

Molecular Beacons: A Real-Time Polymerase Chain Reaction Assay for Detecting *Salmonella*

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Received November 30, 1999

Molecular beacons are oligonucleotide probes that become fluorescent upon hybridization. We developed a real-time PCR assay to detect the presence of *Salmonella* species using these fluorogenic reporter molecules. A 122-base-pair section of the *himA* was used as the amplification target. Molecular beacons were designed to recognize a 16-base-pair region on the amplicon. As few as 2 colony-forming unit (CFU) per PCR reaction could be detected. We also demonstrated the ability of the molecular beacons to discriminate between amplicons obtained from similar species such as *Escherichia coli* and *Citrobacter freundii* in real-time PCR assays. These assays could be carried out entirely in sealed PCR tubes, enabling fast and direct detection of *Salmonella* in a semiautomated format.

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Salmonella is an important food- and water-borne pathogen associated with acute gastrointestinal illnesses around the world. Disease is caused by the penetration of *Salmonella* organisms from the gut lumen into the epithelium of the small intestine and enterotoxin production. The infective dose can be as low as 15–20 cells (1). It is estimated that over 4 million cases of *Salmonella* infections and 1000 deaths occur in the United States annually. Salmonellosis accounts for 60% of all bacterial disease outbreaks in the United States (1).

Rapid methods for detecting *Salmonella* in food products and water resources have been hindered by the requirement that they be extremely sensitive. Conventional cell-culturing methods for detection and isolation involve several time-consuming, labor-intensive

selective enrichment and confirmation steps (2). Immunological assays have also been used to detect the presence of *Salmonella*. These methods generally lack specificity and can take up to 5 days to complete (3).

The advent of molecular biology has led to the developments of new tools such as the polymerase chain reaction for pathogen detection. A number of PCR assays specific for *Salmonella* have been developed (4–6). Typical detection methods of PCR products involve visual detection of an appropriately sized DNA band followed by specific hybridization with a labeled DNA probe, which could take up to 15 h and is very difficult to automate. New improvements in both the sensitivity and speed of the final detection step, preferably real-time monitoring of PCR products within 1 or 2 h, must be developed to realize this powerful PCR-based method for practical applications.

Recently, a new technique, known as molecular beacon (MB),² has been reported for the construction of probes that are useful for real-time detection of nucleic acids (7). These probes are based on single-stranded nucleic acid molecules that possess a stem-and-loop structure (Fig. 1). The loop portion contains sequence complementary to a target, and the stem is formed by annealing of two complementary arm sequences not related to the target. A fluorescent moiety is attached to the end of one arm and a nonfluorescent quenching moiety is attached to the other end. No fluorescence is produced when the two arms are in close proximity, due to the quenching action. When the probe encounters a single-strand target, it forms a hybrid with the target, undergoing a spontaneous conformation change that forces the arm sequences apart and causes fluorescence to occur. The interaction of MBs with their targets is extraordinarily specific. No increase in fluo-

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² Abbreviations used: MB, molecular beacon; CFU, colony-forming unit; MWD, Los Angeles Metropolitan Water District.

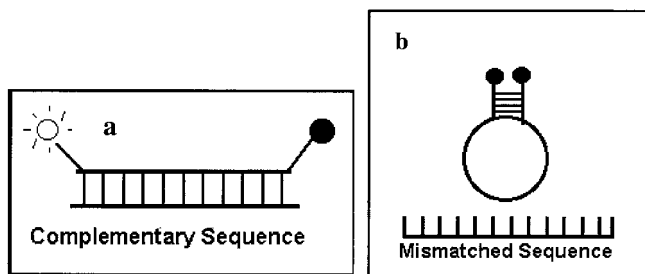


FIG. 1. (a) Molecular beacons anneal to complementary template. This results in separation of the quencher from the fluorophore and increases fluorescence. (b) Molecular beacons do not form stable hybrids with mismatched template sequences. The proximity of the quencher to the fluorophore when the stem portion is hybridized results in very low background fluorescence.

rescence is observed even in the presence of a target strand that contains only a single nucleotide mismatch (7). Since unhybridized beacons do not have to be separated, they can be included in PCR reactions, permitting the progress of the reaction to be followed in real time. In real-time PCR, MBs as well as the primers hybridize to the templates during the annealing stage. Data are collected at each annealing stage, when MBs are hybridized to the target. When the temperature is increased during the extension stage, MBs dissociate from the templates and do not interfere with polymerization. As the target strands synthesized in a reaction accumulate, the fraction of MBs that is bound to targets will increase, causing a brighter fluorescent signal. Recent studies have successfully employed MB for a variety of PCR applications (8–11).

In this paper, we described the detection of *Salmonella* by real-time PCR assay based on MB. A 122-bp region of the *himA* gene was used as the target. We demonstrate that the real-time assay is highly specific with the capability to discriminate even a single base mismatch. As few as 2 colony-forming units (CFU) can be detected with the MB-based PCR assay.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

Salmonella typhimurium LT2, *Escherichia coli* W3110, *Citrobacter freundii* ATCC 8090, and *Enterobacter cloacae* ATCC13047 were obtained from the American Type Culture Collection (ATCC). Seven *Salmonella* isolates were obtained from the Los Angeles County Sanitation Districts. These isolates were identified and labeled: (1) *Salmonella* OC1, (2) *Salmonella* Group C-Factor 7, (3) *S. choleraesuis*, (4) *S. enteritidis*, (5) *S. heidelberg*, (6) *S. newport*, and (7) *S. thompson*. All cultures were grown in Nutrient Broth (DIFCO Laboratories) overnight at 37°C. The cell count of overnight cultures was estimated by enumeration of spread

plate technique and expressed as the number of colony-forming units.

DNA Extraction

One and one-half milliliters of each culture was transferred into Eppendorf tubes and centrifuged at 1400 rpm for 2 min. The pellets were resuspended in 567 μ l of TE buffer. Thirty microliters of 10% SDS and 3 μ l of 20 mg/ml proteinase K were added to each sample. After thorough mixing, the samples were incubated in a water bath at 37°C for 1 h. After incubation, 100 μ l of 5 M NaCl was added and mixed thoroughly. Eighty microliters of a 10% CTAB (hexadecyltrimethyl ammonium chloride) in 0.7 M NaCl solution was added to each sample, followed by incubation in a water bath at 65°C for 10 min. Then 0.8 ml of chloroform:isoamyl alcohol (24:1) was added after incubation. The samples were briefly vortexed and centrifuged for 5 min. The aqueous supernatants were transferred into clean tubes. This was followed by a second extraction. Four hundred microliters of isopropanol was added and each sample was gently mixed until DNA precipitates were visible. The precipitates were pelleted by centrifuging at 1400 rpm for 10 min. The pellets were washed with 200 μ l of ice-cold 70% ethanol. After removing the ethanol, the pellets were allowed to dry in a vacuum oven at 50°C for 5 min and resuspended in 100 μ l of TE buffer. DNA recovery was confirmed by gel electrophoresis. PCR reactions were carried out with each sample to confirm the DNA quality. PCR products were verified by gel electrophoresis. This method provided good yields of PCR quality DNA and was used for subsequent DNA extractions.

Design of Molecular Beacons

MB (BHMA1) 5'-FAM-CGCTATCCGGGGCGTACC-CGTAGCG-3'-DABCYL was designed to be perfectly complementary to the *himA* gene of *Salmonella*. The target sequence contained two nucleotide mismatches, relative to the same region in the *himA* gene of *E. coli*.

MBs were synthesized by MIDLAND Certified Reagent Company. BHMA1 was labeled at the 5' end with fluorescein (6-FAM) and the quencher 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) at the 3' end. The stem sequence was selected so that they would not complement the sequences within the loop region. The length of the beacon was selected so that the annealing temperature is slightly higher than the annealing temperature of the PCR primers. The beacons were resuspended in TE buffer, stored at -20°C, and protected from light. Aliquots (16 mM) were prepared and used for subsequent studies.

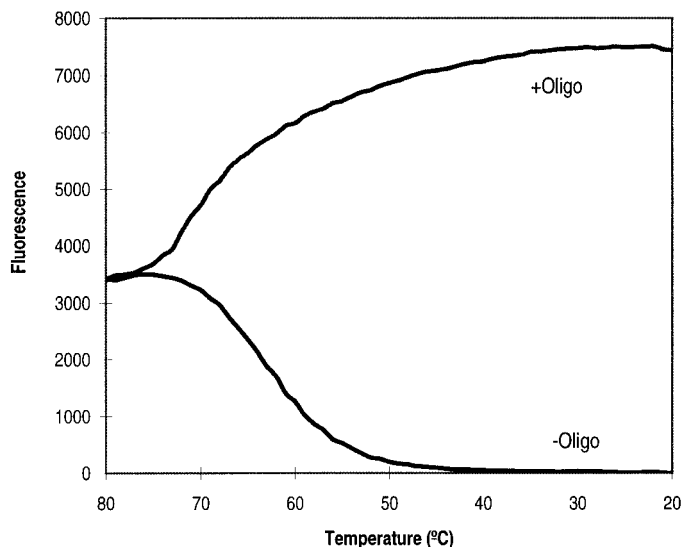


FIG. 2. Thermal denaturation profiles of the molecular beacon.

Thermal Denaturation Profiles

Thermal denaturation profile studies were conducted to determine the optimal annealing temperature for the real-time PCR. The changes in fluorescence of a 50 μ l solution containing 0.3 μ M of the beacon with or without 0.9 μ M of a perfectly complementary single-stranded oligonucleotide were measured. The samples were placed in a Perkin-Elmer ABI Prism 7700 Sequence Detector System and heated to 90°C for 5 min. The temperature was then reduced at 1°C per minute increments to 20°C. Data were recorded at each temperature interval. The optimal hybridization temperatures for each beacon were determined from these plots.

PCR Primers

Primers SHIMAF 5'-CGTGCTCTGGAAAACGGT-GAG-3' and SHIMAR 5'-CGTGCTGTAATAGGAA-TATCTTCA-3', specific for a 122-bp fragment of the *himA* gene, were previously reported by Bej (6). To amplify the *himA* region of *E. coli*, a similar pair of primers (EHIMA-forward, 5'-CGCGCTCTGGAAA-CGG-3'; and EHIMA-reverse, 5'-CGTGCTGTAATGG-GAATATC-3') were designed. The primers used were synthesized by GENOSYS. Primers were resuspended in TE buffer and stored at -20°C. Aliquots (20 mM) of each primer were prepared and used for PCR.

PCR Conditions

The Perkin-Elmer ABI Prism 7700 sequence Detection System was used for real-time analyses. Thermal cycling conditions were specified as follows: initial melting at 96°C for 10 min, followed by 40 cycles of

melting at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. Fluorescent measurements were recorded during each annealing step. At the end of each PCR run, data were automatically analyzed by the system and amplification plots were obtained. For each PCR, 5 μ l of DNA template was added to 45 μ l of PCR master (5 μ l of 1 \times PCR buffer II [Perkin-Elmer], 3.5 mM MgCl₂, 500 nM of each primer, 200 nM dNTPs, 0.5 U of AmpliTaq DNA polymerase, 0.5 mM of beacons, and 25 μ l of water) were added to the PCR tubes. The PCR buffer contained ROX (60 nmol) as the reference dye for normalization of the reactions. Any possible fluctuations in ROX signal are used to correct the sample signal.

RESULTS

Thermal Denaturation Profiles

To explore the optimal hybridization temperature between the beacon and the target sequence, the thermal denaturation profiles of the beacon and the bea-

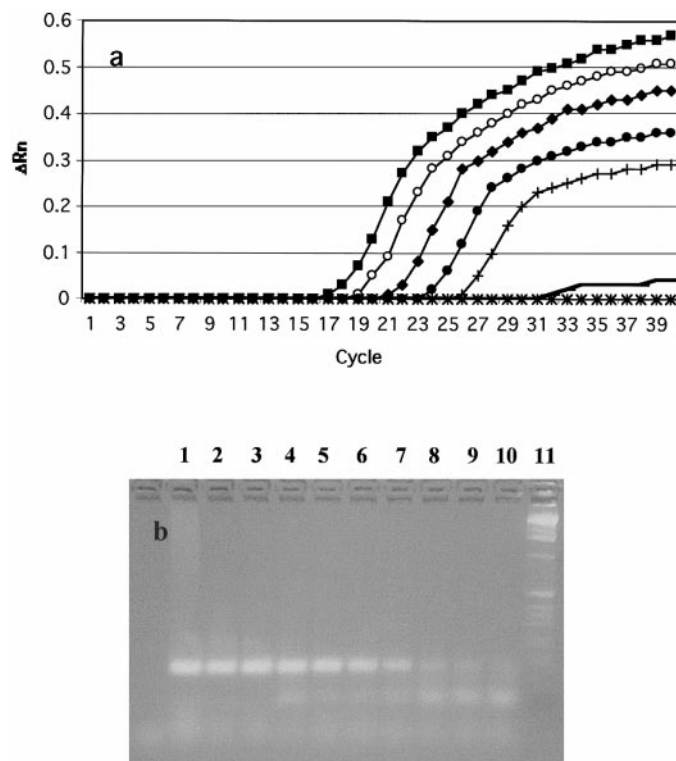


FIG. 3. (a) Real-time PCR. Amplification of the *Salmonella himA* gene, using 10-fold serial dilutions of template DNA. These experiments focused on the detection of low concentrations of *Salmonella* DNA ranging from approximately 18,000 to 1.8 copies. Symbols: ■, 18,000 CFU/PCR; ○, 1800 CFU/PCR; ◆, 180 CFU/PCR; ●, 18 CFU/PCR; +, 2 CFU/PCR; -, no template; *, buffer. (b) Gel electrophoresis. PCR reactions using 10-fold dilutions of template DNA: (1) 180,000,000 CFU/PCR, (2) 18,000,000, (3) 1,800,000, (4) 180,000, (5) 18,000, (6) 1800, (7) 180, (8) 18, (9) 2, (10) no template control, (11) DNA ladder.

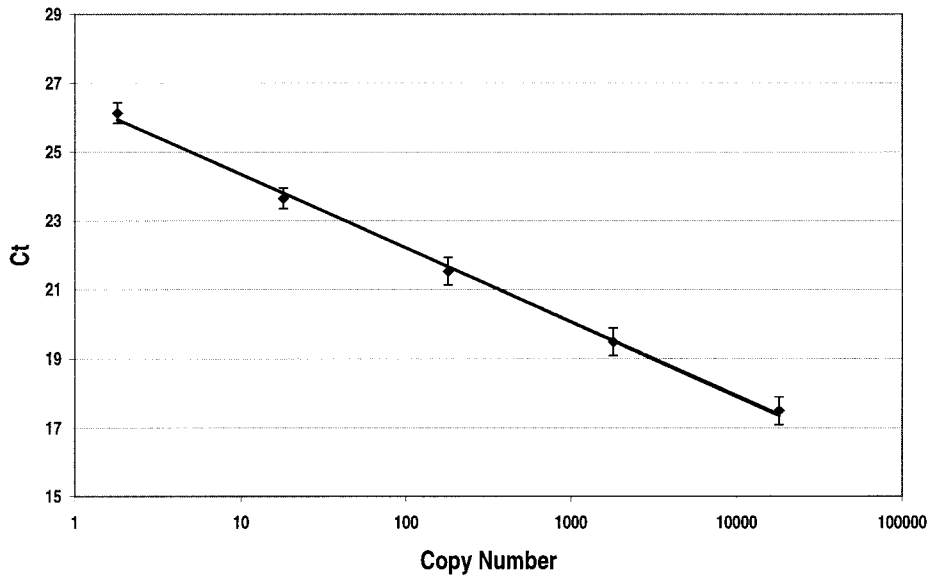


FIG. 4. Critical cycle. The critical cycles for PCR samples containing 10-fold dilutions of template DNA were plotted against the DNA concentration (cells/PCR). The results showed a linear relationship between these values, which can be used to develop a standard quantification curve.

con-target hybrid were determined using a PE/ABI 7700 fluorescence reader. At lower temperatures perfectly complementary beacon-target hybrids fluoresced brightly. As the temperature was raised, a point was reached at which the hybrids dissociated (Fig. 2). This was accompanied by a marked decrease in fluorescence. Conversely, the beacons unravel at higher temperature and exhibited a melting temperature around 62°C. In the temperature interval from 50 to 62°C, the probe-target hybrids elicited significantly stronger fluorescence than the probe alone—thus allowing the detection of target sequence at these temperatures. Initial studies with real-time PCR were conducted between this temperature range and the highest sensitivity was obtained at 57°C. This temperature was chosen as the annealing temperature for subsequent real-time PCR studies.

Real-Time PCR Analysis

The ability of MB to detect *Salmonella* in real-time PCR assays was investigated. Different initial concentrations of template DNA isolated from *S. typhimurium* were used. DNA extracts were diluted with water, in 10-fold serial dilutions. Five microliters of template solution was added to each 50 μ l of PCR reaction. Colony-forming unit was used to represent the initial template concentrations. A no-template-control, in which sterile buffer was substituted for template DNA, was used in each experiment. This control was used to subtract any fluorescence that is not directly related to amplification. Figure 3a shows the

normalized fluorescent measurement vs the PCR cycle. This plot clearly shows the progression of the amplification reaction in each sample. As few as 2 CFU could be detected by the real-time PCR assay (Fig. 3a). Results were also verified by gel electrophoresis (Fig. 3b). Although the intensity of the amplified fragments correlated to the initial amount of template DNA, the visual detection limit was noticeably lower at approximately 180 CFU.

The critical cycle (C_t), defined as the cycle at which a significant increase in fluorescence is first recorded, increases as the initial number of template molecules DNA decreases (12). This was expected because samples containing low concentrations of template DNA would require more PCR cycles to replicate enough copies to produce a significant fluorescent signal. Thus, C_t can potentially be used to quantify the input target concentration. For our real-time PCR assays, the C_t values decrease linearly with increasing target quantity up to 18,000 CFU (Fig. 4). At higher initial target concentrations, the endpoint plateau at a lower fluorescent value than would be expected. This phenomenon has been suggested to be attributable to late cycle inhibition (12).

The variability between different sample preparations was investigated. Five different sets of real-time PCR assay were performed with initial target ranging from 1 to 18,000 CFU. Each amplification was performed in duplicate. Comparison of C_t values for each duplicate sample showed minimal variation, indicating that the PCR amplification is highly reproducible.

TABLE 1
Variation in Real-Time PCR Assay

	C_t				
	2 CFU	18 CFU	180 CFU	1800 CFU	18,000 CFU
Run 1	26.56	24.21	21.85	19.49	18.02
Run 2	26.57	23.70	21.53	19.49	17.50
Run 3	26.14	23.65	21.61	19.43	17.56
Run 4	26.30	24.34	22.10	20.20	18.30
Run 5	26.24	24.04	22.49	20.28	18.21
Mean \pm SD	26.36 \pm 0.17	23.99 \pm 0.27	21.92 \pm 0.35	19.78 \pm 0.38	17.92 \pm 0.33

Note. C_t for each run is the average of two independent samples.

Comparison of C_t values of the five different sets of assay also revealed little variability (Table 1). More importantly, the rate of fluorescent change at each target concentration was similar among the five different assays.

Specificity of the Real-Time Assay

To demonstrate the beacon's specificity for *Salmonella*, primers were designed to specifically amplify the same region of the *himA* gene in *E. coli*. PCR products were examined through gel electrophoresis. A 122-bp product was amplified from both the *E. coli* and *Salmonella* templates. However, real-time PCR studies showed that the MB, specific for *Salmonella*, failed to detect the *E. coli* product (Fig. 5). Because there are two base pair mismatches within the *E. coli* amplicons, the MBs form unstable hybrids that dissociate during the annealing stage, at which fluorescence is recorded—thus demonstrating the ability of MB to discriminate between very similar sequences.

A recent PCR study conducted by the Los Angeles Metropolitan Water District (MWD) on 50 non-*Salmo-*

nella species using the SHIMA primers and a *Salmonella*-specific DNA probe showed negative results for all species tested except for *C. freundii* and *E. cloacae*, which produced false-positive results from both the primers and the probe (R. DeLeon, personal communication). To confirm the specificity of the beacons, DNA isolated from *C. freundii* and *E. cloacae* were obtained from MWD and subjected to the real-time PCR assay. DNA bands of 122 bp were detected for both *C. freundii* and *E. cloacae* (Fig. 6a). However, neither one of these organisms was detected by the beacons (Fig. 6b).

The ability of the beacons to target other *Salmonella* species rather than *S. typhimurium* was tested. Seven different *Salmonella* species isolated by the LA County Sanitation District were analyzed by the real-time PCR assay. All seven targets produced strong fluorescent response ($\Delta R_n > 0.5$ after 40 cycles). The presence of the PCR-amplified *himA* targets was also confirmed by gel electrophoresis (data not shown).

DISCUSSION

The MB-based PCR assay provides the possibility of real-time quantitatively detection of specific target directly in the PCR tube. The reported assay could detect as few as 2 CFU of *S. typhimurium* and also cover a wide dynamic range of detection, all in a real-time manner. Detection of *Salmonella* using MB is also extraordinarily specific. Even in the presence of a similar *himA* sequence from *E. coli*, no increase in fluorescence was detected. Perhaps the most powerful aspects of MB is the capability to distinguish false-positive results from PCR amplification. For example, a 122-bp amplicon was detected from both *C. freundii* and *E. cloacae* using the *Salmonella* primers. Even the use of a linear hybridization probe failed to provide the correct identity. However, no response was produced from both strains in the real-time PCR assay, again demonstrating the highly specific nature of MB.

Analysis of fluorescence data recorded at each annealing stage gives a clear profile of the amplification

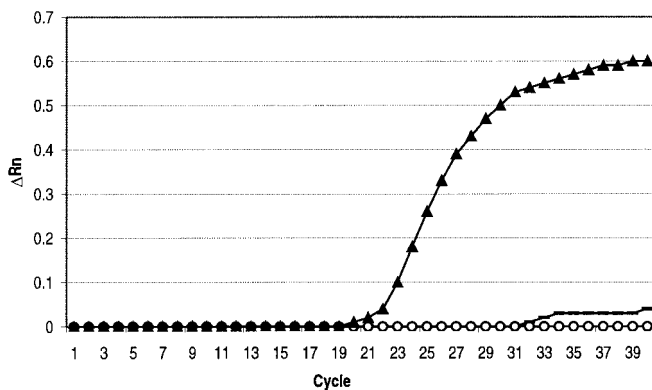


FIG. 5. Target specificity. Real-time PCR amplification of *E. coli* and *Salmonella himA* genes. Beacons specific for *Salmonella* failed to detect the *himA* homologue in *E. coli*. Symbols: \circ , *E. coli*; \blacktriangle , *Salmonella*; —, no template control.

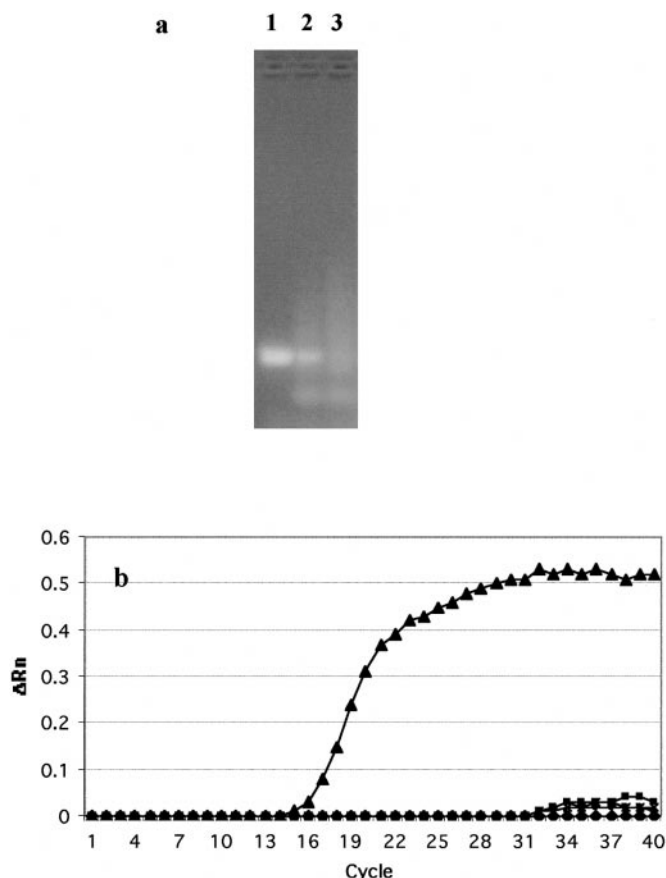


FIG. 6. (a) Gel electrophoresis. Real-time PCR products from reactions using SHIMA primers and DNA from: (1) *Salmonella*, (2) *C. freundii*, and (3) *E. cloacae*. (b) Real-time PCR assay. Even when amplification products were obtained from organisms other than *Salmonella*, they were not detected by the *Salmonella*-specific beacons. ■, no template control; ▲, *Salmonella* + *C. freundii*; ●, *E. cloacae*; ◆, buffer.

process. The number of amplification cycles needed before a significant increase in fluorescence from target-beacon hybrids is detected (critical cycle) can be used to quantify the initial number of template molecules in the reaction. The critical cycle is inversely proportional to the logarithm of the initial number of target molecules. These data can be used to formulate a standard quantification curve for the detection of *Salmonella*. One additional benefit of using the C_t values for quantitation is that a much larger assay range is permitted than directly using total fluorescent emission, which has a dynamic range of approximately 1000.

Real-time PCR detection of *Salmonella* has also been reported using the "Taqman" probes (2), which are linear probes without the hairpin stems. The most critical problem associated with this technology is the distance between the dye and the quencher (13). If they are close together, the likelihood of cleaving the dye

from the quencher drastically decreases, even though a lower background fluorescence is observed. On the other hand, placement of the quencher at the 3' end of the probe increases the signal during PCR assays, but at the expense of a much higher background. As a result, the overall increase in fluorescence (corresponds to sensitivity) caused by the cleavage of the probe during PCR is significantly reduced. Furthermore, because of the hairpin stems, MBs are far more specific than linear probes with the same sequence.

MBs labeled with different color fluorophores can be used simultaneously for sequence analysis, detection of point-mutations, and multiplex PCR for identification of different genes. This unique property has been exploited for a variety of applications, including the detection of point mutations in the methylenetetrahydrofolate reductase gene (8), analysis of an 81-bp region of the *Mycobacterium tuberculosis rpoB* gene for mutations that confer resistance to the antibiotic rifampin (9), ecological studies with ruminal bacteria (14), and real-time assay for HIV viruses (11). MB assays are simple and fast. Reagents are mixed in one step and reactions are carried out in closed tubes, thus preventing contamination. Data are recorded during each cycle and results are automatically analyzed immediately after the reaction is completed, usually 2–3 h. Due to their high specificity and high sensitivity, MB can be effectively incorporated into real-time PCR assays and provide a quick and accurate method for detection of specific nucleic acid sequences in homogeneous solutions. We envision that the speed and sensitivity of bacterial pathogen detection based on PCR-assay method can be greatly enhanced with the application of MB.

ACKNOWLEDGMENTS

This work was funded by the Water Environment Research Foundation (WERF) through a U.S. Environmental Protection Agency Cooperative Agreement No. CR825237-01. We thank Tammy Chen for her help on DNA sequencing and real-time PCR assays. We also thank the Los Angeles County Sanitation Districts and the Metropolitan Water District for providing strains and DNA samples.

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