

Molecular Beacon Polymerase Chain Reaction Detection of *Escherichia coli* O157:H7 in Milk

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ABSTRACT

A fluorescently labeled oligonucleotide probe (molecular beacon) was applied to detect *Escherichia coli* O157:H7 in artificially contaminated skim milk during polymerase chain reaction (PCR) amplification of extracted DNA. The probe was designed to hybridize with a region of the *slt-II* gene coding for the A subunit and to fluoresce when the hairpin-stem conformation was linearized upon hybridization to the target sequence. The molecular beacon was incorporated into PCR reactions containing DNA extracted from artificially contaminated skim milk. The degree of fluorescence was monitored in PCR reactions containing 10^3 , 10^5 , and 10^7 CFU of *E. coli* O157:H7 per ml and was found to correlate with the amount of template in each reaction. Fluorescence significantly increased above background levels by cycle 8, 14, or 14 in reactions containing DNA from the 10^7 -, 10^5 -, or 10^3 -CFU/ml template, respectively ($P < 0.05$). Molecular beacon PCR demonstrated positive results more rapidly than traditional agarose gel electrophoresis analysis of PCR products. Use of molecular beacons allows real-time monitoring of PCR reactions, and the closed-tube format allows simultaneous detection and confirmation of target amplicons without the need for agarose gel electrophoresis and/or Southern blotting. This is the first report of a stem-and-loop molecular beacon being applied for direct detection of a pathogen in food.

Fluorescently labeled oligonucleotides, termed molecular beacons, were recently described (18, 19). These molecules are used in solution-based hybridization assays and have been applied in both clinical and environmental settings to explore gene structure, function, and prevalence (16, 19). Molecular beacons consist of a short probe sequence that is complementary to the target nucleic acid. Flanking the probe region are two GC-rich arm sequences complementary to one another. A fluorescent moiety is conjugated to the end of one arm sequence, and a quencher moiety is attached to the end of the other. In the absence of target DNA sequences, the molecular beacon assumes a hairpin conformation, with the two arm regions hybridizing and the probe sequence forming the loop. In this state, the reporter dye does not fluoresce because of the proximity of the quenching moiety. The energy of the fluorophore is transferred to the quencher and released as heat rather than being emitted as light energy (19). When target DNA is present, however, the hairpin loop structure opens, and the fluorophore and quencher separate. When the molecular beacon is in the open conformation, the fluorophore emits a detectable signal. The intensity of the fluorescent signal is directly correlated with the amount of target template present because the interaction of the molecular beacon with the target is extremely specific, and unbound beacons are completely quenched (18).

A fluorescence-based 5' nuclease (TaqMan) assay has

been developed (9) and applied to detect such food pathogens as *Salmonella* spp. (5), *Listeria monocytogenes* (1), and *Escherichia coli* O157:H7 (13, 21). This assay differs from the traditional hairpin-stem molecular beacon in that the nuclease assay exploits the 5' → 3' nucleolytic activity of Taq DNA polymerase to cleave a reporter dye, such as fluorescein, from the 5' end of a labeled linear probe that has hybridized downstream from the forward polymerase chain reaction (PCR) primer. When this cleavage occurs, the reporter dye is separated from the quencher, and fluorescence can be detected as described above for hairpin molecular beacons (9). Linear probes are not designed to have a secondary structure and depend on the specific cleavage of the 5' reporter for fluorescence to occur, not on an unfolding event, as is the case with hairpin-stem beacons.

One significant advantage of the traditional molecular beacon over the linear probe used in the 5' nuclease assay is that the hairpin stem in the former greatly enhances specificity to target DNA, even to the extent of distinguishing single-base differences (18). Furthermore, depending on the design of the hairpin stem, the 5' and 3' ends of the molecular beacon may be in such proximity to each other that a wide variety of variously colored reporter dyes and quencher moieties can be used on different probe molecules in a simultaneous assay. Each probe can distinguish between targets differing in sequence by only a single nucleotide. This allows multiple allele discrimination in the same tube, with a different color for each target region bound by the appropriate beacon molecule (18).

Fluorescence-based assays such as this allow for real-

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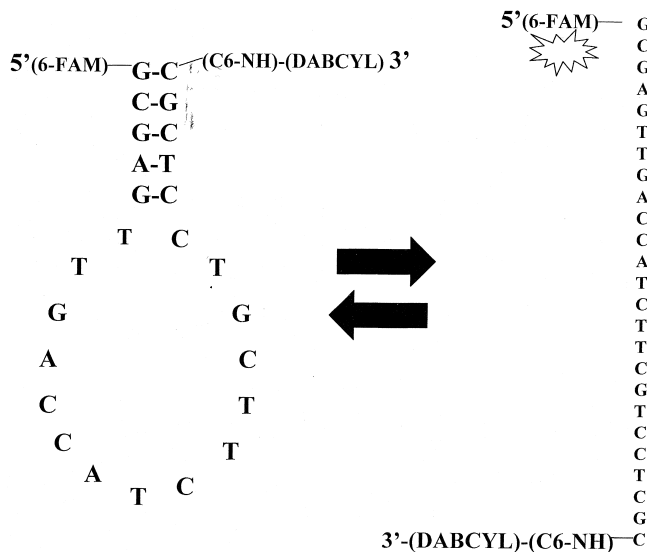
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time detection of target templates. An aliquot of each sample may be removed from the PCR at the desired time (during the annealing stage of each cycle, when probe is bound to the amplified products) and the fluorescence detected in a microplate reader (or read directly in a thermal cycler with the capability of also detecting fluorescence emission) (7). Because amplification and probe hybridization occur within the same tube, amplicon visualization by agarose gel electrophoresis and confirmation by Southern blotting is unnecessary. Multiple samples may be processed relatively easily with little risk of sample contamination. Molecular beacons therefore offer greater speed and higher specificity than traditional PCR-based assays for detection of pathogens in food.

Hairpin molecular beacons have not been used to detect pathogens in food. The goal of the present study was to evaluate and apply molecular beacon technology to the detection of *E. coli* O157:H7 in artificially contaminated skim milk.

MATERIALS AND METHODS

Preparation of artificially contaminated skim milk and extraction of DNA. *E. coli* O157:H7 ATCC 43895 (American Type Culture Collection, Rockville, Md.) was maintained in brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C by daily transfer. For detection experiments, pasteurized skim milk purchased at a local supermarket was first checked for the presence of coliforms by surface plating onto MacConkey agar (Difco). Upon confirmation that <10 coliforms per ml were present in the milk, it was inoculated with *E. coli* O157:H7 at concentrations of 10⁷, 10⁵, or 10³ CFU/ml. Ten milliliters was removed to a 30-ml glass centrifuge tube, and DNA was extracted using a modified version of a previously described bacterial concentration method (10). Briefly, the inoculated skim milk, prepared as described above and containing 1 ml of 25% sodium citrate (Fisher Scientific, Pittsburgh, Pa.), was centrifuged (without added solvent) at 8,000 × *g* for 10 min at room temperature. The pellet was resuspended in 750 μl of sterile 0.9% NaCl (Fisher) and transferred to a 2-ml microcentrifuge tube. An equal volume of titanium hydroxide suspension was added to the tube. For preparation of titanium hydroxide, a 1.3-mM solution was prepared by adding 200 ml of distilled water to 136.6 μl of titanium chloride (Sigma Chemical Co., St. Louis, Mo.). The solution was adjusted to pH 7 (±0.2) by dropwise addition of 2 M ammonium hydroxide (Weber Scientific, Hamilton, N.J.) with continuous stirring. The suspension was washed twice with 5 ml of sterile 0.9% NaCl (Fisher). During washing, the hydroxide was mixed with the sterile saline and allowed to settle for 10 min, and then the clear top phase was decanted. The final volume of the washed titanium hydroxide was approximately 200 ml. The hydroxide suspension (750 μl) was mixed with the resuspended skim milk pellet (containing bacteria) and shaken for 10 min in an elliptical motion to facilitate bacterial immobilization by the metal hydroxide. The tube was centrifuged at 1,500 × *g* for 5 min to pellet the bacteria-metal hydroxide complexes. The pellet was resuspended in 600 μl of guanidinium isothiocyanate solution (containing 4 M guanidinium isothiocyanate, 20 mM sodium acetate [pH 5.2], and 0.1 M dithiothreitol) and 100 μl of 2% Triton X-100 (all from Sigma). The sample was incubated at room temperature for 10 min and occasionally shaken to solubilize the cells. The sample was extracted with phenol/chloroform/isoamyl alcohol (Amresco, Solon, Ohio) as described above, agitated briefly, and centrifuged at



Molecular Beacon

FIGURE 1. Sequence and stem-and-loop conformation of molecular beacon used in this study. When alone in solution, the molecular beacon assumes the closed structure (left), with the quencher (DABCYL) in proximity to the fluorophore (FAM). In the presence of perfectly complementary DNA, the molecular beacon unfolds to hybridize to the target region (right). This conformational change causes the quencher and fluorophore to separate, and fluorescence occurs.

17,000 × *g* for 10 min. The DNA was precipitated from the aqueous layer with 0.1 volume of 3 M sodium acetate and 2.5 volumes of cold 100% ethanol and quantitated spectrophotometrically.

Synthesis of molecular beacon. We designed a molecular beacon containing a 5-bp hairpin region and a 15-nucleotide-long loop. The sequence and conformation are shown in Figure 1. Initial attempts at synthesizing the beacon using the method of Tyagi and Kramer (19) were unsuccessful because the yield after the coupling reactions was low and because the final product after two successive high-performance liquid chromatography steps was too impure to provide reliable hybridization and fluorescence. The molecular beacon was eventually synthesized and modified by TriLink Biotechnologies, Inc. (San Diego, Calif.), and contained a 5' fluorescein moiety and a 3' DABCYL quencher.

Thermal denaturation profile. To demonstrate that the proper secondary structure alterations were occurring, the fluorescence of the molecular beacon was measured as a function of temperature. Triplicate tubes were prepared, and each contained 100 μl of a 250-nM molecular beacon in 4 mM nuclease-free MgCl₂, 10 mM Tris-HCl (pH 7.5), and 0.1 μg of purified target DNA. Fluorescence measurements were made using a CytoFluor 4000 microplate reader (PerSeptive Biosystems, Framingham, Mass.) and black plastic, 96-well microtiter plates (Polyfiltronics, Inc., Rockland, Mass.). Excitation wavelength was 491 nm, and emission was measured at 515 nm. Quadruplicate measurements were made at every 5°C drop in temperature from 80 to 10°C, with a 2-min hold before each reading. Fluorescence intensity at each temperature was plotted on a linear scale that approaches 1. The minimum fluorescence intensity was determined from the fluorescence of buffer alone, and the maximum intensity was determined by adding an excess of target strands to the solution.

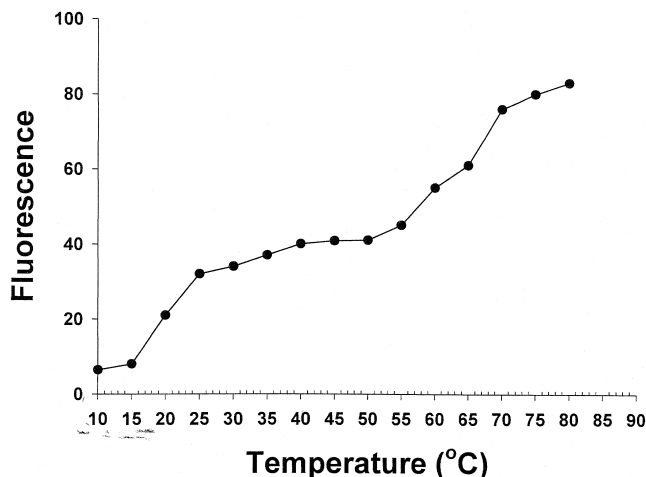


FIGURE 2. Thermal denaturation profile for molecular beacon as a function of temperature. The minimum fluorescence intensity was determined from the fluorescence of buffer alone, and the maximum intensity was determined by adding an excess target DNA to the solution. As the temperature of the solution increases, there is an increase in fluorescence of the molecular beacon due to unfolding of the stem-and-loop conformation.

Monitoring of PCR reactions. The PCR primers used in this study spanned a 400-bp region within part of the *slt-II* gene coding for the A subunit in *E. coli* O157:H7 (forward, positions 180 to 199; reverse, 560 to 580) (10). Sequences of primers were as follows: forward, 5'-TTAAATGGGTACTGTGCCT-3'; and reverse, 5'-CAGAGTGGTATAACTGCTGTC-3' (Life Technologies, Gaithersburg, Md.). The molecular beacon was designed to hybridize to positions 421 through 435 of the *slt-II* A subunit gene (internal to the PCR primers) (11). PCR reactions contained 0.5 μ M molecular beacon, 30 pmol each of the forward and reverse primers, 4.5 mM MgCl₂, 45 μ l of PCR Supermix (Life Technologies), and 2 μ l of purified DNA from skim milk inoculated with either 10⁷, 10⁵, or 10³ CFU of *E. coli* O157:H7 per ml. Control reactions included a tube containing all components except template, a tube lacking both molecular beacon and template, and a tube lacking only molecular beacon. PCR consisted of an initial denaturation step of 94°C for 2 min, 35 amplification cycles (94°C for 15 s, 54°C for 1 min, and 72°C for 2 min), and a final 7-min extension at 72°C and was completed in an Ericomp Delta Cycler I with a 96-place block (Ericomp, San Diego, Calif.). The fluorescence of PCR reactions was measured every two cycles (during the annealing stage) on a CytoFluor Series 4000 instrument by removing the entire 50- μ l reaction volume and transferring it to a black plastic microtiter plate. Fluorescence was measured at 37°C. All PCR reactions were conducted in quadruplicate, with fluorescence plotted as a function of cycle number. Fluorescence intensities were normalized so that 0 represented fluorescence in the absence of target and 1 represented the final level of fluorescence in the presence of excess target DNA. To demonstrate the success of amplification parameters, 10 μ l was removed from each reaction after PCR, analyzed on a 1% (wt/vol) agarose gel, and visualized by ethidium bromide staining and UV light illumination. Fluorescence intensities were plotted as a function of cycle number. The cycle at which fluorescence was significantly higher than the background intensity at each template concentration was determined by analysis of variance of data from triplicate reactions at each cycle (general linear model procedure, SAS software, version 6.12, SAS Institute, Cary, N.C.).

To compare the relative sensitivity of molecular beacon PCR

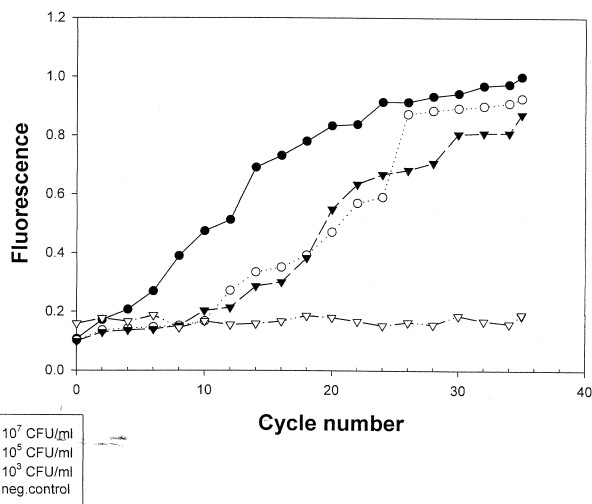


FIGURE 3. Real-time fluorescence of PCR reactions at three different template concentrations. Fluorescence intensities were normalized so that 0 represents fluorescence in the absence of target and 1 represents the final level of fluorescence in the presence of excess target DNA.

with that of traditional agarose gel electrophoresis analysis, aliquots were removed from the sample set containing 10³ CFU of target template per ml at cycles 0, 10, 15, 20, 25, 30, and 35. Ten microliters of each reaction were analyzed on a 1% (wt/vol) agarose gel and visualized by ethidium bromide staining and UV light illumination.

RESULTS

Thermal denaturation profile. The stem-and-loop structure and the unfolded conformation of the molecular beacon used in this study are shown in Figure 1. The thermal transition profile of the molecular beacon is shown in Figure 2. The free probe had little fluorescence until it was denatured by higher temperatures (>50°C), when fluorescence intensity increased. This plot confirms the formation of the hairpin stem by the arms of the molecular beacon at lower temperatures and the unfolding of the beacon as the temperature increases.

Monitoring of PCR reactions. PCR reactions containing three different amounts of target templates were monitored by molecular beacon PCR (Fig. 3). The highest concentration of template, purified from skim milk artificially contaminated with 10⁷ CFU of *E. coli* O157:H7 per ml, resulted in an immediate increase in fluorescence intensity, with values significantly higher than background values at cycle 8. PCR reactions containing the 10⁵- and 10³-CFU/ml templates had a slight lag phase. The fluorescence intensity of the latter two reactions eventually became comparable to the profile seen with high cell numbers, with fluorescence reaching levels significantly ($P < 0.05$) above background values by cycle 14, as determined by analysis of variance of mean values from fluorescence replicates (Fig. 3) (15).

To compare the relative sensitivity of the molecular beacon PCR with that of traditional agarose gel electrophoresis of PCR products, aliquots were removed from the set of tubes containing target DNA from the samples inoculat-

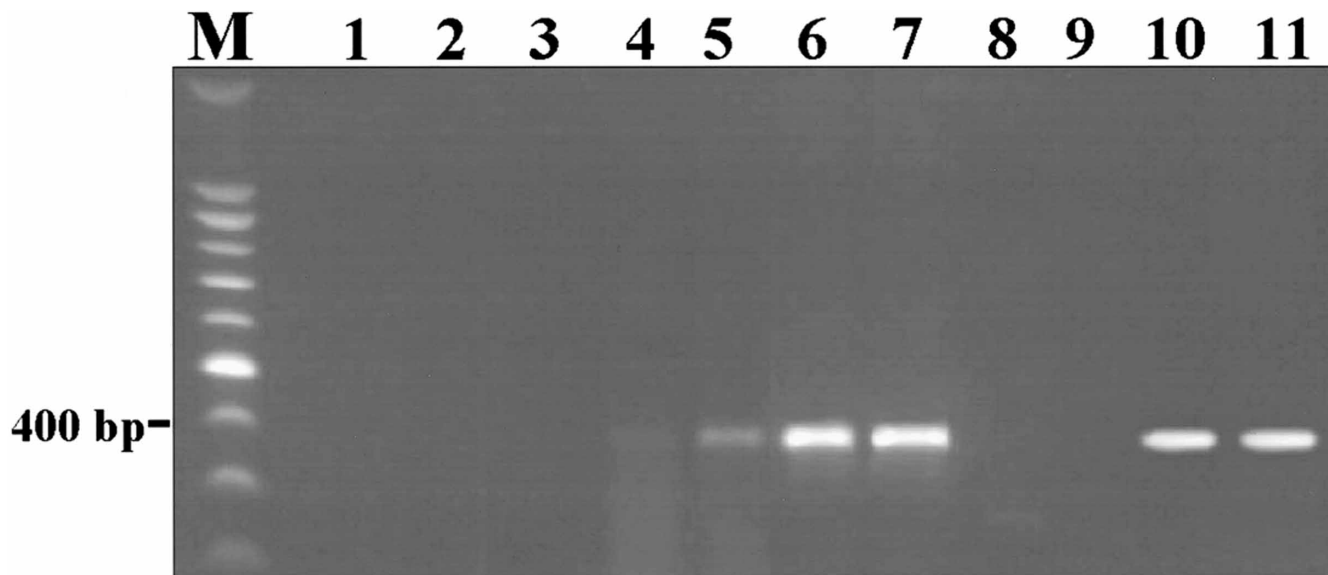


FIGURE 4. PCR products of DNA from template with a concentration of 10^3 CFU *E. coli* O157:H7 per ml. Aliquots were removed at cycles 0, 10, 15, 20, 25, 30, and 35 (lanes 1 to 7, respectively). M = 100-bp DNA ladder (Life Technologies). Lanes 8 to 11 show results for the negative control lacking only template, the negative control lacking both molecular beacon and template, the positive control containing molecular beacon and template, and the positive control lacking molecular beacon, respectively. The positive control template consisted of 1 μ l of log-phase *E. coli* O157:H7 ATCC 43895.

ed with 10^3 CFU of *E. coli* O157:H7 per ml. Results indicated that a detectable signal was present at cycle 20, although the band was always very faint. A more substantial signal was present by cycle 25 (Fig. 4).

DISCUSSION

The degree of fluorescence of the molecular beacon as a function of temperature confirmed the stem-and-loop structure of the molecular beacon at lower temperatures. Proper design of molecular beacons is important to avoid secondary structure formation within the loop while maintaining perfect complementarity to the target DNA (2). It is recommended that the quencher and fluorophore be maximally separated upon probe hybridization for optimal fluorescence (19). This occurs when the loop segment is 15, 25, or 35 nucleotides long and takes advantage of the *trans* configuration of the fluorophore and quencher in relation to the hybridized duplex (19). Our 15-nucleotide-long probe region fits these criteria.

Molecular beacon technology has been used for real-time detection of viral RNA sequences (8, 20), microbial ecology studies (16), clinical applications (12), and in surface array formats for detection of parasites (14). Linear fluorescent probes for food pathogen detection have been developed for *L. monocytogenes* (1), *Salmonella* spp. (5), and *E. coli* O157:H7 (13, 21). These assays are based on the specific cleavage of a fluorescently labeled probe by the 5' \rightarrow 3' exonuclease activity of *Taq* DNA polymerase. The study reported here was the first to use a stem-and-loop molecular beacon for direct detection of food pathogens.

By using skim milk inoculated with serial dilutions of *E. coli* O157:H7, we demonstrated the specific detection and confirmation of *slt-II* in a closed-tube format. The fluorescence assay used in this study positively detected 10^3

CFU of *E. coli* O157:H7 per ml more rapidly than did traditional agarose gel electrophoretic analysis of PCR products. This method has much greater specificity than the commercially available 5' nuclease assays because of the ability of the probe to form a stem-and-loop structure (2). Structural features of the probe may be altered to increase specificity without changing reaction conditions by designing longer stem regions. Longer stems increase the difference in melting temperatures between perfectly complementary duplexes and those containing a single nucleotide mismatch (2). Removal of PCR reaction aliquots during cycles for fluorescence measurement also allows monitoring of fluorescence intensity at various temperatures well below the 54°C PCR annealing temperature.

A number of different fluorophores and DNA-specific dyes have been used in real-time monitoring of PCR reactions, including 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), fluorescein (18), SYBR Green I (22), and ethidium bromide (4). In the study by Cano et al. (4), milk artificially contaminated with *L. monocytogenes* was subjected to a guanidinium thiocyanate/Triton X-100 cell lysis procedure, followed by DNA absorption by a silica matrix. Sensitivities as low as 10 to 100 CFU were obtained. However, ethidium bromide does not allow for specific confirmation of amplicons and may be hazardous to manipulate when large numbers of samples are being processed.

E. coli O157:H7 represents a significant health threat to consumers. Like other gram-negative pathogens, *E. coli* O157:H7 may not be detected by traditional microbiological screening if high concentrations of sublethally injured cells are present (6, 17). The inability of heat- or acid-stressed cells to grow on many selective media necessitates confirmation by molecular-based detection methods, such

as PCR (3). The advantages of real-time PCR monitoring include the ability to process many samples with speed and consistency and single-tube amplification and confirmation of target sequences. This closed-tube format reduces the chances of contamination and eliminates the need to analyze products by agarose gel electrophoresis and Southern blotting.

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