

Detection of *Listeria monocytogenes* from a Model Food by Fluorescence Resonance Energy Transfer-Based PCR with an Asymmetric Fluorogenic Probe Set†

Kai Koo and Lee-Ann Jaykus*

Department of Food Science, College of Life Science and Agriculture, North Carolina State University, and Southeast Dairy Foods Research Center, Raleigh, North Carolina 27695-7624

Received 20 March 2002/Accepted 1 November 2002

It has been shown that fluorescence resonance energy transfer (FRET)-based PCR, including the TaqMan assay and molecular beacons, has potential for rapid detection of pathogens. In these promising real-time detection assays a single internal oligonucleotide probe labeled on both the 5' (reporter) and 3' (quencher) ends is used for selective generation of fluorescence. In this paper, we describe the use of a previously reported novel probe design for FRET-based PCR detection of *Listeria monocytogenes* in pure culture and in a model food commodity. In the assay described here an asymmetric probe set is used; this probe set consists of a long 5' fluorescein-labeled reporter probe and a short, complementary 3' DABCYL-labeled quencher oligonucleotide, which are used in a 5' nuclease amplification and detection assay. By using the listeriolysin O (*hly*) and *p60* (*iap*) genes as amplification targets, the performance of two primer-probe sets in amplification and subsequent detection of target DNA was evaluated. In studies performed with pure cultures of *L. monocytogenes*, the PCR profiles indicated that the relative change in fluorescence intensity was correlated with both the initial number of cells and the accumulation of specific amplicons for both *hly* and *iap* gene fragments. Experiments were also done to determine the applicability of the method to the detection of *L. monocytogenes* by targeting *hly* DNA and its short-lived mRNA product in a model food commodity. Twenty-five-milliliter samples of reconstituted nonfat dry milk (NFDM) were seeded with *L. monocytogenes* and processed to concentrate the bacteria by centrifugation, and this was followed by nucleic acid extraction and amplification with *hly*-specific primers. Endpoint detection of PCR and reverse transcription-PCR amplicons could be achieved at inoculum levels of 10^3 and 10^4 CFU of *L. monocytogenes*/25 ml of NFDM, respectively. This study demonstrated that this asymmetric FRET-based amplification and detection protocol provides an alternative approach for endpoint detection of nucleic acid amplification products as applied to detection of pathogens in a model food.

With the introduction of molecular biological detection methods, such as the PCR, food microbiologists had high hopes that methods would soon be available to overcome some of the common difficulties associated with the detection of pathogens in foods. Of particular interest was the potential to replace time-consuming cultural enrichment steps with specific nucleic acid sequence enrichment, thereby decreasing the total detection time. There have been many reports of PCR-based assays for detection of food-borne pathogens (for a review see reference 27), and several companies market these systems, some of which have been certified recently by the Association of Official Analytical Chemists (23, 24). Unfortunately, all of these methods require prior cultural enrichment, and hence the term rapid remains, in part, a misnomer.

An important rate-limiting step for the PCR lies in the fact that amplification products are detected most commonly by postamplification agarose gel electrophoresis and/or DNA hybridization. In an effort to reduce the time necessary for detection and confirmation, a number of investigators have described methods to simplify or even eliminate the need to

perform postamplification assays. For example, molybdate (7) and some intercalating agents (9) have been used to measure the degree of DNA amplification, but these protocols are limited because they do not consider amplicon sequence specificity. Other investigators have focused their efforts on adapting the fluorescence resonance energy transfer (FRET) phenomenon to the detection of specific PCR amplicons, including AmpliSensor (5), molecular beacons (16, 25), Amplifluor (17), Scorpions primers (26), and adjacent hybridization probes (29). Of particular note in recent years is the TaqMan assay (Applied Biosystems, Foster City, Calif.), which has been applied to the detection of food-borne pathogens, including *Listeria monocytogenes* (1, 6, 8, 19) and *Escherichia coli* O157:H7 (20, 21, 22, 28).

Although many variations of the FRET concept have been reported, in most of them a single oligonucleotide labeled on both the 5' (reporter) and 3' (quencher) ends is used for selective generation of fluorescence. In an effort to reduce the development and assay costs associated with FRET-based PCR assays, we (14) described the use of an inexpensive asymmetric probe set which consists of a long 5' fluorescein-labeled (reporter) oligonucleotide and a short 3' 4-[4'-dimethylaminophenylazo]benzoic acid-labeled (quencher) oligonucleotide (Fig. 1). These probes are able to form an asymmetrical double-stranded conformation at room temperature such that the 5' fluorescein-labeled probe is quenched by the nearby

* Corresponding author. Mailing address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624. Phone: (919) 513-2074. Fax: (919) 515-7124. E-mail: leeann_jaykus@ncsu.edu.

† Paper no. FSR 02-14 of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh.

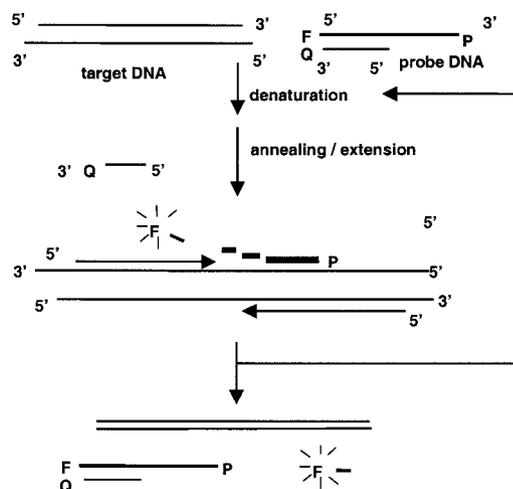


FIG. 1. Detection of PCR products by using 5' nuclease activity and an asymmetric fluorogenic probe set. P, probe; F, fluorescein label; Q, quencher.

quencher oligonucleotide. During the normal annealing-primer extension step of the PCR, the reporter probe molecules are available to hybridize with the target amplicons, and the fluorescein label is digested by the 5' nuclease activity of *Taq* DNA polymerase (10). After the DNA amplification is complete, the residual reporter probe reanneals to the short quencher oligonucleotide at room temperature. The increase in fluorescence at the end of the assay indicates that specific amplicons are produced. Although this assay is not a real-time assay in the sense that the detection of PCR products occurs simultaneously as the PCR is progressing, it is, however, an endpoint detection assay in that specific PCR products can be detected by fluorescence immediately following termination of the PCR. As such, this protocol takes advantage of the lower cost of single labeling (approximately \$225 for 250 nmol of the single-label probe versus \$400 for the same amount of the dual-label probe) and provides a promising alternative for rapid and efficient endpoint detection of PCR amplicons in a single reaction tube.

The purpose of this study was to adapt the previously described asymmetric FRET-based PCR assay to amplification and detection of *L. monocytogenes* in a model food commodity. The listeriolysin O (*hly*) and p60 (*iap*) genes were chosen as the amplification targets. The performance of primer-probe sets in amplification and subsequent detection of target DNA was evaluated. Experiments were also done to determine the applicability of the method for detection of *L. monocytogenes* by targeting *hly* DNA and its short-lived mRNA product in a model food commodity.

MATERIALS AND METHODS

Bacterial strains. Six *Listeria* strains (*L. monocytogenes* ATCC 19115, *L. innocua* ATCC 33091, *L. ivanovii* ATCC 19119, *L. grayi* ATCC 25401, *L. seeligeri* ATCC 35967, and *L. welshimeri* ATCC 35897) were used in various experiments to evaluate gene amplification efficiency and primer specificity. A single colony of each *Listeria* strain was resuspended in 0.3 ml of double-distilled water, and 1 μ l of each cell suspension was used in initial PCR optimization experiments, as well as for evaluation of primer specificity.

A

For *hly* gene

forward primer 5' AGG ATG CAT CTG CAT TCA A 3'
reverse primer 5' GGA TAT CTG CAT TAT TTT GAT T 3'
signal probe 5' fluorescein -TTC TTG GCG GCA CAT TTG TCA CTG CA-p 3'
quencher 3' DABCYL-AAG AAC CGC CG 5'



B

For *iap* gene

forward primer 5' AGG TTC TTC CAA CAA TAA CAG C 3'
reverse primer 5' ATT TAA CGC CAT TGT CTT GC 3'
signal probe 5' fluorescein-TTC ATG GGG TGG TAA CGG ACC AAC TA-p 3'
quencher 3' DABCYL-AAG TAC CCC ACC -5'

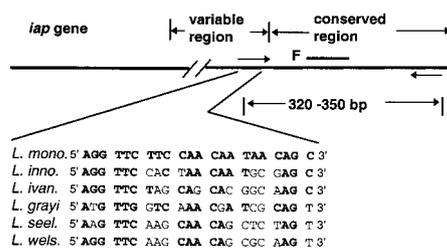


FIG. 2. Oligonucleotide primer and probe sets used for amplification of *hly* and *iap* gene-specific fragments by PCR and RT-PCR. The *hly*-specific primer-probe set was designed on the basis of data obtained from GenBank (accession numbers U25443, U25446, U25449, and U25452) and previously published information (1). The *iap*-specific primer set was also designed on the basis of data obtained from GenBank (accession numbers X52268, M80348, M80349, M80350, M80351, M80352, and M80353) and previous reports (3).

Isolation of *L. monocytogenes* nucleic acids from NFD. Twenty-four-milliliter samples of reconstituted nonfat dry milk (NFD) were each inoculated with 1 ml of a serially diluted overnight culture of *L. monocytogenes* to obtain a final concentration of 1×10^2 to 1×10^5 CFU/25 ml. The samples were pretreated by adding 1.5 ml of 25% (wt/vol) sodium citrate with 10 min of shaking at room temperature, as previously reported (15). After this, cells were collected by centrifugation at $10,000 \times g$ for 10 min at 4°C and extracted for DNA or RNA isolation.

For isolation of DNA, the pellet was resuspended in 0.1 ml of 1% sodium dodecyl sulfate and solubilized by adding 0.4 ml of 60% (wt/vol) guanidinium thiocyanate. The DNA was extracted by adding 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) and was precipitated by using isopropanol. The air-dried DNA pellet was resuspended in 25 μ l of distilled water; 10 μ l of a reconstituted DNA suspension was used in each PCR amplification.

For RNA extraction, the cell pellet was resuspended in 0.1 ml of 1% sodium dodecyl sulfate–0.5% diethyl pyrocarbonate and solubilized by adding 0.4 ml of 60% (wt/vol) guanidinium thiocyanate and 40 μ l of 3 M sodium acetate (pH 4.8). The RNA was extracted by adding 0.4 ml of water-saturated phenol and 0.2 ml of chloroform. To enhance the efficiency of recovery of RNA after isopropanol precipitation, 10 μ g of yeast tRNA (Sigma, St. Louis, Mo.) was added as a coprecipitant. The air-dried RNA pellet was resuspended in 30 μ l of distilled water; 5 μ l of reconstituted RNA was used in each reverse transcription (RT) reaction. Because of the special design of the RT-PCR primer set (13), RNase-free DNase treatment of RNA extracts was not necessary prior to RT.

Oligonucleotide primers and probes. The sequences of amplification primers and detection probes and the sizes of amplicons are shown in Fig. 2. The oligonucleotides were designed based on DNA sequence data from GenBank

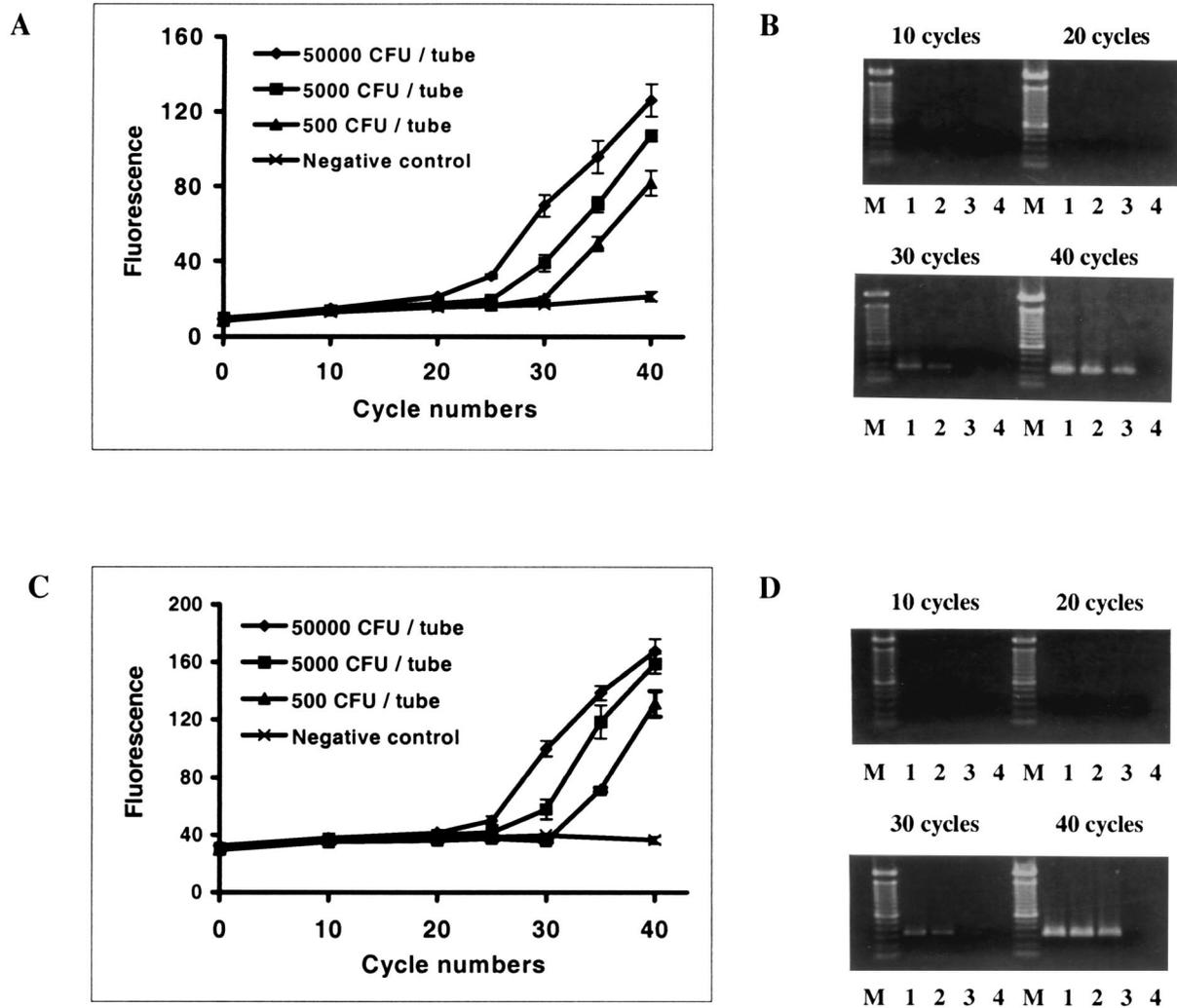


FIG. 3. Amplification of *hly* (A and B) and *iap* (C and D) genes from pure cultures of *L. monocytogenes*. (A and C) PCR profiles for *hly* gene amplification and *iap* gene amplification, respectively, as measured with a fluorescence plate reader. The values are means \pm standard deviations for three replicate samples. (B and D) Agarose (1%) gel electrophoresis and EtBr staining of PCR products for *hly* gene amplification (273 bp) and *iap* gene amplification (330 bp), respectively. Lane M, 100-bp DNA ladder; lanes 1 to 4, PCR products amplified from suspensions corresponding to 5×10^4 , 5×10^3 , 5×10^2 , and 0 CFU of *L. monocytogenes* per reaction mixture, respectively.

and information previously published by Bassler et al. (1) and Bubert et al. (3). To reduce false-positive signals in RT-PCR due to the presence of residual genomic DNA, the reverse primer used for RT-PCR targeting of the *hly* mRNA was modified by incorporating three consecutive mismatched bases near its 3' terminus, as previously reported (13). The oligonucleotide primers and fluorescein-labeled probes were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

Synthesis of cDNA and amplification of target nucleic acids. A 10- μ l RT mixture containing 4 U of avian myeloblastosis virus reverse transcriptase, 10 U of RNasin (Promega, Madison, Wis.), 2 μ l of 5 \times RT buffer (250 mM Tris [pH 8.3], 150 mM KCl, 40 mM MgCl₂, 20 mM dithiothreitol), 5 μ l of RNA from NFDM samples, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, and 1 pmol of RT primer was incubated at 45°C for 1 h and then at 94°C for 5 min to inactivate the enzymes.

For amplification of target gene fragments, 40 μ l of a PCR solution was mixed with 10 μ l of a genomic DNA extract or RT mixture for further amplification. The 50- μ l PCR mixture, which contained 1 \times PCR buffer, 3 mM MgCl₂, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.4 mM dUTP (PCR) or 0.3 mM dUTP plus 0.05 mM dTTP (RT-PCR), 10 pmol of each forward and reverse primer, 5 pmol of each reporter and quencher probe, 1 U of *Taq* DNA polymerase, 1 U of anti-*Taq* antibody (Invitrogen, Carlsbad, Calif.), and 0.2 U of uracil-*N*-glycosylase,

was used for amplification. To digest possible carryover contaminating DNA by uracil-*N*-glycosylase and to denature the target template, 1 cycle of 45°C for 5 min and 94°C for 5 min was used. After this, the DNA was amplified by using 40 cycles of 94°C for 30 s, 60°C (in some experiments, an annealing temperature of 58°C was also tested) for 30 s, and 72°C for 45 s. The PCR was carried out with a GeneAmp PCR system 9600 (Applied Biosystems). Each PCR tube was removed from the thermal cycler after completion of amplification and was equilibrated to room temperature. Forty microliters of each PCR solution was transferred into a 96-well microtiter plate, and the fluorescence was measured (excitation wavelength, 485 nm; emission wavelength, 530 nm) with a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, Mass.). Amplification was confirmed by agarose (1%) gel electrophoresis and ethidium bromide (EtBr) staining.

RESULTS

Detection of *L. monocytogenes* DNA from pure cultures. In a previous study (14), we reported that the amplification profile obtained with the asymmetric probe set-coupled PCR indi-

TABLE 1. Primer specificity for amplification of gene fragments from *L. monocytogenes* compared to other species of the genus *Listeria*

Gene target	Annealing temp (°C)	Strain	Amplicon size (bp)	Fluorescence ^a	Gel ^b
<i>hly</i>	58	<i>L. monocytogenes</i> ATCC 19115	273	115	+
		<i>L. innocua</i> ATCC 33091		28	–
		<i>L. ivanovii</i> ATCC 19119		27	–
		<i>L. grayi</i> ATCC 25401		30	–
		<i>L. seeligeri</i> ATCC 35967		32	–
		<i>L. welshimeri</i> ATCC 35897		30	–
		Water (negative control)		26	–
<i>iap</i>	60	<i>L. monocytogenes</i> ATCC 19115	330	189	+
		<i>L. innocua</i> ATCC 33091		45	–
		<i>L. ivanovii</i> ATCC 19119		43	–
		<i>L. grayi</i> ATCC 25401		44	–
		<i>L. seeligeri</i> ATCC 35967		43	–
		<i>L. welshimeri</i> ATCC 35897		46	–
		Water (negative control)		40	–

^a The values are fluorescence values (excitation wavelength, 485 nm; emission wavelength, 530 nm) obtained with a CytoFluor II plate reader.

^b + or – indicates the presence or absence, respectively, of bands of the expected size as determined by agarose gel electrophoresis and EtBr staining.

cated that there was an overall increase in fluorescence at the end of the assay that reflected the initial copy number of positive control plasmid DNA. In this study, *L. monocytogenes* was used as the template for direct amplification of target DNA fragments. The PCR profiles (Fig. 3A and C) and agarose gel electrophoresis (Fig. 3B and D) revealed changes in fluorescence intensity that were correlated with the initial number of cells and with the accumulation of specific amplicons for both *hly* and *iap* gene fragments, and the detection limits were ≥ 500 CFU/reaction mixture.

Primer specificity tests. The *hly* and *iap* primer-probe sets were further tested for their ability to discriminate *L. monocytogenes* from five different species in the genus *Listeria* (Table 1). The *hly* gene fragment-specific primer set showed excellent specificity for *L. monocytogenes*. This primer set generated the specific amplicon solely from the DNA of *L. monocytogenes* at annealing temperatures as low as 58°C. At the same annealing temperature, the *iap*-specific primer-probe set produced a very faint nonspecific 440-bp amplicon for DNA obtained from an *L. ivanovii* culture (data not shown). When the DNA sequences of *L. ivanovii* (GenBank accession number M80350) were compared to the sequence of the forward *iap* primer, it was found that the nonspecific amplification was most likely caused by a short region exhibiting a high degree of sequence similarity (10 of 12 bases from the 3' end of primer) with this primer. However, this weak nonspecific amplification could be eliminated by increasing the annealing temperature to 60°C (Table 1).

Amplification of *L. monocytogenes* DNA from NFDM samples. As described above, DNA of *L. monocytogenes* could be amplified, detected, and confirmed efficiently from pure cultures of *L. monocytogenes* by using the asymmetric probe set. However, amplification and confirmation of the same gene fragments from a more complex sample matrix remain challenging due to the presence of potential inhibitors and competitors of the PCR. Amplification of DNA isolated from *L. monocytogenes* obtained from seeded NFDM samples was tested as a model system. The results showed that the target DNA extracted from 1,000 CFU seeded in 25 ml of reconstituted NFDM could be specifically amplified and detected ei-

ther by agarose gel electrophoresis or by changes in fluorescence intensity by using the *hly*-specific primer-probe set (Fig. 4A and B). The *iap*-specific primer-probe set generated higher background fluorescence (noise), and use of this set decreased detection sensitivity by at least 1 log (Fig. 4C and D).

Amplification of *L. monocytogenes* mRNA from NFDM samples. Recent studies have demonstrated a better correlation between the presence and amplification of bacterial mRNA and cell viability (12, 19). In this study, we used a modified DNase treatment-free RT protocol (13) along with the asymmetric probe set to detect bacterial mRNA in the NFDM food matrix. The results revealed that the *hly* mRNA obtained from an NFDM sample containing 10,000 CFU/25 ml could be detected and that false-positive signals generated by the amplification of residual DNA were inhibited (Fig. 5). Furthermore, yeast tRNA, the coprecipitant added to improve the recovery of the mRNA, did not interfere with the amplification of specific cDNA under these RT-PCR amplification conditions (data not shown). These experiments also demonstrated the ability of the asymmetric probe set to confirm the presence of specific amplicons (273 bp) even in the presence of relatively high copy numbers of nonspecific amplicons (approximately 200 bp) (Fig. 5B, lanes 1 to 5).

DISCUSSION

In the first report on the use of a fluorogenic PCR-based assay for the detection of *L. monocytogenes*, Bassler et al. (1) described the 5' nuclease TaqMan assay targeting the *hly* gene, which detected as few as 50 to 100 CFU per amplification reaction mixture in less than 3 h. In another report Nogva et al. cited detection limits as low as 6 to 60 CFU per reaction mixture (18), although these authors noted that the DNA extraction efficiency can negatively affect test sensitivity when the test is performed with cell concentrations that are < 500 CFU per reaction mixture. Norton and Batt (19) described a single-tube reverse transcriptase fluorogenic probe-based TaqMan assay targeting *hlyA* mRNA of *L. monocytogenes*, although no specific detection limits were cited. Most recently, Hein et al. (8) described use of the *iap* gene to discriminate between *L.*

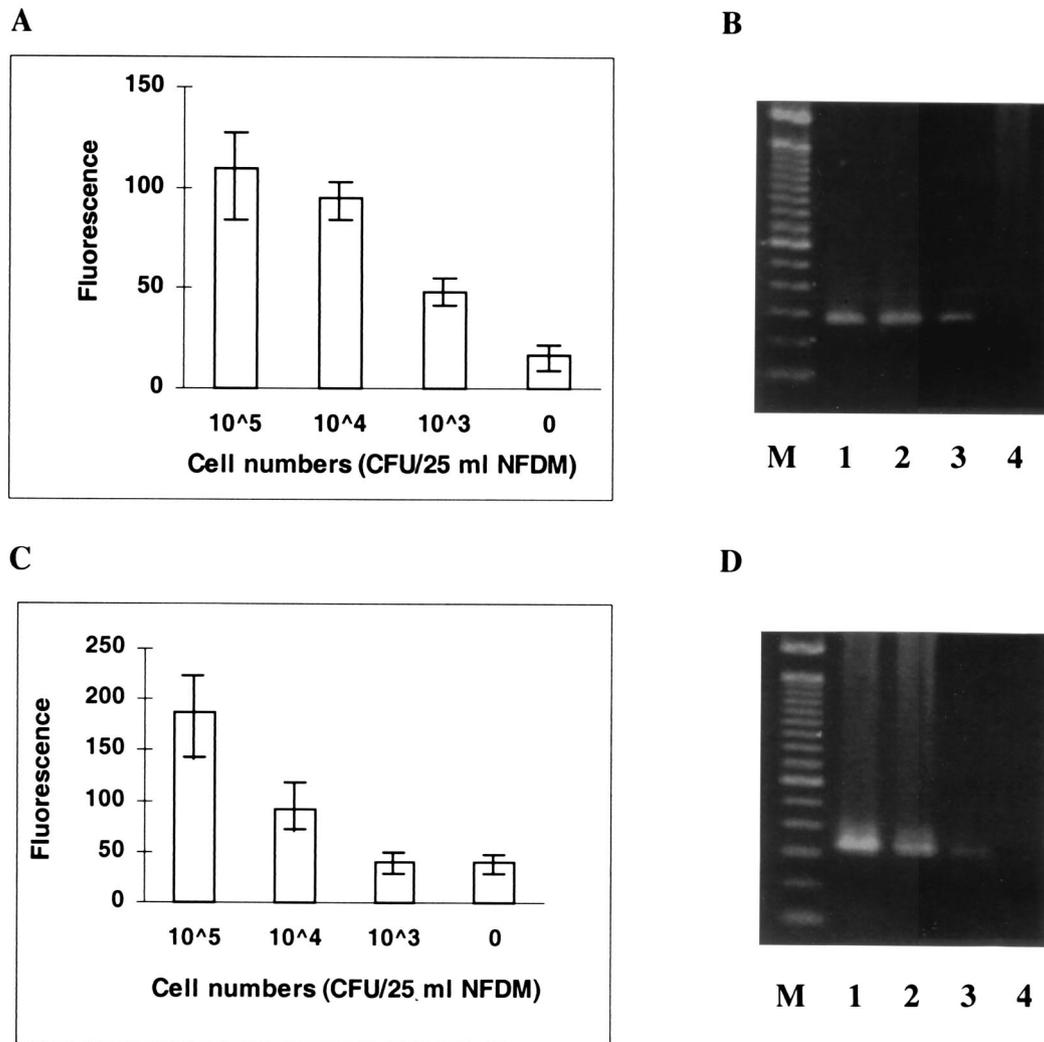


FIG. 4. Detection of *L. monocytogenes hly* (A and B) and *iap* (C and D) genes (genomic DNA) from food samples. (A and C) Fluorescence from *hly*-specific amplification and *iap*-specific amplification, respectively. The values are means \pm standard deviations for three replicate samples. (B and D) Agarose (1%) gel electrophoresis and EtBr staining of PCR amplicons for *hly* gene amplification (273 bp) and *iap* gene amplification (330 bp), respectively. Lane M, 100-bp DNA ladder; lanes 1 to 4, PCR products amplified from DNA extracts corresponding to 10⁵, 10⁴, 10³, and 0 CFU of *L. monocytogenes*/25-ml NFDM sample, respectively.

monocytogenes and *L. innocua* in a quantitative PCR assay, demonstrating a detection limit of six gene copies. These studies and others indicate that the most commonly used PCR amplification targets for the detection of *L. monocytogenes* are the listeriolysin O gene (*hlyA*) and the protein p60 gene (*iap*). Although Klein and Juneja (12) reported that targeting the *iap* gene-specific mRNA yielded the best detection limits with RT-PCR (compared to mRNA from the *hlyA* and *prfA* genes), we obtained better detection limits by targeting the *hlyA* gene product. With PCR detection limits for pure cultures in the range of 500 CFU/reaction mixture, our assay is slightly less sensitive than those described by other workers (1, 18). However, the detection limits and the amplification specificity of both PCR and RT-PCR have been improved recently by adjustments to PCR conditions and nucleic acid extraction protocols in our laboratory (data not shown), and we are hopeful that with further optimization, the detection performance can

be further improved. Furthermore, we have also illustrated that our method is applicable to detection of mRNA and can be applied to a model food matrix.

There are still important issues that must be addressed if rapid fluorogenically based detection of food-borne pathogens is to become a routine reality. Critical methodological issues include, but are not limited to, the following: (i) the need to utilize large realistic sample sizes (≥ 25 ml or ≥ 25 g) to obtain bacterial nucleic acid, compared to requisite PCR amplification volumes (10 to 50 μ l); (ii) the presence of low levels of contaminating pathogens; (iii) the need to assure that nonviable cells are not detected; and (iv) the need to confirm PCR amplification products with more lengthy DNA hybridization assays (2, 27). While in many studies the workers have reported development of PCR and RT-PCR protocols for pure cultures, few researchers have addressed the critical issue of food sample preparation prior to detection. As with the commercial

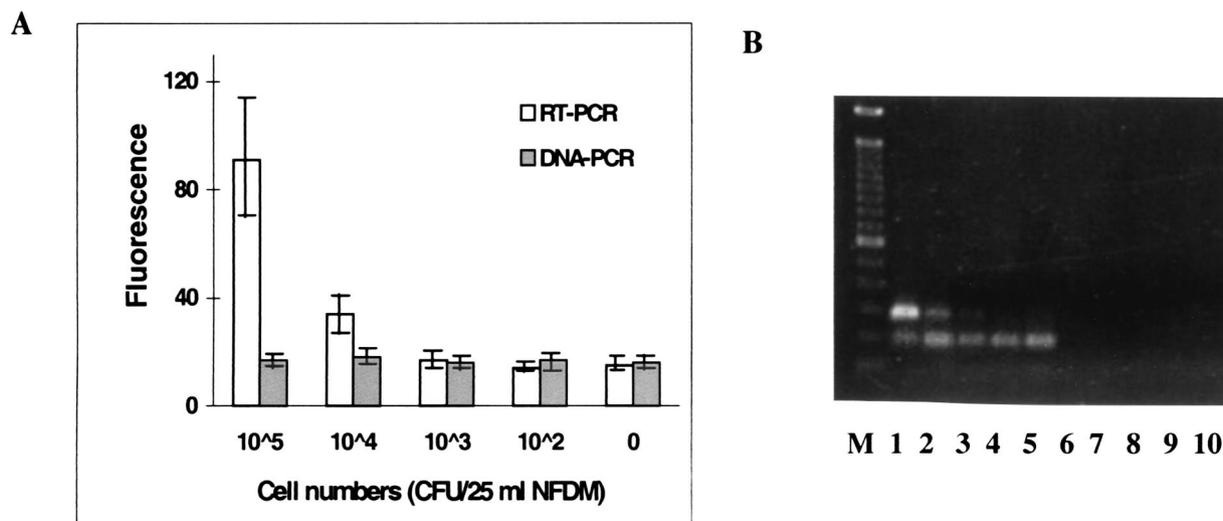


FIG. 5. Detection of *L. monocytogenes hly* gene product (mRNA) from food samples. (A) Fluorescence from *hly*-specific amplification of RNA extracts obtained from seeded 25-ml NFDm samples amplified by RT-PCR (open bars) and PCR (shaded bars). The values are means \pm standard deviations for three replicate samples. (B) Agarose (1%) gel electrophoresis and EtBr staining of amplicons (273 bp). Lane M contained a 100-bp DNA ladder. The amplicons of RT-PCR (lanes 1 to 5) and PCR (lanes 6 to 10) were obtained by amplification performed with RNA extracts corresponding to 10⁵, 10⁴, 10³, 10², and 0 CFU of *L. monocytogenes*/25-ml NFDm sample, respectively.

PCR detection kits for food-borne pathogens (23, 24), in almost all other studies the workers also report the need for cultural enrichment prior to nucleic acid extraction and detection in order to achieve detection limits approaching 1 CFU/25-g food sample (4, 12, 18, 20). In the case of fluorogenic PCR detection of food-borne pathogens, the usual approach is either to extract the nucleic acids from a 1-ml portion of an enrichment culture (4, 6) or to precede the extraction with a paramagnetic capture step (18). For instance, Nogva et al. (18) reported fluorogenic PCR detection limits of 60 to 6,000 CFU of *L. monocytogenes* when the amplifications were preceded by a paramagnetic concentration step done on 1.4 ml of artificially inoculated skim or whole milk. Our approach is unique in that we begin with 25-ml samples and concentrate the bacteria by centrifugation, which is followed by nucleic acid extraction and amplification. Using this type of approach, we obtained detection limits of 10³ to 10⁴ CFU of *L. monocytogenes* per 25-ml sample of our model food commodity without prior cultural enrichment. By targeting mRNA rather than DNA and incorporating the fluorogenic endpoint detection strategy, our approach addresses several of the issues cited above. We recognize that the reconstituted NFDm matrix is compositionally simple and that additional work in matrix processing to concentrate pathogens and to remove inhibitors is critical (15). A next logical step would be to address such matrix issues by adapting this type of technology to the detection of *L. monocytogenes* in at-risk products (cheeses, processed meats) or environmental samples.

Recent studies have demonstrated a better correlation between the presence and amplification of bacterial mRNA and cell viability (12, 19). When mRNA is used as a target template, RNA extraction is considered necessary to avoid coamplification of genomic DNA. Furthermore, a DNase treatment prior to RT is usually employed to further avoid false-positive signals that might occur due to the contamination of RNA

extracts with small amounts of genomic DNA. However, when samples contain low copy numbers of target RNA, the optional DNA removal and subsequent purification steps can cause further loss of RNA, resulting in a decrease in RT-PCR detection limits and overall test sensitivity (11). To address this issue, we (13) described an RT-PCR method designed to reduce false-positive results due to coamplification of contaminating genomic DNA using a *Bacillus cereus* model. Briefly, when a DNA oligonucleotide primer containing three consecutive mismatched bases near its 3' terminus was constructed and RT was performed at a low temperature (40 or 45°C), the cDNA produced contained the mismatched sequence. When this was followed by PCR amplification at high annealing temperatures ($\geq 60^\circ\text{C}$), the amplification of contaminating genomic DNA was hindered relative to the amplification of the cDNA. In the present study, we were able to design RT-PCR primers targeting the *hly* gene mRNA of *L. monocytogenes* using the same general format. This effectively eliminated the need to perform DNase treatment in order to avoid false-positive signals from residual genomic DNA, further simplifying the assay design and most likely improving the detection limit.

For rapid detection of specific amplicons, FRET-based PCR with internal amplicon-specific probes remains an excellent choice. Unlike protocols in which molybdate (7), intercalating agents (9), and fluorescein-labeled amplification primers (17) are used, in which detection is not based on sequence specificity, FRET-based PCR offers exquisite specificity and allows discrimination of nonspecific amplicons from specific amplicons. While the TaqMan assay and molecular beacon approaches are the most popular protocols, they use a dual-label oligonucleotide rather than two singly labeled complementary oligonucleotides for the selective generation of fluorescence. This theoretically simplifies FRET-based assays but also adds expense. The design described here takes advantage of the fact

that the cost of single labeling is about one-half the cost of dual labeling (\$225 versus \$400 for 250-nmol portions of the two probes, respectively), which results in reduced cost in assay development and also maintains the simplicity provided by dual-label probes. For example, a minor change in the sequence or length of the probe during assay optimization would normally necessitate the purchase of a new dual-label probe. In the case of our asymmetric probe design, the use of two separate modules (i.e., a signal module [fluorescein-labeled primer] and a switch module [quencher-labeled short oligonucleotide]) means that only a new signal module would be needed under the same circumstances. Furthermore, the assay can be completed with any PCR thermocycler and fluorescence reader that are routinely available, which means that it is not necessary to purchase expensive real-time amplification equipment. This concept makes our design a good choice for assay development and optimization for a research laboratory on a limited budget.

In this study we demonstrated that 10^3 to 10^4 CFU of *L. monocytogenes* DNA or mRNA in 25 ml of NFDM could be amplified and detected by an asymmetric FRET-based amplification protocol with endpoint detection in about 2.5 h. The ease of use, high specificity, and relatively low cost of this method make it a promising alternative to other fluorescent PCR protocols. However, before any of the methods can be practically used in routine assays of low numbers of pathogens in foods, issues such as pathogen concentration, nucleic acid recovery, and inhibitor removal must be dealt with. Indeed, technical breakthroughs in these issues will be critical to the success of future nucleic acid amplification methods. Currently, research and modification of preamplification processing, amplification optimization, and protocol integration are under way in our laboratory and other laboratories.

ACKNOWLEDGMENTS

This study was funded by Dairy Management, Inc., through the Southeast Dairy Foods Research Center.

The use of trade names in this paper does not imply endorsement by the North Carolina Agricultural Research Service or criticism of similar products not mentioned.

REFERENCES

- Bassler, H. A., S. J. A. Flood, K. J. Livak, J. Marmaro, R. Knorr, and C. A. Batt. 1995. Use of fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **61**:3724–3728.
- Bej, A. K., and M. H. Mahbubani. 1994. Detection of food borne microbial pathogens, p. 341–365. In *PCR technology: current innovations*. CRC Press, Boca Raton, Fla.
- Bubert, A., S. Kohler, and W. Goebel. 1992. The homologous and heterologous regions within the *iap* gene allow genus- and species-specific identification of *Listeria* spp. by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**:2625–2632.
- Chen, S., A. Yee, M. Griffiths, C. Larkin, C. T. Yamashiro, R. Behari, C. Paszko-Kolva, K. Rahn, and S. A. DeGrandis. 1997. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. *Int. J. Food Microbiol.* **35**:239–250.
- Chen, S., R. Xu, A. Yee, K. Y. Wu, C.-N. Wang, S. Read, and S. A. DeGrandis. 1998. An automated fluorescent PCR method for detection of Shiga toxin-producing *Escherichia coli* in foods. *Appl. Environ. Microbiol.* **64**:4210–4216.
- Cox, T., C. Frazier, J. Tuttle, S. Flood, L. Yagi, C. T. Yamashiro, R. Behari, C. Paszko, and R. Cano. 1998. Rapid detection of *Listeria monocytogenes* in dairy samples utilizing a PCR-based fluorogenic 5' nuclease assay. *J. Ind. Microbiol. Biotechnol.* **21**:167–174.
- Gibson, N. J., C. R. Newton, and S. Little. 1997. A colorimetric assay for phosphate to measure amplicon accumulation in polymerase chain reaction. *Anal. Biochem.* **254**:18–22.
- Hein, I., D. Klein, A. Lehner, A. Bubert, E. Brandl, and M. Wagner. 2001. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. *Res. Microbiol.* **152**:37–46.
- Higuchi, R., G. Dollinger, P. S. Walsh, and R. Griffith. 1992. Simultaneous amplification and detection of specific DNA sequences. *Bio/Technology* **10**:413–417.
- Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilization the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* **88**:7276–7280.
- Ivarsson, K., and B. Weijdegard. 1998. Evaluation of the effects of DNase treatment on signal specificity in RT-PCR and in situ RT-PCR. *BioTechniques* **25**:630–638.
- Klein, P. G., and V. K. Juneja. 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Appl. Environ. Microbiol.* **63**:4441–4448.
- Koo, K., and L.-A. Jaykus. 2000. Selective amplification of bacterial RNA: use of a DNA primer containing mismatched bases near its 3' terminus to reduce false positive signals. *Let. Appl. Microbiol.* **31**:187–192.
- Koo, K., and L.-A. Jaykus. 2000. Modified method to detect PCR products by 5' nuclease activity and an asymmetric fluorogenic probe set. *BioTechniques* **29**:690–694.
- Lucore, L. A., M. A. Cullison, and L.-A. Jaykus. 2000. Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods. *Appl Environ Microbiol.* **66**:1769–1776.
- McKillip, J. L., and M. Drake. 2000. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. *J. Food Prot.* **63**:855–859.
- Nazarenko, I. A., S. K. Bhatnagar, and R. J. Hohman. 1997. A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* **25**:2516–2521.
- Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug. 2000. Application of a 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol.* **66**:4266–4271.
- Norton, D., and C. A. Batt. 1999. Detection of viable *Listeria monocytogenes* with a 5' nuclease PCR assay. *Appl. Environ. Microbiol.* **65**:2122–2127.
- Oberst, R. D., M. P. Hays, L. K. Bohra, R. K. Phebus, C. T. Yamashiro, C. Paszko-Kolva, S. J. A. Flood, J. M. Sargeant, and J. R. Gillespie. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (TaqMan) assay. *Appl. Environ. Microbiol.* **64**:3389–3396.
- Sharma, V. K., E. A. Dean-Nystrom, and T. A. Casey. 1999. Semi-automated fluorogenic PCR assays (TaqMan) for rapid detection of *Escherichia coli* O157:H7 and other Shiga toxinogenic *E. coli*. *Mol. Cell. Probes* **13**:291–302.
- Sharma, V. K., and S. A. Carlson. 2000. Simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single-enrichment-broth culture. *Appl. Environ. Microbiol.* **66**:5472–5476.
- Shearer, A. E., C. M. Strapp, and R. D. Joerger. 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**:788–795.
- Stewart, D., and S. M. Gendel. 1998. Specificity of the BAX polymerase chain reaction system for detection of the foodborne pathogen *Listeria monocytogenes*. *J. AOAC Int.* **81**:817–822.
- Tyagi, S., and F. R. Kramer. 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**:303–308.
- Whitcombe, D., J. Theaker, S. P. Guy, T. Brown, and S. Little. 1999. Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* **17**:804–807.
- Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **63**:3741–3751.
- Witham, P. K., C. T. Yamashiro, K. J. Livak, and C. A. Batt. 1996. A PCR-based assay for the detection of *Escherichia coli* Shiga-like toxin genes in ground beef. *Appl. Environ. Microbiol.* **62**:1347–1353.
- Wittwer, C. T., M. G. Herrmann, A. A. Moss, and R. P. Rasmussen. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques* **22**:130–138.