APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1995, p. 3724–3728 Vol. 61, No. 10
0099-2240/95/$04.00+0

Use of a Fluorogenic Probe in a PCR-Based Assay for the Detection of *Listeria monocytogenes*

HEIDI A. BASSLER,[1] SUSAN J. A. FLOOD,[2] KENNETH J. LIVAK,[2] JEFFREY MARMARO,[2] RUTH KNORR,[1] AND CARL A. BATT[1]*

Department of Food Science, Cornell University, Ithaca, New York 14853[1] and Applied Biosystems Division, Perkin Elmer, Foster City, California 94404[2]

Received 20 March 1995/Accepted 27 July 1995

A PCR-based assay for *Listeria monocytogenes* that uses the hydrolysis of an internal fluorogenic probe to monitor the amplification of the target has been formatted. The fluorogenic 5′ nuclease PCR assay takes advantage of the endogenous 5′→3′ nuclease activity of *Taq* DNA polymerase to digest a probe which is labelled with two fluorescent dyes and hybridizes to the amplicon during PCR. When the probe is intact, the two fluorophores interact such that the emission of the reporter dye is quenched. During amplification, the probe is hydrolyzed, relieving the quenching of the reporter and resulting in an increase in its fluorescence intensity. This change in reporter dye fluorescence is quantitative for the amount of PCR product and, under appropriate conditions, for the amount of template. We have applied the fluorogenic 5′ nuclease PCR assay to detect *L. monocytogenes*, using an 858-bp amplicon of hemolysin (*hlyA*) as the target. Maximum sensitivity was achieved by evaluating various fluorogenic probes and then optimizing the assay components and cycling parameters. With crude cell lysates, the total assay could be completed in 3 h with a detection limit of approximately 50 CFU. Quantification was linear over a range of 5×10^1 to 5×10^5 CFU.

Listeria monocytogenes is a food-borne bacterial pathogen responsible for severe and fatal infections in humans. It may enter the food chain by way of carrier animals that shed the organism in their milk and feces. *Listeria* spp. are ubiquitous in nature, but only *L. monocytogenes* poses a significant health risk. Cross-contamination, improper handling, and inadequate cooking all contribute to listeriosis infections in people.

Conventional detection methods include enrichment and isolation with selective media, sometimes in conjunction with incubation at a low temperature (see reference 4 for a review). Cold enrichment in nonselective broth was one of the earliest methods for isolating *Listeria* spp. Samples were held at 4°C for several months, allowing the growth of *L. monocytogenes*. To shorten the isolation time, media used to select for *Listeria* spp. containing ingredients including potassium tellurite, nalidixic acid, acriflavine, cycloheximide, phenylethanol, and lithium chloride have been formulated (4). *Listeria*-selective media, such as *Listeria* enrichment broth, McBride's, lithium chloride-phenylethanol-moxalactam, modified Vogel Johnson, and Oxford, have been developed (4). Confirmation of suspect *Listeria* colonies involves assessing hemolytic and biochemical reactions characteristic of *L. monocytogenes.*

Recent rapid methods for detection of *L. monocytogenes* include those based upon the use of antibodies and nucleic acid sequences that uniquely recognize this pathogen (4). DNA amplification methods based upon ligase chain reaction (15, 16) and PCR (1, 2, 5–8, 12–14) have been reported. These methods usually employ either visual scoring of ethidium bromide-stained agarose gels or post-PCR hybridization-capture assays.

A new fluorogenic PCR-based format which allows homogeneous quantification of the initial template concentration has been developed (11). TaqMan PCR takes advantage of the 5′→3′ nuclease activity of *Taq* DNA polymerase to digest a probe which is labelled with both a fluorescent reporter dye and a fluorescent quencher dye. The concept of utilizing the nucleolytic activity of *Taq* DNA polymerase to generate a quantifiable signal during amplification has been previously described (9, 10).

The Taqman Probe is designed to hybridize to an internal region within the amplicon. It is typically a 20- to 30-bp oligonucleotide with a fluorescent reporter dye (6-carboxyfluorescein [FAM], tetrachloro-6-carboxyfluorescein, or hexachloro-6-carboxyfluorescein) covalently attached to the 5′ end and a fluorescent quencher dye (6-carboxytetramethylrhodamine [TAMRA]) attached two or more bases downstream from the reporter dye. The proximally located quencher dye reduces the emission intensity of the reporter dye. Extension of the TaqMan Probe is prevented by phosphorylation of the 3′ end. The TaqMan Probe is added directly to the PCR mix, and conditions are virtually identical to those that are established for a standard PCR. As *Taq* DNA polymerase extends from the PCR primer, it cleaves the TaqMan Probe only when it is hybridized to the target, separating the reporter dye from the quencher dye. An increase in fluorescence intensity at 518 nm (when FAM is the reporter dye) (due to the release of the quenching effect on the reporter) is the result of TaqMan Probe hydrolysis and is quantitative for the initial amount of the template. The fluorescence intensity specific to the reporter dye increases because of its lack of proximity to the quencher dye. Repeated cycles of denaturation, annealing, and extension result in exponential amplification of the PCR product and of fluorescence intensity.

In this paper, we describe how a PCR assay specific for *L. monocytogenes* was formatted for 5′ nuclease PCR assay detection and analysis.

MATERIALS AND METHODS

Bacterial strain, medium, and culture. *L. monocytogenes* Scott A was used for the initial studies, while other *Listeria* spp. and *L. monocytogenes* strains were used for specificity testing (Table 1). The growth medium was *Listeria* repair

* Corresponding author. Mailing address: 413 Stocking Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-2896. Fax: (607) 255-8741. Electronic mail address: cab10@cornell.edu.

TABLE 1. *Listeria* strains used in the TaqMan PCR detection assay for *L. monocytogenes*

Strain	Source	ΔRQ[a]	yes or no[b]
L. monocytogenes			
Scott A	University of Minnesota	2.49	yes
BR1/93	Cornell University	2.21	yes
BR3/93	Cornell University	0.41	yes
BR17/93	Cornell University	2.39	yes
BR27/93	Cornell University	2.15	yes
L. ivanovii			
19119	American Type Culture Collection	0.01	no
L31	University of Vermont	0.00	no
L. innocua			
P5V5	University of Minnesota	0.00	no
LG5V5	USDA-ARS[c]	0.00	no

[a] The ΔRQ values were determined by using approximately 10^5 CFU per assay.

[b] A yes score is assigned when the ΔRQ is greater than the threshold calculated at the 99% confidence level for the standard deviation observed for a series of no-template controls.

[c] USDA-ARS, U.S. Department of Agriculture-Agricultural Research Service.

broth (3), with the addition of 51.4 mg of cycloheximide and 41.1 mg of nalidixic acid per liter (3). Cultures were grown at 37°C with shaking until they reached an optical density at 600 nm of 0.8.

Template preparations and dilutions. A 0.5-ml aliquot of culture was centrifuged in a 1.5-ml microcentrifuge tube at 12,000 × *g* for 10 min, and the supernatant was discarded. The cell pellet was resuspended in 95 μl of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), and lysozyme (2 mg/ml) was added. Cells were lysed at room temperature for 15 min. Proteinase K (200 μg/ml) was added, and the lysates were incubated at 55°C for 1 h; this was followed by boiling for 8 min. Lysates were stored at −20°C. Prior to centrifugation, the cultures were serially diluted in modified *Listeria* repair broth. CFU were enumerated by plating 0.1 ml of each dilution onto Oxford agar (Unipath, Ltd.) and incubating at 37°C for 2 days.

TaqMan Probe design and synthesis. The TaqMan Probes are listed in Fig. 1. The linker arm nucleotide phosphoramidite used for the coupling of the quencher dye was obtained from Glen Research (Sterling, Va.). Standard DNA phosphoramidites, 6-carboxyfluorescein (6-FAM) phosphoramidite, 6-carboxytetramethylrhodamine succinimidyl ester (TAMRA succinimidyl ester), and Phosphalink for attaching the 3′ blocking phosphate were obtained from the Applied Biosystems Division of Perkin Elmer (Foster City, Calif.). Oligonucleotide synthesis was performed with an ABI Model 394 DNA Synthesizer (Applied Biosystems Division, Perkin Elmer). TaqMan Probes were synthesized with 6-FAM phosphoramidite at the 5′ end, and the linker arm nucleotide replaced one of the thymidines in the sequence (Fig. 1). The Phosphalink was used to block the 3′ end. Following deprotection, the TAMRA succinimidyl ester was coupled to the linker arm nucleotide-containing oligonucleotide in 250 mM sodium bicarbonate buffer (pH 9.0) at room temperature. The unreacted dye was removed by using a PD-10 Sephadex column, and the probe was purified by preparative high-performance liquid chromatography with an Aquapore C_8 column operated with a linear gradient of 8 to 20% acetonitrile in 0.1 M triethylamine acetate.

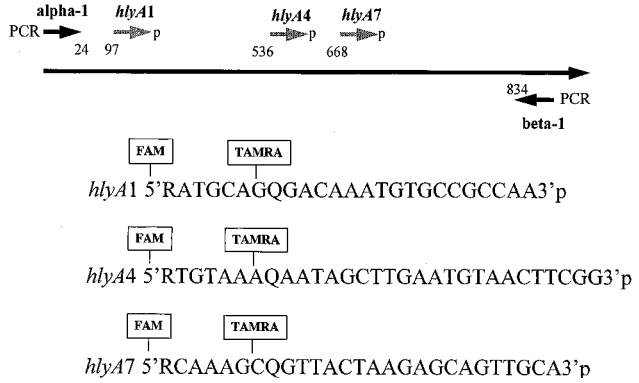

FIG. 1. Locations of PCR primers and TaqMan Probes used for amplification of the *L. monocytogenes hlyA*. FAM, reporter dye; TAMRA, quencher dye; p, phosphate cap. Numbers refer to the 3′ end of the PCR primers and to the 5′ end of the TaqMan Probes.

The TaqMan Probes were designed on the basis of some general rules as outlined by the manufacturer (Applied Biosystems Division, Perkin Elmer) that include keeping the G+C content in the 40 to 60% range and avoiding extensive hairpins or self-complementary regions. Runs of identical nucleotides, especially G's, and extensive regions of complementarity between probe and either PCR primer should be avoided. The probe should be designed so that the predicted T_m is at least 5°C higher than the T_m of the PCR primers. Finally, a G should not be the 5′-end nucleotide. TAMRA was placed 8 nucleotides to the 3′ end of the probe.

5′ nuclease PCR assay. The PCR conditions were a modification of the assay described by Bsat and Batt (2); however, the signal generated from the hydrolysis of a fluorogenic probe substituted for reverse dot blot detection. The location and sequence of TaqMan Probes as well as primers α-1 and β-1 are depicted in Fig. 1. PCRs were performed in 25-μl volumes, using MicroAmp vials, as follows. The assay mixture consisted of 1 μl of cell lysate; $MgCl_2$ (from 3.5 to 5.0 mM); 2.5 μl of 10× PCR buffer; 460 nM (each) primers α-1 and β-1; 200 μM (each) dATP, dCTP, dGTP; 400 μM dUTP, 0.625 U of *AmpliTaq* DNA polymerase (Perkin Elmer, Norwalk, CT); 0.25 U of uracil-*N*-glycosylase (AmpErase UNG; Perkin Elmer), and 200 nM TaqMan Probe. A thermocycler (GeneAmp PCR System 9600; Perkin Elmer) was used for amplification. All cycles began with 2 min at 50°C and then 2 min at 95°C. Following these initial steps, two-step cycles were 15 s at 95°C and 90 s at 60°C. The three-step cycles were 15 s at 95°C, 30 s at 60°C, and 72°C for 30 to 90 s. All samples were held at 72°C once cycling was complete, and all assays were performed at least in triplicate.

Post-PCR analysis. After PCR cycling, the fluorescence intensities of the reporter (i.e., FAM) and quencher (i.e., TAMRA) dyes were quantified. A 20-μl aliquot of the PCR product was transferred into a 96-well flat-bottomed white microtiter plate (Perkin Elmer) and diluted with 20 μl of water. The emission intensities were measured in a Perkin Elmer LS-50B luminescence spectrophotometer equipped with a microtiter plate reader. The entire plate was scanned at 518 nm (FAM) and then at 582 nm (TAMRA), with an excitation wavelength of 488 nm. The increase in the emission intensity at 518 nm when the template is added is due to the release of the FAM dye as the probe is hydrolyzed. The intensity at 582 nm remains relatively constant, as the emission intensity from the TAMRA dye is unaffected by hydrolysis of the probe. Data acquisition and analysis were handled by a personal-computer-based system by using the Fluorescence Data Manager (Perkin Elmer) and EXCEL (Microsoft Corporation, Redmond, Wash.) spreadsheets. The degree of probe hydrolysis was estimated by calculating the ΔRQ as follows: $\Delta RQ = RQ^{+} - RQ^{-}$, where

$$RQ^{+} = \frac{\text{Emission intensity of reporter (i.e., FAM } \lambda_{em} = 518 \text{ nm)}}{\text{Emission intensity of quencher (i.e., TAMRA } \lambda_{em} = 582 \text{ nm)}}$$

$$RQ^{-} = \frac{\text{Emission intensity of reporter (no template)}}{\text{Emission intensity of quencher (no template)}}$$

The ΔRQ is an increase in the ratio of the emission intensity of the unquenched reporter dye to that of the quencher dye after the release of the reporter dye from the TaqMan Probe (RQ^{+}) minus the average baseline emission intensity of the quenched reporter dye on the intact TaqMan Probe (RQ^{-}) after PCR in three no-template controls. A series of three no-template controls was used to establish the baseline ratio of emission intensities of the quenched reporter dye on the intact TaqMan Probe (RQ^{-}). A yes or no decision was assigned for each sample based upon the ΔRQ threshold calculated at a 99% confidence level, using the standard deviation obtained from the three no-template controls.

The PCR product was verified with ethidium bromide-stained agarose gels. Diluted PCR samples (10 μl) were mixed with 2 μl of loading dye (2.5 mg of bromophenol blue per ml, 2.5 mg of xylene cyanol per ml, 150 mg of Ficoll type 400 per ml) and loaded on 1.5% agarose gels. After the fluorescence spectra were measured, the PCR products were electrophoresed at 100 V for approximately 30 min, stained with ethidium bromide, and photographed.

RESULTS

Probe design and performance. The 5′ nuclease PCR assay is a modification of standard PCR by the addition of a fluorogenic probe to quantify PCR product formation during amplification. In formatting a 5′ nuclease PCR assay, the unique characteristics of the TaqMan Probe must be considered in both the design of the probe and the amplification conditions. The length of the probe as well as the separation between the FAM reporter and the TAMRA quencher can be varied. We

have found that these variations can affect quenching and/or the specificity of the probe.

The *L. monocytogenes* hemolysin (*hlyA*) was used as a target sequence, and PCR primers α-1 and β-1 which generate an 858-bp amplicon have been described previously (2). Three TaqMan Probes *hlyA*1, -4, and -7, which were 23, 29, and 26 nucleotides, respectively, were evaluated for detection of *L. monocytogenes* (Fig. 1). Based upon G+C content, the T_ms of TaqMan Probes *hlyA*1, -4, and -7 were calculated to be 70, 76, and 74°C, respectively. Each TaqMan Probe is labelled with FAM at the 5′ end, and a TAMRA was placed 7 nucleotides 3′ to FAM after oligonucleotide synthesis, using a succimide ester linkage.

Initial trials using *Listeria* enrichment broth revealed that acriflavine, a selective ingredient in the medium, had an emission intensity that overlapped with that of FAM (data not shown). The high emission intensities at 518 nm that were observed for non-*L. monocytogenes* templates were probably due to the acriflavine, which intercalates into DNA. Therefore, a modified *Listeria* repair broth which does not contain acriflavine was used; however, cycloheximide plus nalidixic acid were used as selective agents.

Initial trials with *hlyA*1, -4, and -7 revealed that only the *hlyA*1 TaqMan Probe yielded a significant ΔRQ upon the addition of the *L. monocytogenes* template. The ΔRQ for the *hlyA*1 probe was 2.42, compared with 0.14 and 0.22 for the *hlyA*4, and -7 probes, respectively. The RQ^- values for the three probes *hlyA*1, -4, and -7 were 1.0, 0.31, and 0.81, respectively, indicating that the degree to which FAM was quenched by TAMRA was not the source of the lower ΔRQs for *hlyA*4 and -7. The differences in the ΔRQs could be a function of their sequence (and T_m) as well as their distance from the α-1 PCR primer. *hlyA*1 was, however, initially selected for assay optimization.

Effect of $MgCl_2$ concentration, extension time, and number of cycles on ΔRQ. The TaqMan Probe is a phosphorylated, nonextendable oligonucleotide, and therefore, its T_m does not increase during amplicon extension as is the case for standard PCR primers (11). Also, the quenching ability of TAMRA is enhanced as the $MgCl_2$ concentration is increased (11). Both the annealing and extension temperatures as well as the $MgCl_2$ concentration can, therefore, affect not only the amplification of the target sequence but also the annealing of the TaqMan Probe. Only when the TaqMan Probe is hybridized to the target will it be hydrolyzed by the *Taq* DNA polymerase as it proceeds along the target strand. The effect of $MgCl_2$ concentration, extension time, and number of cycles on ΔRQ was evaluated for the *hlyA*1 probe. When the $MgCl_2$ concentration was varied from 3.5 to 5.0 mM, the ΔRQ increased from 2.29 to 2.89 for a three-step PCR after 40 cycles. The RQ^- values over this $MgCl_2$ concentration did not vary; therefore, the differences in the ΔRQ values were a function of probe hydrolysis. Increasing the extension time from 30 to 60 s resulted in an increase in ΔRQ from 1.68 to 2.29. No proportional increase in ΔRQ was realized when the extension time was lengthened to 90 s (ΔRQ = 2.40).

The 5′ nuclease PCR assay is quantitative, since in theory, each time the *Taq* DNA polymerase extends from the PCR primer and encounters the fluorogenic probe, it will release the FAM reporter dye. Therefore, the ΔRQ should increase with each cycle until one of the PCR components becomes limiting. The ΔRQ after 40 cycles was compared with that after 50 cycles, using the *hlyA*1 probe in a three-step PCR (Fig. 2). After 40 cycles, the ΔRQ increased from 0.04 to 2.20 as the template concentration was increased from 5×10^1 to 5×10^6 CFU, while after 50 cycles it increased from 0.00 to 2.96 over the same range of template concentrations. The ΔRQ increased proportionately with increasing starting template concentration. After 50 cycles, the ΔRQ values were 0.3- to 10-fold higher than after 40 cycles, with the largest differences observed at the higher template concentrations.

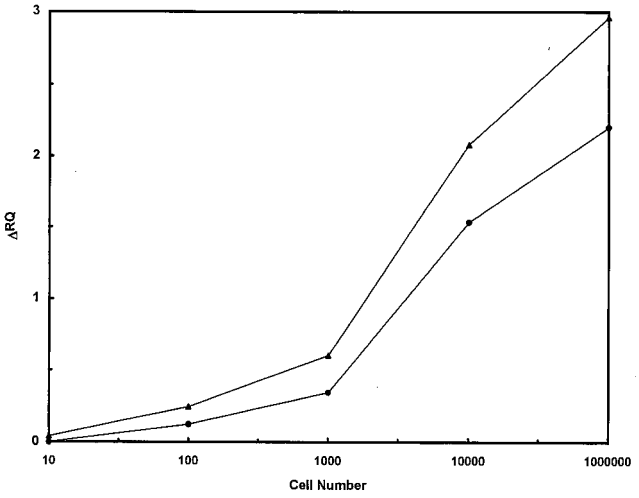

FIG. 2. Effect of the number of cycles on ΔRQ. The PCR was carried out with the *hlyA*1 probe, 5 mM $MgCl_2$, 10^1 to 10^6 CFU of *L. monocytogenes*, and a three-step cycle for a total of 40 (●) or 50 (▲) cycles. Samples were run in triplicate. Cell numbers were determined by plate counts on *Listeria* enrichment broth agar.

Effect of two-step versus three-step PCR cycling. In order to get cleavage of the TaqMan Probe and generate an increased fluorescent signal, the probe must be hybridized to the template, while the *Taq* DNA polymerase extends from the primer through the target sequence (9, 10). Due to the TaqMan Probe's inability to be extended by the *Taq* DNA polymerase, its T_m does not increase. Therefore, as the PCR temperature is increased between the annealing and extension steps, the TaqMan Probe may not remain hybridized during extension if the temperature of the latter step is higher than its T_m. A two-step PCR was compared with a three-step PCR to determine its effect on the ΔRQ for the *hlyA*1, -4 and -7 probes. In the two-step PCR, the annealing and extension steps were combined and carried out at 60°C. In the three-step PCR, the annealing step was at 60°C, and the extension step was at 72°C. The three-step method yielded a higher ΔRQ relative to the two-step method for TaqMan Probe *hlyA*1. The ΔRQ values were 2.17 for the two-step method and 2.42 for the three-step method. In contrast, the ΔRQs were higher for the two-step method than for the three-step method with TaqMan Probes *hlyA*4 and -7. The ΔRQ values for TaqMan Probe *hlyA*4 were 0.37 and 0.14, and for TaqMan Probe *hlyA*7 they were 0.54 and 0.22, for the two-step and three-step PCRs, respectively. Regardless of whether two-step or three-step cycling was used, TaqMan Probe *hlyA*1 consistently generated higher ΔRQ values upon hydrolysis than either TaqMan Probe *hlyA*4 or -7.

TaqMan sensitivity and specificity. The relative sensitivity and specificity of the *L. monocytogenes* 5′ nuclease PCR assay optimized for the *hlyA*1 probe was examined. The effect of the initial *L. monocytogenes* cell template concentration on the ΔRQ was measured. Four independent sets of *L. monocytogenes* culture dilutions varying from 5×10^0 to 5×10^6 CFU were prepared, and assays were carried out and then replicated on different days. Figure 3 shows a positive linear relationship ($r^2 = 0.91$) between the log number of cells and ΔRQ, over a

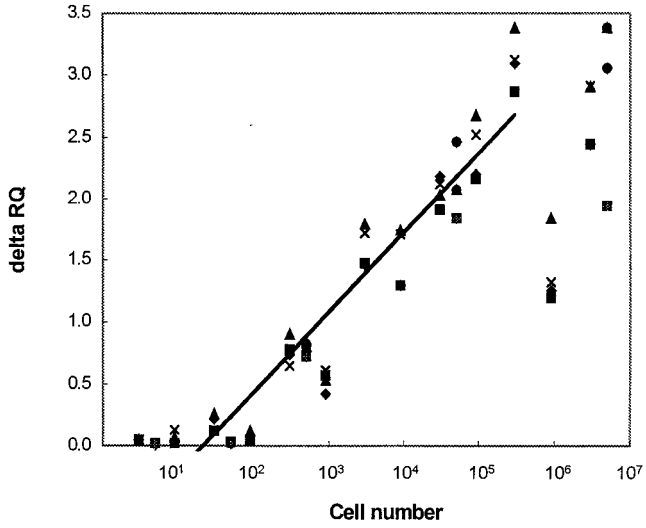

FIG. 3. Quantification of *L. monocytogenes* cells. The PCR was carried out with the *hlyA*1 probe, 5 mM $MgCl_2$, 10^0 to 10^6 CFU of *L. monocytogenes*, and a total of 40 three-step cycles. Lysates were prepared from two different cultures at different times. These templates were assayed on two different days in duplicate. The first number refers to the day, the letter refers to the time, and the last number refers to the experiment number. ◆, 1-A-1; ■, 1-A-2; ▲, 2-A-1; x, 2-A-2; ●, 1-B-1; +, 1-B-2; ○, 2-B-1. A line calculated by linear regression of the data excluding $>5 \times 10^5$ CFU of *L. monocytogenes* is shown. Cell numbers were determined by plate counts on *Listeria* enrichment broth agar.

range of 5×10^0 to 5×10^5 CFU. Good reproducibility was observed in this range with different template concentrations and between assays carried out on different days. Above 5×10^5 CFU, the ΔRQ was highly variable between replicates, presumably because of template saturation.

A yes or no decision is rendered on the basis of the significance of the ΔRQ for a given template preparation. This is calculated at a 99% confidence interval, using the standard deviation for RQ^- that is obtained for 5′ nuclease PCR carried out in the absence of template. A yes score was consistently assigned for *L. monocytogenes* down to 100 CFU, suggesting that this calculated threshold was suitable for concluding that a sample was positive for *L. monocytogenes*. In all cases where a yes score was assigned, an 858-bp band was observed after gel electrophoresis (Fig. 4). In contrast, for all 5′ nuclease PCR assays receiving a score of no, no PCR product was observed, suggesting that the yes-no threshold was functionally equivalent to a visual scoring of the PCR product by gel electrophoresis.

The specificity of the 5′ nuclease PCR assay for *L. monocytogenes* was determined by using a limited number of other *Listeria* spp., including *L. innocua* and *L. ivanovii* (Table 1) (2). All templates were initially diluted 1:50, to approximately 10^5 CFU per assay. For both the *L. innocua* and *L. ivanovii* assays, the ΔRQ values were less than 0.01, which was less than the threshold ΔRQ, and hence they received a score of no. The assays containing template prepared from different *L. monocytogenes* strains ranged from 0.41 to 2.49 and all received a score of yes. The lowest value was for *L. monocytogenes* BR3/93, while all of the other ΔRQs were between 2.15 and 2.49. Differences in the ability of these strains to be lysed by lysozyme may explain the low ΔRQ for the BR3/93 strain. At a 1:10 template dilution (5×10^5 cells), *L. innocua* P5V5 had a ΔRQ of 0.05, and, therefore, using the ΔRQ threshold, this strain would receive a score of yes, while the other *L. innocua* strain and both *L. ivanovii* strains received scores of no. If the ΔRQ threshold is to be used to score positive and negative samples, some initial template dilution is required to bring the non-*L. monocytogenes* ΔRQs to below this value. Alternatively, a ΔRQ threshold of 0.1 (at a 99% confidence level) would easily segregate the *L. monocytogenes* from the non-*L. monocytogenes* results, with a sensitivity of approximately 50 cells as a conservative estimate.

DISCUSSION

In the present study a TaqMan system was applied to detect *L. monocytogenes*, and the assay has a number of unique features that makes it attractive for routine usage. The specificity of the PCR primers and reaction which were used in this assay has already been documented and was supported by our data (2). Significant ΔRQs and amplification products, the latter based on ethidium bromide-stained agarose gels, were observed only for *L. monocytogenes* and not for other *Listeria* spp. Integrated into the 5′ nuclease PCR assay is uracil-*N*-glycosylase (AmpErase UNG), which prevents carryover contamination, a potential problem when any PCR-based assay is used in routine testing. The PCR can be quantified immediately after thermocycling by transferring the contents to a microtiter plate. Scans of the emission spectra at 518 and 582 nm can be accomplished in less than 10 min, and the software has been configured to allow rapid data analysis. A total of 92 samples (plus the three no-template controls) can easily be handled, using multichannel pipettors, and analyzed in a single microtiter plate. The EXCEL-based spreadsheet assigns a yes or no decision to each sample; the threshold ΔRQ for this decision is based upon a 99% confidence level, using the standard deviation of the three no-template controls.

The linearity of the TaqMan system using the *hlyA*1 probe was good over a range of 5×10^0 to 5×10^5 CFU of *L. monocytogenes. L. monocytogenes* could be easily detected at approximately 50 CFU, while template concentrations became saturating above 5×10^5 CFU. The ΔRQ data for $>5 \times 10^5$ CFU are scattered and not reproducible. Saturation presumably occurs because one or more reagents in the PCR mix become exhausted within the first few cycles, which limits amplification and TaqMan Probe hydrolysis. The yes-no score assignment appears to be accurate in scoring positive and negative samples; however, an initial template dilution is required, since non-*L. monocytogenes* samples can yield ΔRQ values sufficiently high as to receive a score of yes. It should, however, be noted that these represent in excess of 5×10^5 copies of the template. This is, however, based upon the assumption that CFU equates with template copy number.

The cycling parameters for each 5′ nuclease PCR assay must be optimized to maximize the ΔRQ. Possible factors contributing to an assay's performance include the T_m of the TaqMan

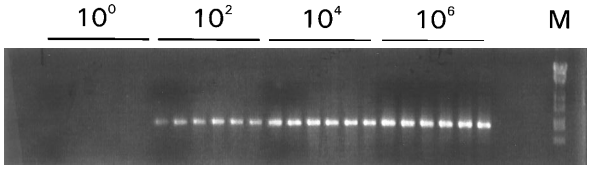

FIG. 4. Gel electrophoretic analysis of amplification products from TaqMan PCR assay using from 10^0 to 10^6 CFU of *L. monocytogenes*. Six replicates of the PCR after fluorometric scanning were separated on a 1.5% agarose gel at 100 V in Tris-acetate-EDTA buffer. λ DNA digested with *Eco*RI-*Hin*dIII was used as a size marker (M). The gel was stained with 1 mg of ethidium bromide per ml and illuminated with long UV-irradiation. The stained PCR products were documented by using a charge-coupled device camera (17).

Probe and its distance from the upstream PCR primer. TaqMan Probes *hlyA*1, -4, and -7 are located 73, 512, and 644 bases downstream from the α-1 PCR primer, respectively. Their predicted T_ms are 70, 76, and 74°C, respectively. In a standard PCR, each amplification cycle usually consists of three steps: denaturation of template, annealing of the primer to the target, and extension of the primer. The extension temperature is typically 72°C, the optimal temperature for *Taq* DNA polymerase activity, while the annealing temperature is set below the T_m of the primers. After the annealing step, as the temperature approaches 72°C, the primers begin to extend and their T_m increases. The TaqMan Probe, however, is not extended and may dissociate from the target as the reaction temperature increases above its T_m before it is hydrolyzed by *Taq* DNA polymerase. A TaqMan Probe with a T_m lower than the set extension temperature would be more likely to dissociate during this stage, thereby preventing its hydrolysis. This problem was believed to be the source of the relatively low ΔRQs with TaqMan Probes *hlyA*4 and -7 during our initial experiments and was overcome by a two-step PCR, where the annealing and extension steps were combined and were both carried out below the probe T_m. The ΔRQ values for the two-step PCR for TaqMan Probes *hlyA*4 and -7 were 2.64- and 2.45-fold higher than those for the 3-step PCR, respectively. The three-step PCR was better for TaqMan Probe *hlyA*1, despite its having the lowest predicted T_m of the three TaqMan Probes used. The ΔRQ for the three-step PCR with probe *hlyA*1 was slightly higher than that for the two-step PCR. TaqMan Probe *hlyA*1 is located only 73 bases downstream from the α-1 primer, and it may be hydrolyzed by *Taq* DNA polymerase either during the 60°C annealing step or before the temperature reached 72°C in the extension step. Obviously, amplification and therefore probe hydrolysis must occur at 60°C, since the two-step PCR functions at that temperature. The kinetics of probe hydrolysis need to be examined further, but as a general rule in this PCR system, it appears that the TaqMan Probe should be positioned as close to the upstream PCR primer as is feasible.

Magnesium chloride concentration and extension time were adjusted to maximize ΔRQ values. The $MgCl_2$ affects annealing and the T_m of both the TaqMan Probe and the PCR primers. It also affects the activity of *Taq* DNA polymerase and FAM quenching by TAMRA on the probe. Increasing the $MgCl_2$ concentration sometimes enhances TAMRA quenching by allowing the probe to form stable secondary structures, thus bringing TAMRA closer to FAM. This alters the RQ^- and changes the ΔRQ for the assay (11). No effect of $MgCl_2$ concentration on the RQ^- was observed with the *hlyA*1, -4, and -7 TaqMan Probes, which may be because of the limited spacing between FAM and TAMRA. Therefore, the effect of $MgCl_2$ concentration was due to either probe or PCR primer binding, with the former being more likely because of an overall consistency in the amount of PCR product formed (data not shown). Among other factors affecting assay performance, increasing the PCR extension time to 60 s lengthened the overall assay time to 3 h but increased the relative ΔRQ values.

The TaqMan system has been optimized for the detection of *L. monocytogenes*. A major obstacle of other nucleic acid-based detection methods is the difficulty in quantifying the initial template concentration. In this assay, the TaqMan system reliably demonstrated its ability to detect and quantitate *L. monocytogenes* in pure culture. The addition of competing organisms does not affect the assay until the ratio of competing-to-target organisms exceeds 10^6 (data not shown). Above this level, nonspecific binding of either the PCR primer or the probe is likely to adversely affect the performance of the assay. The TaqMan system is not only fast and easy to perform but also unique in its ability to quantify the amount of template. This method is robust and can probably be applied to the detection of other food and environmental pathogens, easily substituting for the current detection scheme in standard PCR assays.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of the Applied Biosystems Division of Perkin Elmer and the enthusiastic support of Mike Lucero and Lincoln McBride.

REFERENCES

1. **Bessesen, M. T., Q. Luo, H. A. Rotbart, M. J. Blaser, and R. T. I. Ellison III.** 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. Appl. Environ. Microbiol. **56:**2930–2932.
2. **Bsat, N., and C. A. Batt.** 1993. A combined modified reverse dot-blot and nested PCR assay for the specific non-radioactive detection of *Listeria monocytogenes*. Mol. Cell. Probes **7:**199–207.
3. **Busch, S. V., and C. W. Donnelly.** 1992. Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocua*. Appl. Environ. Microbiol. **58:**14–20.
4. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
5. **Fitter, S., M. Heuzenroeder, and C. J. Thomas.** 1992. A combined PCR and selective enrichment method for rapid detection of *Listeria monocytogenes*. J. Appl. Bacteriol. **73:**53–59.
6. **Fluit, A. C., R. Torensma, M. J. C. Visser, C. J. M. Aarsman, M. J. J. G. Poppelier, B. H. I. Keller, P. Klapwijk, and J. Verhoef.** 1993. Detection of *Listeria monocytogenes* in cheese with the magnetic immuno-polymerase chain reaction assay. Appl. Environ. Microbiol. **59:**1289–1293.
7. **Furrer, B., U. Candrian, C. H. Hoefelien, and J. Luethy.** 1991. Detection and identification of Listeria monocytogenes in cooked sausage products and in milk by in vitro amplification of haemolysin gene fragments. J. Appl. Bacteriol. **70:**372–379.
8. **Golsteyn-Thomas, E. J., R. K. King, J. Burchak, and V. P. J. Gannon.** 1991. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. Appl. Environ. Microbiol. **57:**2576–2580.
9. **Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand.** 1991. Detection of specific polymerase chain reaction product by utilizing the 5′→3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. USA **88:**7276–7280.
10. **Lee, L. G., C. R. Connell, and W. Bloch.** 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. **21:**3761–3766.
11. **Livak, K. J., S. J. A. Flood, J. Marmaro, W. Giusti, and K. Deetz.** 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. **4:**357–362.
12. **Niederhauser, C., U. Candrian, C. Hoefelein, M. Jermini, H. P. Buehler, and J. Luethy.** 1992. Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Appl. Environ. Microbiol. **58:**1564–1568.
13. **Rossen, L., K. Holmstrom, J. E. Olsen, and O. F. Rasmussen.** 1991. A rapid polymerase chain reaction (PCR)-assay for the identification of *Listeria monocytogenes* in food samples. Int. J. Food Microbiol. **14:**145–152.
14. **Starbuck, M. A. B., P. J. Hill, and G. S. A. B. Stewart.** 1992. Ultrasensitive detection of *Listeria monocytogenes* in milk by the polymerase chain reaction (PCR). Lett. Appl. Microbiol. **15:**248–252.
15. **Wiedmann, M., F. Barany, and C. A. Batt.** 1993. Detection of *Listeria monocytogenes* with a nonisotopic polymerase chain reaction-coupled ligase chain reaction assay. Appl. Environ. Microbiol. **59:**2743–2745.
16. **Wiedmann, M., J. Czajka, F. Barany, and C. A. Batt.** 1992. Discrimination of *Listeria monocytogenes* from other *Listeria* species by ligase chain reaction. Appl. Environ. Microbiol. **58:**3443–3447.
17. **Winans, S. C., and M. J. Rooks.** 1993. Sensitive, economical laboratory photodocumentation using a standard video camera and thermal printer. Biotechniques **14:**902.