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Molecular beacons: A real-time polymerase chain reaction assay for detecting *Escherichia coli* from fresh produce and water

S. Sandhya, Wilfred Chen, Ashok Mulchandani*

Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, United States

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ABSTRACT

Molecular beacons (MBs) are oligonucleotide probes that fluoresce upon hybridization. The development of a real-time polymerase chain reaction (PCR) assay to detect the presence of *Escherichia coli* using these fluorogenic reporter molecules is reported. MBs were designed to recognize a 19-bp region of the *uid A* gene, coding for an enzyme β-glucuronidase. The specificity of the MB-based PCR assay was evaluated for various *E. coli* strains as well as bacteria species that are present in nature. The capability of the assay to detect *E. coli* in drinking water and produce was demonstrated. Positive detection of *E. coli* was demonstrated when $>10^1$ CFU mL^{-1} (colony forming unit) was present in the water samples and fresh produce after 18 h of enrichment. These assays could be carried out entirely in sealed PCR tubes, enabling rapid and semiautomated detection of *E. coli* in food and environmental samples.

1. Introduction

Escherichia coli is a widely used indicator of contamination from domestic sewage mainly originated from warm blooded animals. Elevated levels of *E. coli* in natural waters can indicate the presence of fecal pollution introduced through animals or human waste. The organism was used as an index of water quality as well as indicator microorganism for a fecal source tracking as it is easily culturable [1]. Previously, *E. coli* has been identified via biochemical and or culture based approaches. Recently, *E. coli* identification is based on polymerase chain reaction (PCR) for various pathogenic or nonpathogenic genes, genotyping involving repetitive extragenic palindromic PCR (REP-PCR), enterobacterial repetitive intergeneric PCR (ERIC-PCR) and poly ethylene glycol E (PEGE), or sequencing of regions of genes of interest. Real-time PCR with fluorogenic probes is faster than traditional PCR and offers the user the ability to simultaneously identify and quantify specific genes, thus making real-time PCR a diagnostic tool of choice for measuring bacteria in food, water, fecal and tissue supply [2–4]. As the reaction is homogeneous, the risk of cross contamination is minimized and downstream analysis is eliminated.

Molecular beacons (MBs) are single-stranded nucleic acid sequences that possess a stem loop structure that is double labeled with fluorescent dye and a universal quencher at the 5′ and 3′ ends, respectively [5]. When the internal probe hybridizes to its target sequence, undergoing a spontaneous conformation change that forces the arm sequences apart and causes fluorescence to occur. Perhaps the biggest advantage of molecular beacon is their extraordinary specificity. No

* *Corresponding author*. Present address: NEERI, CSIR Complex, Chennai, India. Tel.: +90 4422541964; fax: +90 44 22541964.
E-mail address: sswami_in@yahoo.com (S. Sandhya).
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increase in fluorescence is observed even in the presence of a target strand that contains only a single nucleotide mismatch [6]. The application of real-time Synergy Brands, Inc. (SYBR) green PCR assay for detection of food and water borne pathogens was shown by Fukushima et al. [7]. Robinson et al. [8] have used reverse transcription PCR (RT-PCR) and melting curve analysis for rapid sensitive and discriminating identification of *Naegleria* sp.

β-Glucuronidase activity is observed in approximately in 95% of naturally occurring *E. coli* [9]. Sequence based source tracking of *E. coli* is based on genetic diversity of β-glucuronidase and forms the basis of Colilert test, m Coli Blue and modified m total *E. coli* (mTEC) methods for enumerating *E. coli* [10]. The entire 1812-nucleotide gene for β-glucuronidase, known as *uid A* or *gus* A has previously been sequenced in strain *E. coli* K-12 [11,12], four pathogenic strains of *E. coli* [13,14] and two strains of closely related *Shigella flexneri* [15,16]. The presence of a substituted amount of variation in β-glucuronidase gene suggested that it therefore might be useful as a genetic tool for tracking source of *E. coli* in the environment.

The aim of this study was to develop a real-time fluorogenic PCR assay for detection of *E. coli* from water and fresh produce samples. In this study we examined the ability of the *uid A* gene, which codes for the β-glucuronidase enzyme, to serve as a target for fluorogenic PCR detection of *E. coli*. A 140 bp region of the *uid A* gene present in all *E. coli* was used as target.

2. Materials and methods

2.1. Bacterial strains, medium, and culture conditions

Bacterial strains evaluated in this paper are listed in Table 1. *E. coli* strain JM109 ATCC 43895 was used as the reference strain in all optimization and sensitivity experiments. Bacterial strains were cultivated at 37 °C and 300 rpm in LB medium (Difco Laboratories, Detroit, MI). Prior to DNA extraction, 100 mL of 10-fold serial dilutions of freshly grown cells was spread on an LB plate for colony enumeration.

2.2. DNA preparation

The CTAB (hexadecyltrimethylammonium chloride) phenol/chloroform (CPC) method was used for the preparation of chromosomal DNA [17]. The CPC method was performed as follows: 1.5 mL of overnight grown bacteria was spun at 1400 rpm for 2 min and resuspended in 600 μL TE buffer (pH 8.0) containing 1% SDS and 1 mg mL^{-1} of proteinase K. Cells were allowed to lyse for 1 h at 37 °C. One hundred microliters of 5 M NaCl was added to adjust the salt concentration to 0.5 M. The solution was subjected to three cycles of freezing (−70 °C) and thawing (65 °C), followed by the addition of 80 μL of 10% CTAB in 0.7 M NaCl solution (1% final) and incubation at 65 °C for 10 min. After the first extraction with 800 μL of phenol:chloroform:isoamyl alcohol (25:24:1), the solution was briefly vortexed and spun at 12,000 × *g* for 15 min. The supernatant was transferred to a clean tube and subjected to a second chloroform:isoamyl alcohol (24:1) extraction. Chromosomal DNA was precipitated by adding 400 mL of isopropanol. The pellet was washed with 500 μL of iced 70% ethanol, dried, resuspended in 20 μL TE buffer, and stored at −20 °C.

2.3. Target gene, PCR primers, and molecular beacon

The forward primer (764–774) F5′-AGC CAA AAG CCA GAC AGA GT-3′ and reverse primer (886–900) R 5′-CATGACGACCAAAGCCAGTA-3′, designed to amplify a 140-bp fragment of the *uid A* gene and the MB 5′-FAM-CGCTAT GCATCCGGTCAGTGGCAGTATAGCG -826-DABCYL-3′, were purchased from Integrated DNA Technologies, INC. (Coralville, LA). The 19-bp probe moiety was designed to perfectly match the *uid A* gene of *E. coli* strain 86-24 and was flanked by two 6-bp arms. Fluorescein (6-FAM) was chosen as the chromophore (excitation at 488 nm and emission at 518 nm) and DABCYL was used as the quencher. Beacons were resuspended in TE buffer, stored at −20 °C, and protected from light.

2.4. Thermal denaturation profiles

The thermal denaturing profile and signal-to-background ratio (S/B) of the molecular beacon were investigated to determine the optimal recording temperature of the fluorescent "probe–target" hybrid for real-time PCR. The changes in fluorescence of a 50 μL solution containing varying amount of $MgCl_2$ and 0.5 mM beacon with or without 0.9 mM of a perfectly complementary single-stranded oligo were measured. Solutions were heated in the Icycler iQ sequence detector System (Bio-Rad, CA) at 95 °C for 10 min and gradually cooled to

Table 1 – Variation in real-time PCR assay

	C_t					
	8 CFU	80 CFU	800 CFU	8000 CFU	80,000 CFU	800,000 CFU
Run 1	ND	25	24	23	21	19
Run 2	ND	25	24	24	22	20
Run 3	ND	25	24	24	21	19
Run 4	ND	25	24	24	21	19
Run 5	ND	25	24	24	22	20
Mean ± S.D.	–	25	24	23.8	21.4	19.4

ND is not detected.

20 °C and the fluorescence intensity was recorded. The (S/B) ratio was then determined using the relation:

$$\frac{(F_{\text{probe–target}} - F_{\text{buffer}})}{(F_{\text{hairpin}} - F_{\text{buffer}})},$$

where $F_{\text{probe–target}}$, F_{buffer}, F_{hairpin} are the fluorescence of the probe–target hybrid, the buffer, and the hairpin structure, respectively.

2.5. PCR conditions

For each PCR, 2.5 μL of template DNA was added to 27.5 μL of PCR master mix (1.5 mM $MgCl_2$, 0.5 mM of each primers, 0.2 mM dNTPs mix, 1 mM MB, 5 U Taq DNA polymerase, 2.5 μL Taq buffer, and the rest water). Both the polymerase and the amplifying buffer were purchased Bio-Rad, CA. All reactions were performed in the 50 μL Bio-Rad optical tubes sealed with the optical seal. The Bio-Rad IQ Cycler system was used for real-time analyses. For the PCR reactions, samples were heated at 95 °C for 2 min, followed by 40 cycles of melting at 94 °C for 45 s, fluorescent measurement at annealing temperature of 53 °C for 45 s, and extension at 72 °C for 45 s. At the end of each PCR run, data were automatically analyzed by the system and amplification plots were obtained. The threshold (C_t) cycle of each amplification reaction was calculated based on the first PCR cycle at which the fluorescence was 10-fold higher than the standard deviation of the mean baseline emission.

2.6. Detection of E. coli in water samples

Drinking water sample about 100 mL was artificially contaminated with 10^2–1 cells which were filtered through membrane filter (0.45 μm), then transferred to 10 mL nonselective LB broth and incubated overnight (18 h) at 37 °C. Cells were collected and DNA was extracted for RT-PCR run.

2.7. Detection of E. coli in produce samples

Fresh produce-spinach, obtained from local grocery shop, (25 g) was inoculated with 10^2–1 cells in saline using pure culture of *E. coli*, shaken for 1 h and processed for pre-enrichment. Twenty grams of produce was combined with 10 mL of saline in sterile stomacher bags then transferred to 10 mL nonselective LB broth and incubated overnight (18 h) at 37 °C. Cells were collected and DNA was extracted for RT-PCR run.

3. Result

3.1. Thermal denaturation profiles

To determine the optimal recording temperature for the real-time PCR assay, the thermal denaturing profiles of the beacon in the presence and absence of a perfectly complementary oligo were investigated using an IQ Cycler fluorescence reader. The fluorescence intensity of the molecular beacon in the presence or absence of an excess amount of complementary oligo was monitored at different temperatures. The temperature range that provided the highest signal-to-background ratio was used as the annealing temperature for the PCR. Fig. 1 shows the fluorescence variation as a function of temperature. MB *uid A* remained as a dark hairpin at temperatures below 39 °C while the probe–target hybrid emitted high intensity of fluorescence. The maximum signal-to-background ratio was recorded at around 39 °C. As the temperature increased from 39 °C to 73 °C, the stem gradually melted and the signal-to-background ratio decreased dramatically. At 73 °C, the probe–target hybrid denatured as indicated by a drop in the fluorescence. This result indicates that the optimal recording temperature window of MB *uid A* should be at a temperature lower than 55 °C.

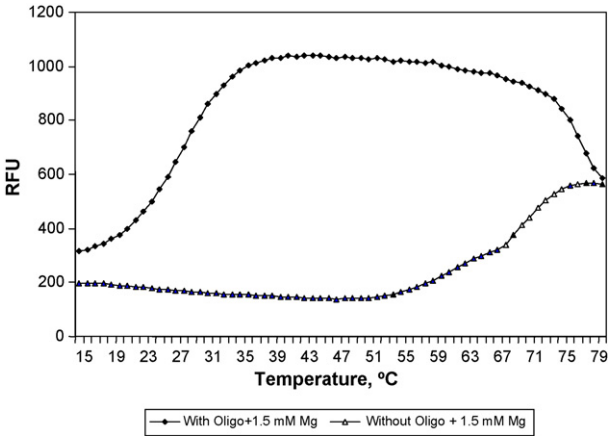

Fig. 1 – Thermal denaturation profile for molecular beacon.

3.2. Development of real-time PCR assays

The ability of MB *uid A* to detect *E. coli* in real-time PCR assays was investigated. DNA isolated from *E. coli* JM109, ATCC 43985 was used as the template. A three-step PCR with a recording temperature and an annealing step at 53 °C was found to provide better sensitivity. Fig. 2 shows the normalized fluorescent measurement vs. the PCR cycle using the three-step PCR. This plot clearly shows the progression of the amplification reaction. The real-time PCR assays were further optimized by monitoring the relative fluorescence intensity (DRn) and the threshold cycle (C_t). We first adjusted the concentration

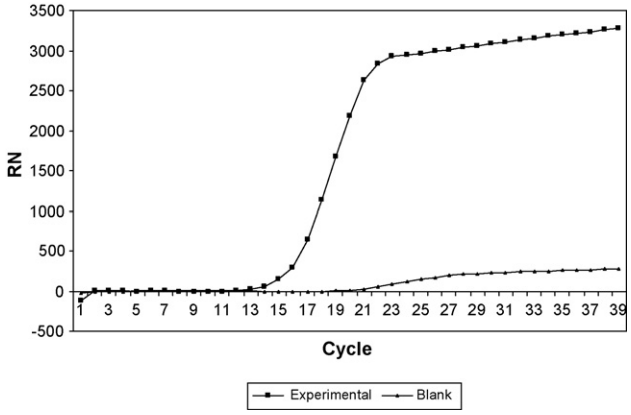

Fig. 2 – Real-time PCR amplification of the *E. coli uid A* gene.

of $MgCl_2$ to 1.5 mM, which provided the maximum signal-to-background ratio without drastically compromising the yield and specificity. Varying MB concentration from 0.33 mM to 1.66 mM resulted in a 3.5-fold increase in the DRn value but had very little effect on the C_t value (Fig. 2). Optimal conditions were determined as stated under Section 2.

3.3. *Sensitivity of the real-time PCR assay*

The sensitivity of the real-time PCR assay was evaluated. Ten-fold serial dilutions of template DNA isolated from *E. coli* JM109 strain ATCC 43985 were used for the assay (Fig. 3a). Any fluorescent signal that is 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection. Using this criterion, the detection limit of the real-time PCR assay was determined to be 8×10^3 CFU m L^{-1}, a value comparable to that reported using other fluorogenic PCR assays for *E. coli* [18,19]. Since the C_t value represents the PCR cycle at which the fluorescent intensity rises above the threshold, it can be used to quantify the input target concentration (Fig. 3b). This quantification proved to be linear over a wide range of initial target concentrations (from 10^2 to 10^7 CFU m L^{-1}).

The variability between different samples was investigated. Five different sets of PCR assay were performed with initial target ranging from 8 CFU to 80×10^5 CFU. Each amplification is highly reproducible. Comparison of C_t values of the five different sets of assay also reveals little variability (Table 1). More importantly the rate of fluorescent change at each target

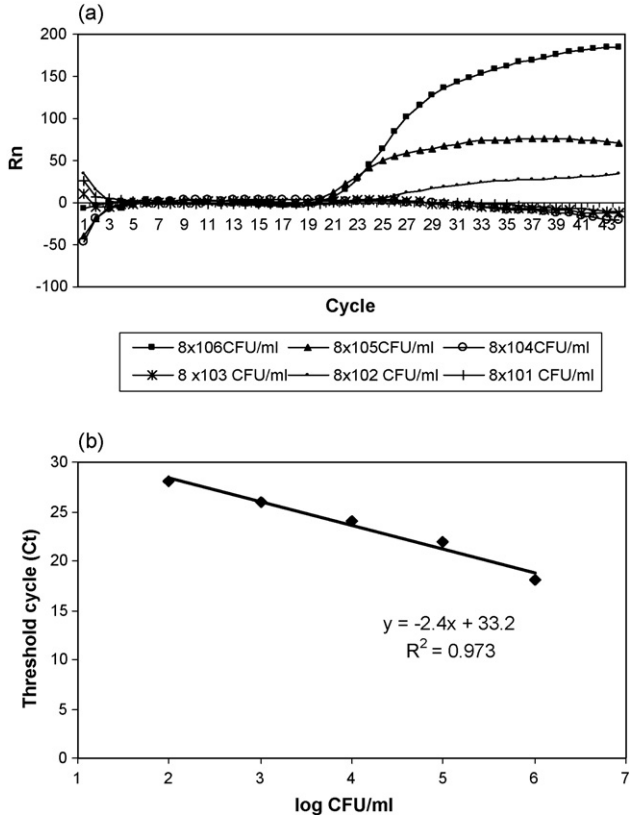

Fig. 3 – (a) Sensitivity of real-time PCR assay. (b) Standard curve for the real-time PCR assay.

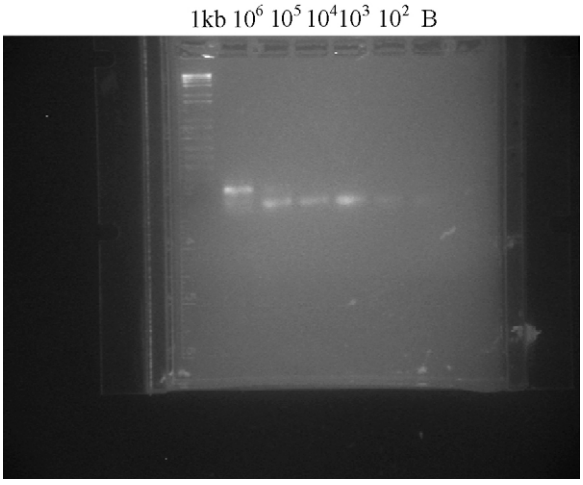

Fig. 4 – Real-time PCR products from reactions using *uid A* primers.

was similar among the five different assays. Fig. 4 shows the detection of amplified product on agarose gel.

3.4. *Specificity of the real-time PCR*

The specificity of the MB-based PCR assay relies on the selected sequence of the primer set and the probe moiety of the beacon, both of which were based on the *uid A* of *E. coli* strain coding 807–826 nucleotide gene region. [20]. All of the nine references strains of *E. coli* were positively recognized with the MB. Three non-*E. coli* strains were not positively detected. In contrast, the remaining non-*E. coli* strains did not emit fluorescence upon PCR amplification, regardless of their genetic profiles (Table 2).

3.5. *Detection of E. coli in water samples and produce*

Application of the assay to detect *E. coli* in water and produce samples was assessed by using water and spinach seeded with known amount of *E. coli* cells and further enriched on selec-

Table 2 – *E. coli* serotypes tested for the specificity of the molecular beacons

Strain	Detection	Source
E. coli JM109	+	ATCC
E. coli H7,35150	+	ATCC
E. coli 026:h7,5710	+	1
*E. coli*0103:*H7*,13C62	+	1
E. coli B6-914	+	ATCC
E. coli 0157:HM,13120	+	ATCC
E. coli 0103:H2,5702	+	ATCC
*E. coli*13180-23	+	2
*E. coli*055:H7,5906	+	ATCC
Pseudomonas putida	–	ATCC
Salmonella typhimurium	–	ATCC
Salmonella typhimurium LT2	–	ATCC

1 ARS, USDA, Ames, IA.
2 ARS, USDA, Philadelphia, PA.

Table 3 – Detection of *E. coli* in water and produce samples by real-time PCR assay

CFU mL	C_t values after enrichment	
	Water	Produce
120	12.5	12.5
12	13.5	14.5
1.2	18.5	18.5

Average of three experiments.

tive LB medium. In combination with the selective enrichment step, the real-time PCR assay could detect even 1 CFU mL^{-1} in water and produce only after 18-h enrichment (Table 3). Although many pretreatment methods are available for the enrichment of *E. coli*, the above method was selected because of its simplicity. These results demonstrate the potential of molecular beacons for rapid assay and quantification of *E. coli* in contaminated water and food.

4. Discussion

A rapid and quantitative technique to detect the presence of *E. coli* from contaminated food and water samples is essential to provide rapid diagnostic capability during emergency time. Although traditional biochemical method has been replaced by PCR, but quantification is not possible at an earliest. The MB-based PCR assay provides the possibility of real-time quantitative detection of specific target directly in the PCR tube. Reagents are mixed in one step and reactions are carried in closed tubes, thus preventing contamination. Data are recorded during each cycle and results are automatically analyzed immediately after the reaction is completed, usually within 2 h. The use of MB has previously been shown to detect very low concentrations of *Salmonella* species [21] and viruses [22]. The present MB-based assay is sensitive and specific for *E. coli*, and as little as 10 CFU mL^{-1} can be detected. Analysis of fluorescence data recorded at each annealing stage gives a clear profile of the amplification process. Detection of *E. coli* using MB is extraordinary specific, as it did not show signals with other pathogenic and nonpathogenic microorganisms tested. The most powerful aspects of MB are its capability to distinguish false positive results from PCR amplification. The critical cycle (C_t) is inversely proportional to the logarithm of the initial number of target molecules. This data can be used to formulate a structural quantification curve for detection of *E. coli*. Perhaps this will be most popular tool to overcome false positive results.

The aim of this study was to develop technique for routine testing of food product and drinking water. The water samples were always filtered incubated with nonselective peptone broth and DNA was extracted for PCR. The speed and sensitivity of bacterial pathogens detection based on PCR assay method can be greatly enhanced with the application of MB.

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