Detection of *Listeria monocytogenes* in Fresh Produce Using Molecular Beacon—Real-time PCR Technology

S.H. LIMING, Y. ZHANG, J. MENG, AND A.A. BHAGWAT

ABSTRACT: The capability of an assay to detect *Listeria monocytogenes* from artificially inoculated fresh-cut produce such as cantaloupe and mixed salad was demonstrated. An oligonucleotide probe that becomes fluorescent upon hybridization to the target DNA (Molecular Beacon, MB) was used in a real-time polymerase chain reaction (PCR) assay. As few as 4 to 7 colony-forming units (CFU) of *L. monocytogenes* per 25 g of artificially contaminated produce could be detected. A comparison of 2 commercially available kits using MB-PCR (iQ-Check, Bio-Rad Laboratories) and conventional PCR (BAX, Dupont Qualicon) was performed on artificially inoculated produce. The time required to detect *L. monocytogenes* (from produce to PCR) was considerably shorter for the iQ-Check protocol (approximately 26 h) compared with the BAX-PCR (approximately 52 h). The iQ-Check protocol was also used to confirm the identity of the *L. monocytogenes* isolates obtained during a microbiological screen of conventional and organic leaf lettuce and alfalfa sprout samples from local supermarkets. The iQ check protocol was successful in differentiating *L. monocytogenes* isolates from other *Listeria* spp. such as *L. welshimeri*, *L. innocua*, and *L. ivanovii*. This is the 1st report of the application of the MB probe being used for real-time detection of *L. monocytogenes* in whole and fresh-cut produce.

Keywords: microbial food safety, ready-to-eat produce, foodborne pathogens

Introduction

The detection of *Listeria* species by regulatory agencies is still primarily based on traditional microbiological culture methods that may take several days to complete. The incidence of foodborne infections caused by *Listeria monocytogenes* continues to be a problem in the United States (Burnett and Beuchat 2001; Gombas and others 2003). Several outbreaks of listeriosis in the United States and Canada have been linked to fresh produce (Francis and others 1999; Burnett and Beuchat 2001; Sewell and Farber 2001). The results of surveys to determine the prevalence of *L. monocytogenes* in various foods indicated that the highest prevalence rate (4.7%) was observed for seafood salads, whereas a relatively lower prevalence rate was reported for deli salads and bagged salads at 2.4% and 0.74%, respectively (Gombas and others 2003).

Most of our knowledge of routes of foodborne transmission of *Listeria* and other pathogens has been acquired through the study of epidemiological data from various prevalence studies and outbreak investigations (Farber and Peterkin 1991; Donnelly 2001; Zhao and others 2001). Therefore, determining the precise source of contamination is crucial when devising strategies to reduce future outbreaks. However, only 2 of the 27 outbreak investigations on fresh produce clearly identified a point of contamination, which underscores the importance and need for rapid and accurate pathogen identification methods (NACMCF 1999).

Most polymerase chain reaction (PCR) assays, although rapid and sensitive, rely on visualizing the amplification product by ethidium bromide or SYBR® Green staining of agarose or polyacrylamide gels (Golsteyn-Thomas and others 1991; Shearer and others 2001; Cocolin and others 2002). More recently, modifications have been introduced whereby PCR was monitored in "real time" by the addition of a fluorogenic dye such as SYBR Green (Bhagwat 2003, 2004). Although this modification enabled the simultaneous and rapid detection of 3 foodborne pathogens in a high-throughput format, the fluorogenic reporter dye lacked specificity for the desired target molecule. However, a new fluorogenic PCR-based format, Molecular Beacon probe PCR (MB probe PCR) has been developed that uses an internal fluorogenic probe that is specific to the target gene (Chen and others 2000; Hoorfar and Radstorm 2000; Liming and Bhagwat 2004). During the PCR assay, the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety (Tyagi and Kramer 1996).

The successful application of fluorogenic as well as conventional PCR assays to food samples, particularly to fresh produce, has been hindered by the presence of inhibitory compounds of plant origin that interfered with PCR biochemistry, resulting in false-negative data (Shearer and others 2001; Bhagwat 2003; Liao and Shollenberger 2003). In this article, we have evaluated and compared the MB probe PCR method (iQ-Check *L. monocytogenes* kit from Bio-Rad Laboratories, Hercules, Calif., U.S.A.) with the BAX PCR (Qualicon Inc, Wilmington, Del., U.S.A.) and conventional microbiology methods (Johnson 1998) to detect *L. monocytogenes* from fresh and fresh-cut produce (cantaloupe and mixed salad), which was artificially contaminated at the level of 4 to 7 colony-forming units [CFU]/25 g. The iQ-Check protocol was also used to confirm the identity of the *L. monocytogenes* isolates and to differentiate from other *Listeria* spp. (*L. welshimeri, L. innocua,* and *L. ivanovii*) obtained during a microbiological screen of conventional and organic

MS 20040223 Submitted 4/9/04, Revised 5/25/04, Accepted 6/9/04. *Authors Liming and Bhagwat are with Produce Quality and Safety Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Dept. of Agriculture, 10300 Baltimore Ave., Bldg. 002, BARC-W, Beltsville, MD 20705-2350. Authors Zhang and Meng are with Dept. of Nutrition and Food Science, Univ. of Maryland, College Park, Md. Direct inquiries to author Bhagwat (E-mail: bhagwata@ba.ars.usda.gov).*

leaf lettuce and alfalfa sprout samples from local supermarkets. This is the 1st report of the application of the MB probe being used for real-time detection of *L. monocytogenes* in fresh-cut produce.

Materials and Methods

Experimental design

This study examined the sensitivity and efficacy of 2 PCR-based methods (iQ-Check *L. monocytogenes* kit, Bio-Rad Laboratories and BAX-PCR, *L. monocytogenes* kit). The data from the PCR protocols were compared with the traditional culture methods using pure cultures of *L. monocytogenes* as well as other *Listeria* spp., such as *L. welshimeri, L. innocua,* and *L. ivanovii*. Each experiment was performed at least twice, and each experiment contained 3 subsamples per produce. Artificially inoculated fresh produce (cantaloupe and prepacked mixed salad) at a low inoculum dose of 4 to 7 CFU per 25 g was used in this investigation.

Bacterial strains and media

L. monocytogenes ATCC 13932, *L. welshimeri* ATCC 35897, *L. innocua* ATCC 33090, and *L. ivanovii* ATCC 19119 were used as reference strains in this study. Cultures were routinely started from freezer stocks for growth on tryptic-soy agar (TSA) medium (Difco Laboratories, Detroit, Mich., U.S.A.). After overnight incubation at 37 °C, a single colony was selected and inoculated into 10 mL tryptone-soya broth in a 125-mL Erlenmeyer flask. Cells were grown for 20 to 22 h at 37 °C with shaking at 200 rpm (Lab-Line Instruments, Melrose Park, Ill.) to obtain stationary-phase cultures. The cells were harvested by centrifugation at 4000 × *g* for 10 min (Eppendorf 5410R, Hamburg, Germany), washed once with 3 volumes of saline (0.85% NaCl), and suspended in saline at a cell density of 10^9 cells/mL. Cells were further diluted in saline to achieve the desired cell density. Final cell numbers were confirmed by determining viable cell counts on TSA plates.

Inoculation of fresh produce with *L. monocytogenes*

Whole cantaloupes and prepacked mixed salad (made up of approximately 80% leaf lettuce, 10% red cabbage, and 10% carrot by weight) were obtained from local grocery stores. The cantaloupes were brushed and rinsed with warm tap water, sprayed with 70% ethanol, and allowed to dry in a microbiological hood before cutting into small portions. Fresh produce (25 g) was inoculated with approximately 4 to 7 cells in 50 μL saline using either pure culture of *L. monocytogenes* and processed within 10 min for preenrichment. For each experiment, non-inoculated produce was used as a control and was found to be free of *L. monocytogenes* by PCR as well as culture methods.

Enrichment procedures

Experimentally inoculated samples were subjected to 2 different enrichment protocols per the recommendations specific for the 2 PCR methods. For the BAX-PCR, the manufacturer's recommended enrichment protocol was followed (Shearer and others 2001). Briefly, 25 g of produce was combined with 225 mL of Demi-Fraser broth (Difco Laboratories) in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender (Seaward, London, U.K.). After stomaching, the samples were incubated for 24 ± 2 h at 30 °C. The next day, the samples were pummeled again for 2 min in a stomacher, and 0.1 mL of the slurry was transferred to 9.9 mL of prewarmed (35 °C) MOPS (3-N-Morpholino propanesulfonic acid)-buffered *Listeria* enrichment broth and further incubated for 24 ± 2 h at 35 °C. After the 2nd enrichment period, a 5 μL sample was taken for DNA isolation.

To analyze artificially contaminated produce samples with the iQ-check *L. monocytogenes* kit, a 1-step enrichment protocol recommended by the manufacturers was followed. Briefly, 25 g of produce was combined with 225 mL of ½-strength Demi-Fraser broth in sterile stomacher bags and pummeled for 2 min in a stomacher 400 Lab Blender. The samples were incubated for 24 ± 2 h without shaking at 30 °C. After the incubation period, 1 mL of the sample was withdrawn from the top without disturbing the food debris and processed for DNA isolation.

DNA extraction procedures

For the BAX-PCR method, 5 μL of the sample from the secondary enrichment broth was transferred to an eppendorf tube containing 200 μL of BAX cell lysis reagent. DNA was then isolated according to the manufacturer's protocol. Briefly, cells were incubated in the BAX-lysis buffer for 1 h at 55 °C and then for 10 min at 95 °C (to inactivate the proteases in the lysis buffer). For the iQ-Check protocol, 1-mL samples from the half-strength Fraser broth were centrifuged for 12000 × *g* for 5 min; the pellet was suspended in 200 μL of lysis reagent (iQ-Check-*L. monocytogenes*, Bio-Rad Laboratories) and vortexed. Lysis was carried out by incubating the suspension at 100 °C for 15 min.

For isolating DNA from *L. monocytogenes* and *Listeria* spp., 10-fold serial dilution of known quantities of viable cells (10^9 to 10^5, measured as CFU/mL) were mixed in individual tubes containing lysis buffer and used as standards in the respective PCR assays.

Detection of *L. monocytogenes* by PCR

For BAX-PCR, 50 μL of DNA preparations were transferred to *L. monocytogenes* BAX-PCR tubes containing the lyophilized pellet of all PCR reagents except target DNA. The thermocycler was programmed for 94 °C for 2 min, (94 °C for 15 s, 70 °C for 3 min) × 38 cycles. Fifteen microliters of loading dye was added and mixed to each PCR tube. The PCR mixture was processed by performing agarose (2.0%) gel electrophoresis and the 400 base pair target DNA was visualized by ethidium bromide staining (Shearer and others 2001).

In some experiments, a modified protocol of the BAX-PCR that enables real-time detection of amplified target DNA was also performed using a pure culture of *L. monocytogenes* (Bhagwat 2003).

For the iQ-Check PCR protocol, samples were examined in duplicate at 2 concentrations of template DNA. For each sample, 5 μL of 1:10 and 1:25 diluted DNA were mixed with 40 μL of amplification mixture and 5 μL of fluorogenic oligonucleotide molecular beacon probe labeled with Texas Red at the 5′-end and DABSYL at the 3′-end as the quencher. The fluorogenic MB-probe from the iQCheck-kit (Bio-Rad Laboratories) targets an internal section of the PCR product of the *hlyA* (sulfhydryl-activated hemolysin) gene specific to *L. monocytogenes*, also known as listeriolysin (Gaillard and others 1986; Menguad and others 1988; Bessesen and others 1990). To monitor successful DNA amplification in each reaction tube, the kit provides a synthetic DNA (at suboptimal concentration) as a part of the reaction mixture that works as an "internal control." This control DNA was amplified with a specific probe at the same time as the *L. monocytogenes* target DNA sequence and detected by a 2nd fluorophore (FAM). The thermocycler was programmed for 50 °C for 2 min, 95 °C for 5 min (95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s) × 50 cycles, and 72 °C for 5 min. Data were collected after each annealing step (that is, after 55 °C for 30 s) using an excitation and emission wavelength of 575 nm and 620 nm for Texas Red (for the target DNA). Concurrently, the data for the "internal control" DNA amplification was also monitored using an excitation wavelength of 490 nm and an emission wavelength of 530 nm for FAM as specified by BioRad Laboratories.

Detection of *L. monocytogenes* by conventional microbiology methods

The protocol recommended for *L. monocytogenes* by the U.S. FDA Bacteriological Analytical Manual was followed (Johnson 1998). After an enrichment step, the broth was streaked on Bacto-Modified Oxford agar (MOX) and Bacto-PALCAM agar (Difco Laboratories) to identify *L. monocytogenes*.

For the isolation of *L. monocytogenes* from fresh retail organic and conventional produce (alfalfa sprouts and romaine lettuce), after initial enrichment (½-strength Fraser broth, 24 h, 30 °C), samples were plated on Oxford agar and *L. monocytogenes* plating medium (LMPM; BCM, Inc., Naperville, Ill., U.S.A.) and incubated for 48 h at 30 °C and 35 °C, respectively. Five colonies typical of *L. monocytogenes* on LMPM were selected and streaked onto *L. monocytogenes* confirmatory medium (LMCM, BCM, Inc.). Presumptive *L. monocytogenes* colonies were examined using the Gram stain and catalase test, and API *Listeria* strips (bioMerieux, Hazelwood, Mich., U.S.A.). The isolates were further confirmed using an iQCheck- *L. monocytogenes* PCR kit by isolating DNA from a single colony from LMCM plate into 200 μL of iQcheck lysis buffer as described previously.

Results and Discussion

Detection of *L. monocytogenes* by conventional PCR and molecular beacon real-time PCR

We examined the sensitivity of the MB probe, real-time PCR methodology with reference to conventional PCR. As a 1st step, a pure culture of *L. monocytogenes* ATCC 13932 grown to stationary phase in tryptone-soya broth was serially diluted 10-fold in saline; viable counts were confirmed by growing aliquots on tryptone-soya agar medium. DNA isolation was performed from samples with varying quantities of cells ranging from 10^8 CFU/mL to 10^2 CFU/mL. A no-template-control, in which sterile saline was substituted for template DNA, was used in each experiment. This control was used to subtract any fluorescence that was not directly related to amplification. Figure 1 shows the normalized fluorescence measurement from the MB probe versus the PCR cycle collected in real-time for ATCC 15313 serotype strain 1/2a. Fluorescence from the MB-probe increased as the target DNA (*hlyA* gene) accumulated at the end of each successive round of amplification. All data collected during the 30-s annealing cycle were used in the analysis and for quantifying the amplification of target DNA. Using the MB-probe with real-time detection, it was possible to detect up to 10^2 to 10^3 CFU of *L. monocytogenes*.

The conventional PCR method was performed using reagents specific to *L. monocytogenes* in the BAX-PCR kit, and the amplification product was examined after agarose gel electrophoresis. Using this protocol, the detection limit appears to be 10^3 CFU/PCR assay when the agarose gels were stained with ethidium bromide and examined under UV light for the presence of 400 bp PCR product (data not shown). Recently, we modified this AOAC-approved PCR protocol to achieve real-time detection by including a fluorescent reporter dye, SYBR Green I (Bhagwat 2003) in the PCR assay. Using this modified protocol, it was possible to collect data in real time. However, as reported, there was no change in the sensitivity of the assay, and the lower detection limit remained at 10^3 CFU/PCR assay.

Quantitation of *L. monocytogenes* based on the target DNA amplification

The threshold cycle (C_t) is defined as the cycle at which a significant increase in fluorescence is 1st recorded. The C_t value increases as the initial number of the available template molecules decreases. Thus, C_t values can potentially be used to quantify input target molecules. For MB real-time PCR assays using *L. monocytogenes*, C_t values decreased linearly with increasing target quantity 10^1 to 10^4 CFU per PCR assay (Figure 2), with a correlation coefficient of 0.991 for ATCC 15313 serotype strain 1/2a. The amplification plot of the experiment generated a slope of –4.23, corresponding to a 72.3% efficiency of the PCR assay, using the formula, E (efficiency) = $(10^{-1/\text{slope}}) - 1$ (Higuchi and others 1993). Similarly amplification plots for ATCC 19114 serotype strain 4a and ATCC 13932 sero-

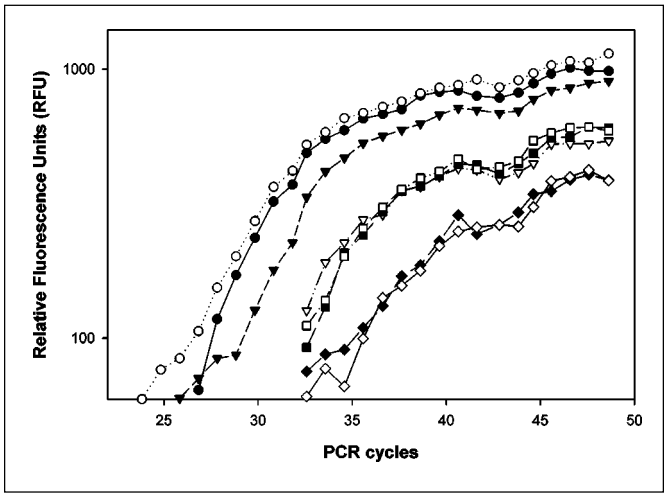

Figure 1—Amplification plot of a 10-fold serial dilution series of *Listeria monocytogenes* ATCC 19114. Sample replicates (open and closed symbols) with cells per assay 10^2 (△, ◆); 10^3 (□, ■); 10^4 (▽, ▼); and 10^5 (○, ●) using a molecular beacon probe and real-time PCR assay. Real-time detection was done by measuring the fluorescence of Texas Red during the annealing step of each PCR cycle (x axis). Relative fluorescence units (RFU) are plotted on the y axis.

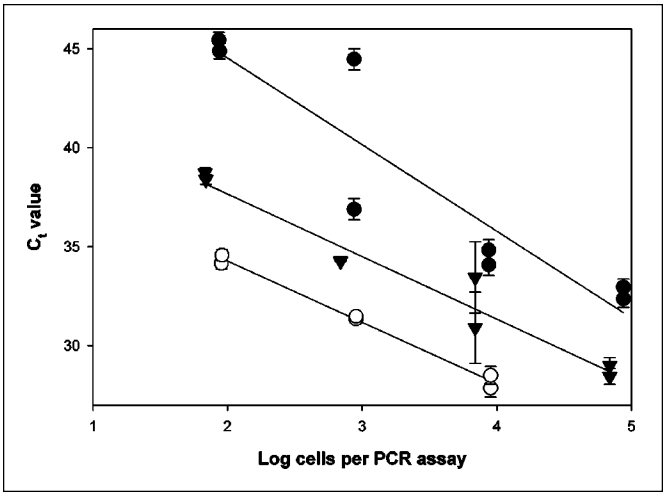

Figure 2—Standard curve for a 10-fold serial dilution series of *Listeria monocytogenes* serotype 4a (ATCC 19114), ○; serotype 4b (ATCC 13932), ●; and serotype 1/2a (ATCC 15313), ▽. Approximately 10^5 to 10^2 cells per assay were used (in duplicate), and data are plotted as the threshold cycle (C_t) on the y axis. The target copy number per assay is on the x axis.

Table 1—Interpretation of sample results by MB PCR iQCheck *Listeria monocytogenes* assay

Sample	*L. monocytogenes* detection (Texas Red)	Internal control DNA detection (FAM)	Interpretation
Negative control	C_t = N/D[a]	C_t > 20	Experimental-set up free of *L. monocytogenes*
Positive control	C_t > 10	Not significant	PCR kit components in good condition
Negative test	C_t = N/D	C_t > 20	Absence of *L. monocytogenes*, PCR reaction successful
Positive test sample	C_t > 10	Not significant	Positive identification for *L. monocytogenes*
False-negative test sample	C_t = N/D	C_t = N/D	Inhibition of PCR reaction

[a]N/D = none detected.

Table 2—Comparative analyses of detection frequencies of *Listeria monocytogenes* from artificially inoculated fresh produce by 2 PCR methods

		Detection of *L. monocytogenes*					
		One-step enrichment (24 h)			Two-step enrichment (24 h × 2)		
			Molecular Beacon PCR			Conventional PCR	
Type of fresh produce	Contamination level (CFU/25 g)	Detection frequency (selective media)	Detection frequency (iQ-Check)	Quantitation (C_t value[a])	Detection frequency (selective media)	Detection frequency (BAX-PCR)	Quantitation
Cantaloupe	5.3 ± 3.1	6/6	6/6	17.74 ± 0.28	6/6	5/6	NA[b]
Mixed salad	3.8 ± 1.7	9/9	8/9	19.16 ± 2.02	6/6	1/1	NA

[a]C_t value is defined as the cycle at which a significant increase in fluorescence is 1st recorded.
[b]CFU = colony-forming unit; NA = not applicable.

type strain 4b had correlation coefficients of 0.991 and 0.839, respectively (Figure 2). Modification of the BAX-PCR method with the inclusion of SYBR Green I dye allowed the quantitative detection of the amplified target in the range 10^3 to 10^6 CFU per PCR assay and had a high correlation coefficient of 0.98 (data not shown).

Interpretation of C_t values using iQCheck-MB PCR. In the iQCheck MB-PCR method, amplification of the internal control DNA greatly facilitated differentiating samples with PCR-inhibitory compounds (that is, false negative) and samples truly lacking the template DNA (that is, absence of the pathogen) (Table 1). When no C_t value was obtained for Texas Red (*hlyA* beacon probe), then the interpretation of the result relied on amplification of internal control DNA (C_t value obtained for FAM). The control DNA template, beacon probe, and primer concentrations are kept intentionally several-fold lower compared with those of *hlyA*. This parity in concentration enables detection of the control DNA (particularly in the absence of *hlyA* template) at a C_t value of approximately 30 (Table 1). The sample was considered negative for *L. monocytogenes* if there was no C_t value for Texas Red and the C_t value for FAM was > 20. If the C_t value for FAM was also undetected, then it was not possible to interpret the result, and such data probably indicate an inhibition of the PCR reaction. For positive *L. monocytogenes* samples, the C_t value for FAM is not significant because concentration of the control DNA template could get several-fold lower compared with the *hlyA* target DNA as PCR cycles progress.

Evaluation of detection limits using artificially contaminated produce

The ability to detect *L. monocytogenes* from fresh produce was tested using artificially contaminated cantaloupe and mixed salad. The 2 PCR assays and conventional microbiological protocols were performed in parallel for each produce sample (Table 2). For each experiment, non-inoculated produce was used as a control and was found to be free of *L. monocytogenes* by PCR as well as culture methods. MB probe real-time PCR assays and microbial selective media (Bacto-MOX and Bacto-PALCAM agar) were able to detect contamination of *L. monocytogenes* from both produce types and at a low level of contamination in all samples tested (approximately 4 CFU 25/g produce). BAX-PCR assays were also able to detect *L. monocytogenes* contamination in 5 of 6 cantaloupe samples. However, 5 of 6 samples of mixed salad gave false-negative results, even though the sample tested positive when analyzed using selective growth media.

Confirmation of *L. monocytogenes* isolates obtained from naturally contaminated produce

Over 100 fresh produce samples (alfalfa sprouts and romaine lettuce) were obtained from retail supermarkets and screened for the presence of *L. monocytogenes* by conventional microbiology procedures (unpublished work, data not shown). The incidence of *L. monocytogenes* was 3.6% among organic produce and 5.3% in conventional produce. During the survey, other *Listeria* spp., such as, *L. welshimeri, L. innocua,* and *L. ivanovii*, were also identified from produce samples. We wanted to determine whether the iQCheck MB-PCR method would differentiate among the species and identify natural isolates of *L. monocytogenes*. A total of 4 colonies were picked per isolate, and DNA was isolated from each colony using the lysis reagent. The iQCheck protocol was successful in differentiating all *L. monocytogenes* isolates from other *Listeria* species (Table 3).

Discussion

As a result of the public health significance of *L. monocytogenes*, U.S. regulatory agencies, such as the Food and Drug Administration and the Food Safety and Inspection Service, established a policy under which ready-to-eat foods contaminated with the organism at a detectable level (1 CFU/25 g food) are deemed adulterated. Because the establishment of this "zero tolerance" policy, the food industry has implemented several preventive measures in an effort to eradicate the organism from foods and food-processing environments. Despite these efforts, *L. monocytogenes* contamination continues to occur. One of the factors that makes *L. monocyto-*

genes particularly difficult to control in foods is that, unlike most foodborne pathogens, it can grow at cold temperatures at which produce is typically stored (5 °C to 15 °C). Reduction in foodborne illnesses by 50% caused by *L. monocytogenes* is one of the goals of the Healthy People 2010 initiative (Anonymous 2003). Rapid detection methods with high sample throughput capabilities will be of immediate help in designing better microbiological screening procedures.

The fluorogenic detection of target DNA during PCR provides the possibility of real-time quantitative detection of a specific pathogen directly in the PCR tube, thus making it compatible for a high-throughput format (Higuchi and others 1993; Tyagi and Kramer 1996). Many studies have used PCR for the rapid detection of *L. monocytogenes* from laboratory cultures, as well as from meat and poultry products (Bessesen and others 1990; Golsteyn-Thomas and others 1991; Niederhauser and others 1992; Fluit and others 1993; Shearer and others 2001). Using a fluorogenic linear probe targeting the *hlyA* gene of *L. monocytogenes* and end point (instead of real-time) detection, Bassler and others (1995) reported a detection limit of 50 CFU in pure cultures. However, no challenge studies were performed, and the assay lacked an internal DNA amplification control (Liming and Bhagwat 2004) or melt curve analysis of the PCR product (Bhagwat 2003). Using a combination of immunomagnetic separation of *L. monocytogenes* from enrichment broths to remove inhibitors of *Taq* polymerase (discussed subsequently) and PCR, Fluit and others (1993) reported a detection limit of 1 CFU/g of cheese after total enrichment of approximately 55 h.

In the present study, we examined the efficacy of MB probes to detect *L. monocytogenes*-specific amplicons from whole and fresh-cut produce. Given the low incidence (about 3% to 5%) of *L. monocytogenes* in ready-to-eat fresh and fresh-cut produce (Zang and Meng, unpublished observations), we used artificially contaminated samples to examine the efficacy of pathogen detection. The MB beacon probe used in this study was able to detect *L. monocytogenes* from fresh and fresh-cut produce (cantaloupe and mixed salad) at a very low level of contamination (that is, at 4 to 7 CFU/25 g of produce) (Table 2). Several previous reports, which used PCR for the detection of pathogens from produce samples, have attributed observed false-negative results to the presence of inhibitory compounds of plant origin (Shearer and others 2001; Bhagwat 2003; Liao and Shollenberger 2003). In the MB-PCR assay, an internal control DNA was included in each reaction tube to monitor for successful DNA amplification. The internal control DNA was amplified at the same time as the *L. monocytogenes* target DNA (that is, *hlyA* gene), except that the amplification was detected by a second fluorophore (FAM). Thus, it was easy to interpret the lack of *hlyA* gene amplification as either the potential absence of *L. monocytogenes* or inhibition of PCR (Table 1). The occurrence of false-negative results have been attributed to the poor quality of target DNA, or insufficient enrichment of the target pathogen (Miller 2001; Shearer and others 2001; Bhagwat 2003; Heller and others 2003; Liao and Shollenberger 2003). In this study, enrichment in half-strength Fraser broth and taking samples *without* disturbing the media at the end of enrichment helped keep inhibition by plant pigments to a minimum (comparative data with and without shaking the enrichment broth before sampling for DNA isolation not shown). Further, the dilution of the DNA template (1:10 and 1:25) before the assay and the presence of a suspended gel-matrix in the lysis buffer helped to reduce the concentration of inhibitory compounds to a level that did not affect DNA amplification and allowed detection of *L. monocytogenes*.

L. monocytogenes can be differentiated on the basis of serology, and to date, more than 14 serotypes have been designated (Graves and others 1999). Despite the widespread occurrence of *L. monocytogenes* in nature, only 3 serotypes (4b, 1/2a, and 1/2b) account for 96% of human infections reported in the United States (Tappero and others 1995). Although the serotype 4b strain tested had a lower correlation coefficient (0. 839) compared with the serotype strain 1/2a (0.991) (Figure 2), both strains showed a linear-inverse relationship for the C_t value and the log starting quantity of specific DNA during PCR reaction. The specific reasons for a lower correlation coefficient for the serotype strain 4b are not clear at this time. The previous study using a linear fluorogenic PCR probe and end-point detection did not examine different serotype strains for their detection efficacy (correlation coefficient) (Bassler and others 1995). A detailed study involving more *L. monocytogenes* strains from each of the 3 serotypes as well as others needs to be undertaken to determine the efficacy of the MB-PCR assay.

Table 3— Molecular beacon-polymerase chain reaction (MB-PCR) analysis (threshold cycle values, C_t) of different *Listeria* spp.

	Threshold cycle value (C_t) in iQCheck-MB PCR	
Strain description[a]	Texas Red (*hlyA* DNA)	FAM (control DNA)
Listeria monocytogenes ATCC 13932	22.5	N/D[b]
L. monocytogenes isolate nr 27 from organic alfalfa sprouts	26.7	N/D
L. monocytogenes isolate nr 84 from organic romaine lettuce	23.9	N/D
L. welshimeri isolate nr 75 from organic romaine lettuce	N/D	34.0
L. welshimeri ATCC 35897	N/D	34.4
L. innocua ATCC 33090	N/D	34.2
L. ivanovii ATCC19119	N/D	34.5

[a]DNA was isolated from colonies of isolates growing on *Listeria* selective agar.
[b]N/D = none detected.

Although automated real-time PCR assays are rather complicated and require costly equipment, an increasing number of reference laboratories are converting traditional gel-based detection PCR to real-time, fluorescence-based detection to conduct microbial quality control and handle a high sample volume (Hoorfar and Radstorm 2000; Daum and others 2002). It required approximately 5 to 6 d to obtain definitive results from microbial selective media, about 26 h using MB-probe real-time PCR and 52 h using the BAX-PCR protocol. This study illustrates for the 1st time the feasibility of using MB probe real-time PCR detection technology for detection and/or surveillance of *L. monocytogenes* involving whole and fresh-cut fruits and vegetables.

Conclusions

The oligonucleotide molecular beacon probe detected target DNA specific to *L. monocytogenes* in real-time PCR assay, with sensitivity of 4 to 7 CFU/25 g of produce. The total detection time (from produce to PCR) was reduced to 26 h compared with conventional PCR assays and microbiological selective media. The inclusion of an internal amplification control provided ability to differentiate false-negative tests from general failure of PCR reaction due to inhibitory compounds or inactive assay components.

Acknowledgments

Mention of brand names does not constitute an endorsement by the U.S. Dept. of Agriculture above others of a similar nature not mentioned. We thank Ingrid Berlanger and Irving Newman for excellent technical assistance. One of the authors (SHL) was supported by the Overseas Industrial Attachment Program of the School of Life Sciences & Chemical Technology, Ngee Ann Polytechnic, Singapore. The study was supported in part by a cooperative research agreement with Bio-Rad Laboratories, Hercules, Calif., U.S.A. We also thank William Conway, Kenneth Gross, James Linday, and Jitu Patel for critically reading the manuscript and helpful suggestions.

References

Anonymous. 2003. Healthy people 2010 initiative. Washington, D.C.: Office of Disease Prevention and Health Promotion, U.S. Dept. of Health and Human Services. Available from: http://www.health.gov/healthypeople. accessed 28 Sept. 2004.

Bassler HA, Flood SJA, Livak KJ, Marmaro J, Knorr R, Batt CA. 1995. Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. Appl Environ Microbiol 61:3724–8.

Bessesen MT, Luo Q, Rotbart HA, Blaser MJ, Ellison III RTI. 1990. Detection of *Listeria monocytogenes* by using polymerase chain reaction. Appl Environ Microbiol 56:2930–2.

Bhagwat AA. 2003. Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. Int J Food Microbiol 84:217–24.

Bhagwat AA. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. Food Microbiol 21:73–8.

Burnett SL, Beuchat LR. 2001. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. J Ind Microbiol Biotechnol 27:104–10.

Chen W, Martinez G, Mulchandani A. 2000. Molecular beacons: a real-time polymerase chain reaction assay for detecting *Salmonella*. Anal Biochem 280:166–72.

Cocolin L, Rantsiou K, Iacumin L, Cantoni C, Comi G. 2002. Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. Appl Environ Microbiol 68:6273–82.

Daum LT, Barnes WJ, McAvin JC, Neidert MS, Cooper LA, Huff WB, Gaul L, Riggins WS, Morris S, Salem A, Lohman KL. 2002. Real-time detection of *Salmonella* in suspect foods from a gastroenteritis outbreak in Kerr county, Texas. J Clin Microbiol 40:3050–2.

Donnelly CW. 2001. *Listeria monocytogenes*. In: Labbe R, Garcia S, Labbe R, Garcia S. Guide to foodborne pathogens. New York: John Wiley & Sons, Inc. p 99–132.

Farber JM, Peterkin PI. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev 55:476–511.

Fluit AC, Torensma R, Visser MJC, Aarsman CJM, Poppelier MJ, Keller BHI, Klapwijk P, Verhoef J. 1993. Detection and identification of *Listeria monocytogenes* in cheese with the magnetic immuno-polymerase chain reaction assay. Appl Environ Microbiol 59:1289–93.

Francis GA, Thomas C, O'Beirne D. 1999. The microbiological safety of minimally processed vegetables. Int J Food Sci Technol 34:1–22.

Gaillard JL, Berche P, Sansonetti P. 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect Immunol 52:50–5.

Golsteyn-Thomas EJ, King RK, Burchak J, Gannon VPJ. 1991. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. Appl Environ Microbiol 57:2576–80.

Gombas DE, Chen Y, Clavero RS, Scott VN. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. J Food Prot 66:559–69.

Graves LM, Swaminathan B, Hunter SB. 1999. Subtyping *Listeria monocytogenes*. In: Ryser ET, Marth EH, Ryser ET, Marth EH, editors. Listeria, listeriosis and food safety. New York: Marcel Dekker. p 279–80.

Heller LC, Davis CR, Peak KK, Wingfield D, Cannons AC, Amuso PT, Cattani J. 2003. Comparison of methods for DNA isolation from food samples for detection of Shiga toxin-producing *Escherichia coli* by real-time PCR. Appl Environ Microbiol 69:1844–6.

Higuchi R, Fockler C, Dollinger G, Watson R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Bio/Technology 11:1026–30.

Hoorfar J, Radstorm P. 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J Clin Microbiol 38:3429–35.

Johnson JL. 1998. Isolation and identification of *Listeria monocytogenes* from meat, poultry, and egg products. In: Dey BP, Lattuada CP, editors. USDA/FSIS microbiology laboratory guidebook. Washington, D.C.: Government Printing Office. p 8.1–17.

Liao C-H, Shollenberger LM. 2003. Detection of *Salmonella* by indicator agar media and PCR as affected by alfalfa seed homogenates and native bacteria. Lett Appl Microbiol 36:152–6.

Liming SH, Bhagwat AA. 2004. Application of a molecular beacon—real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. Int J Food Microbiol 95:177-86.

Menguad J, Vicente MF, Chenevert J, Pereira JM, Geoffrey C, Gicquel-Sanzey B, Baquero F, Perez-Diaz JC, Cossart P. 1988. Expression in *Escherichia coli* and sequence analysis of the lysteriolysin O determinant of *Listeria monocytogenes*. Infect Immunol 56:766–72.

Miller DN. 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. J Microbiol Methods 44:49–58.

[NACMCF] Natl. Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10:117–43.

Niederhauser CU, Candrian U, Hoefelein C, Jermini M, Buehler HP, Luethy J. 1992. Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Appl Environ Microbiol 58:1564–8.

Sewell AM, Farber JM. 2001. Foodborne outbreaks in Canada linked to produce. J Food Prot 64:1863–77.

Shearer AEH, Strapp CM, Joerfer RD. 2001. Evaluation of a polymerase chain reaction- based system for detection of *Salmonella* Enteritidis, *Escherichia coli* O157:H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. J Food Prot 64:788–95.

Tappero JWA, Schuchat A, Deaver KA, Mascola L, Wenger JD. 1995. Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts? J Am Med Assoc 273:1118–22.

Tyagi S, Kramer FR. 1996. Molecular beacon: probes that fluoresce upon hybridization. Nature Biotechnol 14:303–8.

Zhao C, Ge B, Villena JD, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D.C., area. Appl Environ Microbiol 67:5431–6.