx 2	F, ggcactgtctgaaactgctcc	H_2N-atcctctccccgatactccggaagc
	R, tcgccagttatctgacattctg	acatt
hlyA	F, gcatcatcaagcgtacgttcc	H_2N-gagccatgcctgataaagcaatcc
	R, aatgagccaagctggttaagct	ccgtaa
eaeA	F, gacccggcacaagcataagc	H_2N-gcggccttcatcatttcgctttcaga
	R, ccacctgcagcaacaagagg	actg
flicH7	F, gcgctgtcgagttctatcgagc	H_2N-cgggtcaggcgattgctaaccgttt
	R, caacggtgacttatcgccattcc	tactt
rfbE	F, aaatataaaggtaaatatgtgggaacatttgg	H_2N-tgatcgcggtgtcagttcttt
	R, tggcctttaaaatgtaaacaacgg tcat	
uidA	F, tgatcgcggtgtcagttcttt	H_2N-gtggaattgagcagcgttggt
	R, attgcccggctttcttgta	

Inc., USA) at a resolution of 5 μm. The fluorescent signals from each spot were measured and compared by using QuantArray software (GSI Lumonics). Analysis of collected data was performed on the basis of total fluorescence intensities measured from a fixed circular area of each oligonucleotide spot. Fluorescent signals with a statistically significant difference (P<0.01) from the background level were considered to be positive.

RESULTS AND DISCUSSION

The principle of the streptavidin-Cy3 staining method for detecting hybridized DNA on microarray is illustrated in Figure 1. First, the target DNA is labeled with biotin by the incorporation of biotion-16-dUTP during the multiplex PCR. After target hybridization on the array, streptavidin-Cy3 is added for specific binding to biotin. Six pairs of primers were selected with reference to published sequence data from *rfbE* (Fortin, N. Y., et al. 2001), *fliCh7* (Gannon, V. P., et al., 1997), *stxI*, *stx II*, *eaeA*, and *hlyA* (Paton, A. W. and Paton, J. C.1998), and primers for *uidA* were designed by us in this study. To standardize the conditions for the multiplex PCR assay, the relative ratio

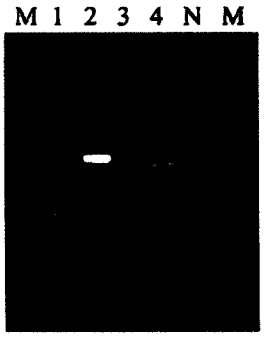

Figure 2. Agarose gel analysis of nucleic acids released from E.coli by multiplex PCR on electrophoresis. The biotin-labeled PCR products were generated in the presence of primers to detect the *uidA, rfbE, Stxl, stx II, eaeA, hlyA,* and *flicH7*. Lanes M, DL2000 molecular size DNA marker. Lanes N, Negative control. Lane numbers corresponds to the bacterial sample number in the Table1.

of the seven sets of primers and the optimal annealing temperature and Mg^{2+} concentration were determined using the genomic DNA (20ng reaction^{-1}) of strain 83460 as template. The ideal concentrations of each primer pair that yielded seven distinct bands were 200, 300, 450, 250, 250, 250, 200 nM for *fliCh7*F/*fliCh7*R, *rfbE*F/*rfbE*R, *stx I*F/*stx I*R, *stx II*F/*stx II*R, *eaeA*F/*eaeA*R, *hlyA*F/*hlyA*R, and *uidA*F/*uidA*R, respectively. The standardized conditions were used in subsequent preparation of biotinylated target DNA. We obtained the biotin-labeled targets by incorporation the biotin-16-dUTP during multiplex PCR. To obtain the higher incorporation efficiency of biotin label into PCR products, the biotin-16-dUTP and the relative molar ratio of dTTP and biotin-16-dUTP were selected and determined using serial two-fold dilution. The molar ratio 19:1 of dTTP and biotin-16-dUTP provided the optimal incorporation efficiency in this study. Herein we printed a pattern containing 3×3 array in quadruplicate as a model system by an arrayer depicted in Figure 3A. From left to right, the bottom row represent *uidA*, *rfbE*, *Stx1*; the second last row 2 represent *Stx2*, *hlyA, eaeA*; the top row are *flicH7*, buffer and positive control, respectively. Figure 3 (B)-(E) displays a quarter of the fluorescent image of the arrays hybridized at 37℃ with different biotinylated targets DNA generated from four various *E.coli* strains. The results indicate that this condition not only had no effect on the yield of PCR products (Figure 2), but also was less expensive than the other methods of synthesis of fluorescent targets. As anticipated, four isolates of *E.coli* serotype were correctly identified, and results are consistent with the results of conventional methods. The image demonstrates the selectivity of the PCR products of different genes for the corresponding oligonucleotide probes immobilized on the slides. As expected, the result of this image were nearly identical with the data from electrophoretic pattern shown in Figure 2, what is more, the confusion between the *uidA* and *Stx 2* gene analyzed on agarose gel disappear. The uneven intensities of the fluorescence image on the difference spots on the array were also observed as same as the uneven intensities of the electrophoresis brands. There would be interpreted by the following discussion. Firstly, because of various amplification efficiency of the set of primer pairs during the multiplex PCR, DNA product intensities are typically variable and unrelated to the quantity of target sequence in the samples. Secondly, may be the probability that hybridization process seems to depend on the distance of the oligonucleotide position from the end of the DNA target (Chizhikov, et al. 2001). Figure 4 displays the quantification of raw data presented in fluorescence image above. This graph indicates the good specificity of the hybridization assay. The incorporated efficiency is the most important for preparation of fluorescent-labeled target DNA during PCR amplification, especially during the multiplex PCR amplification.

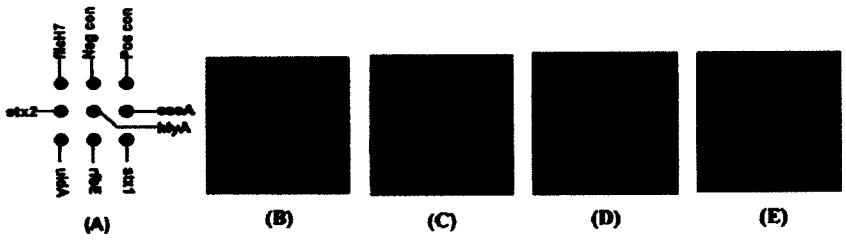

Figure 3. Hybridization arrays layout and hybridization images of the presence of specific genes in the four isolates of the *E.coli*. (A) Hybridization arrays layout of a quarter region of the fluorescence image of the four *E.coli* strains. Different oligonucleotides probes or controls were spotted on glass slides in low densities array format as indicated in the figure by depositing 500pl droplets at 300μm spacing. The target element name and the corresponding gene are shown in the layout. (B)- (E) are the fluorescence image correspond to the sample of *E.coli* ATCC 25922, *E.coli O1:H7*, *E.coli O157:H19* and *E.coli O157:H7* (882364) strain, respectively.

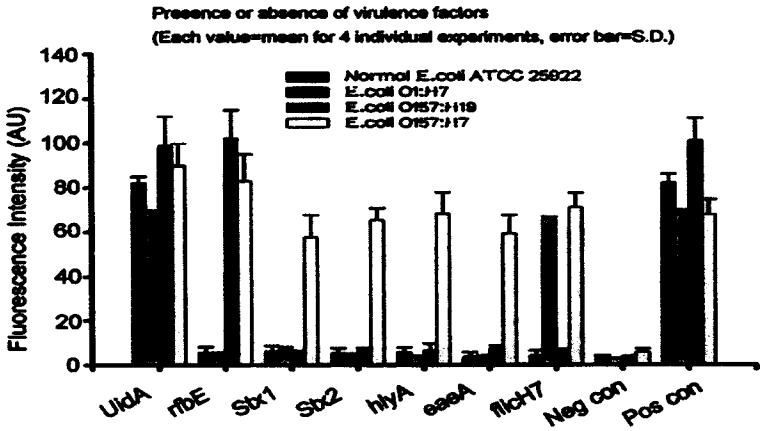

Figure 4: Fluorescence intensities of raw data presented in fluorescence image shown above. QuantArray software will process this raw image data and produce a single value for each dot's intensities. From left to right, the probes are *uidA,rfbE ,Stx 1, Stx2,h lyA, eaeA, flicH7*, buffer and positive control, respectively.

Traditionally, direct incorporation of fluorescence dyes in a PCR synthesis reaction has been used to prepare fluorescence-labeled target DNA for hybridization to microarrays. In this method the fluorescence dyes are not efficiently incorporated into the target DNA, and the low incorporation efficiency limits the sensitivity of the labeled targets. In addition to, the addition of an internal fluorescent label was found to reduce the hybridization stability (Mineno et al., 1993). Here a biotion-dUTP is incorporated into the target DNA during the PCR amplification. The biotin-dUTP exhibit relatively low steric hindrance in the PCR reaction, which results in target DNA with biotin-dUTP Red and black representation reveals a presence or absence of the specific genes of the different bacterial *E.coli* incorporated at a higher frequency than a fluorescence dye

dUTP. This makes the labeling method highly sensitive to detect low copy number genes and the specificity of hybridization can be improved at the same time. In conclusion, a hybridization assay with this array was carried out to identify successfully seven specific genes of the *E.coli O157:H7*. Our result indicated that the array provide a relatively fast and reliable method to simultaneous analysis of thousands of sequences of DNA for genomic research and diagnostics applications. The array format is adaptable to a wide variety of nucleic acid detection applications, including rapid diagnostic testing for infectious disease panels, DNA typing for forensic application, and antibiotic resistance panels.

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