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Transcriptional Enhancement of the *Listeria monocytogenes* PCR and Simple Immunoenzymatic Assay of the Product Using Anti-RNA:DNA Antibodies†

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A method was developed to enhance the sensitivity of a *Listeria monocytogenes* PCR detection system by in vitro transcription of amplicons incorporating bacteriophage T7 RNA polymerase promoter sequences in one of the priming oligonucleotides. The resulting transcript can be detected by hybridization with a DNA probe immobilized in the wells of a microtiter plate, followed by immunoenzymatic assay of the RNA-DNA hybrids with an anti-RNA-DNA hybrid antibody. This highly sensitive method was reactive in the assay of various *L. monocytogenes* isolates but not with other *Listeria* or non-*Listeria* species.

The amplification of specific DNA sequences by the PCR technique has been used widely for the rapid and sensitive detection of bacterial food pathogens (3, 7, 12). Although PCR has great potential as a very sensitive and specific technique, in some instances permitting the detection of fewer than 10 cells (e.g., enterotoxigenic *Escherichia coli*) per reaction (17), it has not always been possible to achieve this level of sensitivity in its application to the detection of pathogens in enrichment cultures of food and environmental samples. Limitations on the ability of the PCR to detect pathogens may result partly from the presence of inhibitors in the enrichment broth and sample matrix (13) and other more mundane possibilities, such as the quality of the *Taq* DNA polymerase used and the limited volume of sample (e.g., a few microliters) which can be introduced into the PCR mixture. The sensitivity of the PCR can be enhanced by detection of the product with DNA probes targeting the amplified sequences (4, 8). However, such methods usually involve labor-intensive DNA-DNA hybridization procedures and require the use of probes labelled with detectable chemical or radioactive moieties, which can be costly to prepare and difficult to standardize. Furthermore, DNA probes targeting the PCR product sometimes produce only a moderate improvement in the sensitivity of the test, which may still require more than 10 to several hundred cells per reaction in order to give a detectable signal (1, 5). Therefore, there is a great need for simple and inexpensive methods to augment the sensitivity and reliability of PCR tests, particularly for the food industry and other users who must routinely process large numbers of samples.

Before the introduction of the automated PCR amplification technique with the thermostable enzyme *Taq* DNA polymerase (14), an elaborate method was developed to enhance the sensitivity of a human immunodeficiency virus PCR system. This method involved appending bacteriophage promoter sequences to one of the priming oligonucleotides, resulting in the generation of amplicons, which were subsequently transcribed in vitro to give further amplification of the PCR product (11). Transcripts incorporating a radioactively labelled ribonucleotide were sensitively analyzed by combined electrophoresis and autoradiography. Despite the resulting improvement in the detection ability of the PCR, this method would be of limited use for routine PCR analyses, especially for the food industry and other large-scale users, since it is difficult to automate, requires extensive manipulation of the sample in carrying out the amplification, and relies on the use of a hazardous radioisotope.

This article describes a simple method for direct transcriptional enhancement of the automated PCR and a rapid, inexpensive immunoenzymatic detection system that uses an antibody recognizing the RNA-DNA hybrids formed upon hybridization of the transcription product with probe DNA immobilized in the wells of a microtiter plate. The availability of antibodies recognizing RNA-DNA hybrid helices (15, 16) obviates the need to introduce chemical labels on either of the reacting nucleic acid strands in this assay, since the specific hybridization of the RNA transcripts with the immobilized complementary DNA probe should form a suitable structure for immunospecific detection (2). As an example of the applicability of this transcriptional enhancement and immunoenzymatic assay system in automated PCR analyses, the amplification of DNA sequences from the *Listeria monocytogenes*-specific *hlyA* gene encoding the well-known virulence factor listeriolysin O (9) was studied.

Bacterial strains and media. The bacteria used in this study included 2 *L. monocytogenes* reference strains (nonhemolytic type strain ATCC 15313 and ATCC 43256) and 11 *L. monocytogenes* isolates from egg, dairy, and environmental samples collected by Canadian Government inspection staff and submitted for routine microbiological analysis by the Laboratory Services Division, Agriculture and Agri-Food Canada. Unless otherwise stated, experiments were routinely carried out with an *L. monocytogenes* isolate from cheese. Other *Listeria* spp. examined include three strains of *L. innocua*, two strains of *L. ivanovii*, two strains of *L. seeligeri*, and one strain each of *L. welshimeri*, *L. murrayi*, and *L. grayi*. Additionally, several gram-positive and gram-negative non-*Listeria* organisms were examined, including *Streptococcus thermophilus* ATCC 19258, *Lactobacillus casei* ATCC 393, *Lactococcus lactis* ATCC 19257, *Micro-*

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coccus luteus ATCC 9341, *Enterococcus faecalis* ATCC 19433, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 10145, *E. coli* ATCC 11775, *Salmonella typhimurium* LT2 (ATCC 19585), and *Yersinia enterocolitica* ATCC 9610. All bacteria were routinely grown by inoculating a single colony from brain heart infusion (BHI; Difco) agar into tryptic soy broth (TSB; BDH) and shaking for 24 h at 30°C for *Listeria*, *Lactococcus*, *Micrococcus*, *Bacillus*, and *Yersinia* spp. and at 37°C for all other bacteria. Viable-cell counts were obtained by plating serial dilutions of the broth cultures on BHI agar.

PCR. Bacterial lysates and purified chromosomal DNA were prepared for PCR as previously described (2). Primers for the PCR were selected from the published nucleotide sequence of the *hlyA* gene (9). A 730-bp fragment spanning nucleotides 680 to 1411 (encompassing the two *Hin*dIII sites) was amplified by using a 21-mer forward primer, 5′-CATTA GTGGAAAGATGGAATG-3′ (primer A), and a 20-mer reverse primer, 5′-GTATCCTCCAGAGTGATCGA-3′ (primer B). The synthesis of these primers and the conditions used in the PCR amplification of *hlyA* sequences were described previously (2). To minimize the possible inhibitory effects of enrichment broth components (13), 10 μl of sample was routinely mixed with PCR mixture to give a total volume of 100 μl.

PCR-TE. For PCR with transcriptional enhancement (PCR-TE), the PCR was performed as described above except that the forward primer was replaced with an identical oligonucleotide having an additional 26 nucleotides corresponding to the T7 RNA polymerase promoter sequences appended to the 5′ end, giving the 47-mer forward primer 5′-AATTTAATACGACTCACTATAGGGATCATTAGTG GAAAGATGGAATG-3′ (primer A-T7). (The T7 RNA polymerase binding and preferred transcription initiation sites [6] are indicated by underscoring.) The use of this primer in combination with reverse primer B yielded a 756-bp amplicon which was transcribed by T7 RNA polymerase as follows: 25 μl of PCR product was mixed with 25 μl of transcription reaction mix (TRM; RNase-free deionized distilled water containing 80 mM Tris-HCl [pH 7.5], 12 mM $MgCl_2$, 4 mM spermidine, 20 mM NaCl, 20 mM dithiothreitol, 1 mM each of the four ribonucleotides [Promega; no. P1221], 10 U of T7 RNA polymerase [Promega; no. P2075], and 20 U of RNasin [Promega; no. N2511] per 25 μl) and incubated at 37°C for 2 h. The PCR-TE product was then analyzed by subjecting 10 μl of the mixture to agarose gel electrophoresis or by immunoenzymatic assay on a microtiter plate, as described below.

Preparation of probe DNA and immobilization on microtiter plate. An *hlyA*-specific DNA capture probe for the immunoenzymatic assay of the PCR-TE products was prepared by PCR with primers A and B and 10 ng of purified *L. monocytogenes* chromosomal DNA as the template. The 730-bp PCR product was digested with *Hin*dIII to give a 657-bp fragment devoid of primer-complementary sequences. This fragment (probe DNA) was purified on Magic PCR Preps columns (Promega; no. A7170) and stored as a 1-μg/μl stock in deionized distilled water at −20°C. The wells of a microtiter plate (Dynatech Laboratories, Inc.; no. 011-010-3350) were coated with probe DNA by a modification of the method of Morrissey and Collins (10). The probe DNA was denatured by heating at 100°C for 10 min and then diluted to 2 μg/ml in ice-cold coating buffer (0.3 M Tris-HCl [pH 8.0] containing 0.5 M $MgCl_2$ and 1.5 M NaCl). Microtiter plate wells were incubated with 100 μl of this coating buffer solution containing 0.2 μg of probe DNA at 37°C for 16 h. The wells were emptied and air dried, and the DNA was then cross-linked by exposure to UV light (254 nm) for 3 min. The wells were then washed three times with wash buffer (0.1 M Tris-HCl [pH 8.0] containing 2 mM $MgCl_2$, 1 M NaCl, and 0.1% [vol/vol] Tween 20) and blocked by incubation with 100 μl of hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% [wt/vol] protein blocking reagent, 0.1% [wt/vol] *N*-lauroylsarcosine, and 0.02% [wt/vol] sodium dodecyl sulfate) at 37°C for 1 h. The wells were then washed three times with 0.01 M phosphate-buffered saline (pH 7.2) (0.85% NaCl) containing 0.05% (vol/vol) Tween 20 (PBST) and air dried, and the plate (probe DNA plate) was stored sealed at 4°C until use.

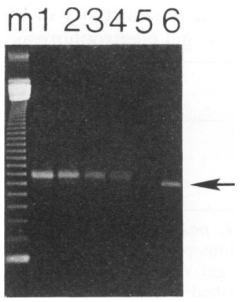

FIG. 1. Optimum reaction time for transcriptional enhancement of PCR-amplified *hlyA* sequences. PCR buffer (25 μl) containing 0.1 μg of purified 756-bp template DNA was mixed with 25 μl of TRM and incubated at 37°C for various periods of time; 10 μl of product was analyzed by electrophoresis on a 1.2% agarose gel as described in the text. Incubation times: lane 1, 3 h; lane 2, 2 h; lane 3, 1 h; lane 4, 0.5 h; lane 5, 0 h. Lane m, 123-bp ladder DNA marker; lane 6, 0.07 μg of 756-bp template DNA (arrow).

Immunoenzymatic assay. Samples (50 μl) of PCR-TE product were mixed with 50 μl of hybridization solution containing 50% (vol/vol) formamide in the wells of a probe DNA plate. The plate was incubated at 56°C for 1 h and then washed with PBST as described above. Subsequent reactions were carried out at room temperature. To detect RNA-DNA hybrids formed on the plate, the wells were incubated with 100 μl of goat anti-RNA-DNA immunoglobulin G (IgG) (16) at 5 μg/ml in PBST containing 0.2% (wt/vol) protein blocking reagent (PBST-B) for 20 min, washed with PBST, and incubated for a further 20 min with 100 μl of anti-goat IgG-peroxidase conjugate (Sigma; no. A-3540) diluted 1:2,000 in PBST-B. After a final wash with PBST, the wells were incubated with 100 μl of tetramethylbenzidine (TMB) microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Inc.; no. KP-50-76-00) for 20 min. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4, and the A_{450} of the wells was measured with a scanning microtiter plate autoreader (Bio-Tek Instruments, model EL900).

Time course of the TE reaction. The optimum reaction time for the transcriptional enhancement of a 756-bp DNA template generated by PCR amplification of *hlyA* sequences with primers A-T7 and B was determined by incubating a fixed quantity of the PCR DNA with TRM for various periods of time. Figure 1 shows that the product of the transcription reaction obtained at all incubation times (Fig. 1, lanes 1 to 4) was of a uniform size, suggesting that the transcripts were complete. The amount of transcript produced increased with the incubation time, with maximum production (as judged by the intensity of the transcript bands in the agarose gel)

TABLE 1. Comparative sensitivity of PCR-based methods with agarose gel electrophoresis analysis[a]

Primers	Transcriptional enhancement	Minimum no. of cells[b]
A + B	No	980 ± 185
A-T7 + B	No	1,090 ± 210
A-T7 + B	Yes	185 ± 50

[a] Serial dilutions of *L. monocytogenes* cells in TSB were subjected to PCR amplification with various primers, as indicated. The PCR product was then analyzed by agarose gel electrophoresis before and after transcriptional enhancement, as described in the text.

[b] Minimum number of cells per reaction giving a visible product (mean ± standard deviation, $n = 3$).

occurring after 2 to 3 h of incubation with the TRM (Fig. 1, lanes 1 and 2). No qualitative difference in the amount of transcript produced was discernible after 3 h of incubation (data not shown). Subsequent experiments were performed with a 2-h incubation.

The specificity of initiation of the transcription reaction at the T7 promoter sequences was confirmed by incubation of the TRM with 0.1 μg of a 730-bp amplicon (devoid of the T7 promoter sequences) generated by PCR amplification of *hlyA* sequences with primers A and B. No detectable transcript was produced after a 2-h incubation (data not shown).

Comparative sensitivity of the TE reaction. The improvement in the sensitivity of detection of *L. monocytogenes* cells by transcriptional enhancement of the PCR product was studied by agarose gel electrophoresis analysis of the amplification products and hybridization of the TE reaction products with *hlyA* probe DNA immobilized in the wells of a microtiter plate and subsequent immunoenzymatic assay with an anti-RNA-DNA hybrid antibody. Table 1 shows that the limit of detection for *L. monocytogenes* cells was essentially the same (ca. 1,000 cells) whether *hlyA* sequences were amplified by PCR with primer set A plus B (730-bp product) or A-T7 plus B (756-bp product) when the products were analyzed by agarose gel electrophoresis. When the PCR product obtained by using primers A-T7 and B was subjected to the TE reaction, the RNA transcript from as few as ca. 185 cells could be visualized on the gel (Table 1). No evidence of the 756-bp PCR DNA could be discerned at this cell level. A minimum of 10 to 12 ng of purified 730-bp and 756-bp PCR DNA could be visualized on a gel, whereas the TE reaction product from a minimum of 1.2 ng of 756-bp DNA could be visualized on the same gel (data not shown). Thus, transcriptional enhancement of the PCR and analysis of the products by agarose gel electrophoresis increased the sensitivity ca. 5-fold for the detection of *L. monocytogenes* cells and ca. 8- to 10-fold for the detection of purified 756-bp PCR DNA.

The detection of TE reaction product by hybridization with an *hlyA* DNA probe immobilized in the wells of a microtiter plate and immunoenzymatic assay of the resulting RNA-DNA hybrids with an anti-RNA-DNA detector antibody was studied. The presence of as little as 0.15 ng of purified 756-bp PCR DNA in the TE reaction gave a detectable response (A_{450}) above the background (no DNA) in the immunoenzymatic assay of the TE reaction product (Fig. 2). This represents a ca. 10-fold improvement in the overall sensitivity of the procedure compared with analysis by agarose gel electrophoresis. The specificity of this assay was confirmed by omitting the 756-bp PCR DNA from the TE reaction and adding either 0.1 μg of purified bacteriophage

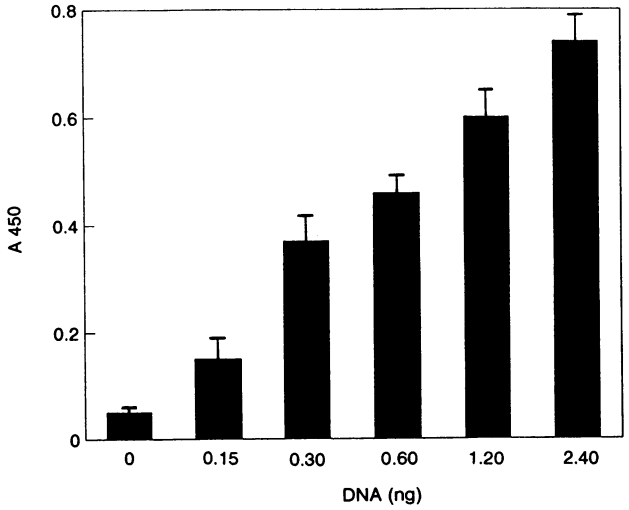

FIG. 2. Sensitivity of detection of the PCR-generated DNA template by combined transcriptional enhancement and immunoenzymatic assay. Purified 756-bp PCR-generated template DNA was serially diluted in PCR buffer. The dilutions were mixed with TRM, giving different amounts of DNA in each reaction, and then subjected to transcriptional enhancement followed by immunoenzymatic assay as described in the text. The results are presented as mean A_{450} ± standard deviation ($n = 3$).

lambda DNA (Boehringer Mannheim; no. 236 250) or 0.1 μg of 16S and 23S *E. coli* rRNA (Boehringer Mannheim; no. 206 398). No detectable response was observed in either instance (data not shown), indicating that the nonspecific DNA or RNA in the sample did not form the necessary RNA-DNA hybrids with the immobilized *hlyA* probe DNA. When this combined transcriptional enhancement and immunoenzymatic system was used to assay PCR-generated *hlyA* se-

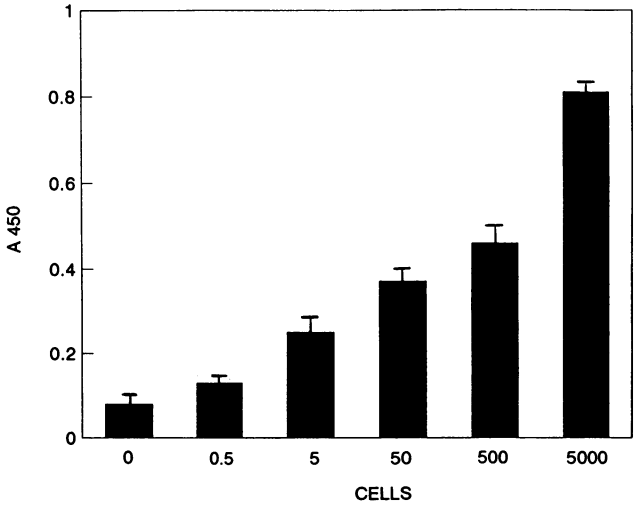

FIG. 3. Sensitivity of detection of *L. monocytogenes* cells by combined transcriptional enhancement of the PCR and immunoenzymatic assay. Various numbers of *L. monocytogenes* cells suspended in TSB were subjected to PCR amplification with primers A-T7 and B. The PCR product was then transcriptionally enhanced and processed in the immunoenzymatic assay as described in the text. The results are presented as mean A_{450} ± standard deviation ($n = 3$).

TABLE 2. Specificity of combined transcriptional enhancement of PCR-generated *hlyA* DNA and immunoenzymatic assay for *Listeria* spp.

Organism	Origin or source	A_{450}[a]
L. monocytogenes	ATCC 15313	1.50 ± 0.08
	ATCC 43256	1.23 ± 0.15
	Cheese	1.38 ± 0.17
	Cheese	1.30 ± 0.11
	Cheese	1.11 ± 0.09
	Egg	1.55 ± 0.10
	Egg	1.46 ± 0.16
	Cream	1.35 ± 0.10
	Environmental	1.30 ± 0.09
	Environmental	1.25 ± 0.09
	Environmental	1.23 ± 0.12
	Environmental	1.16 ± 0.10
	Environmental	1.25 ± 0.21
L. innocua	HPB[b]	0.08 ± 0.02
	HPB	0.06 ± 0.02
	Cheese	0.06 ± 0.01
L. ivanovii	HPB	0.04 ± 0.0
	ATCC 19119	0.06 ± 0.01
L. seeligeri	HPB	0.09 ± 0.02
	Cheese	0.09 ± 0.01
L. welshimeri	HPB	0.08 ± 0.01
L. murrayi	HPB	0.05 ± 0.02
L. grayi	HPB	0.04 ± 0.0

[a] Mean A_{450} ± standard deviation (n = 2).
[b] HPB, Health Protection Branch, Health and Welfare Canada.

quences from whole *L. monocytogenes* cells, a detectable assay response was obtained with fewer than five cells added to the initial PCR mixture (Fig. 3). This represents a minimum ca. 200-fold improvement in sensitivity over PCR without transcriptional enhancement (Table 1).

Specificity of the TE reaction. The specificity of this system for *L. monocytogenes* was first examined by its use to assay a small number of different *Listeria* and non-*Listeria* spp. organisms. Broth cultures of the different bacteria containing ca. 5×10^8 cells per ml were lysed and then subjected to PCR with primers A-T7 and B, followed by transcriptional enhancement of the product and immunoenzymatic assay. Table 2 shows that all of the *L. monocytogenes* isolates tested (including two ATCC strains) and none of the other *Listeria* species gave a strong response in the assay. None of the non-*Listeria* organisms tested produced a detectable response in this assay (data not shown).

These experiments demonstrate that it is possible to significantly increase the sensitivity of an automated PCR method for the detection of *L. monocytogenes* by transcriptional enhancement of an amplicon incorporating bacteriophage T7 promoter sequences. The sensitivity could be further increased by hybridization of the RNA transcript with an immobilized DNA probe followed by immunoenzymatic assay of the RNA-DNA hybrids. Although additional time was required to complete the transcriptional enhancement, hybridization, and assay reactions, the higher sensitivity achieved should permit the detection of *L. monocytogenes* at lower cell densities in enrichment broth during cultivation of this organism from foods and other samples, possibly reducing the total time for the analysis.

On the basis of the small number of strains tested, this detection system seemed to be specific for *L. monocytogenes*. This system offers two junctures at which the specificity of the test is potentially ensured: (i) annealing of the priming oligonucleotides with the target DNA and (ii) hybridization of the transcriptional enhancement product with the probe DNA. However, before this system can be used for routine screening of samples for *L. monocytogenes*, its specificity needs to be further evaluated with a larger number of *Listeria* and non-*Listeria* strains. The use of this test for the detection of *L. monocytogenes* in naturally contaminated samples (e.g., foods and environmental samples) and in the presence of mixed populations of bacteria will also require study.

In the present experiments, probe DNA for the hybridization reaction was prepared by PCR amplification of *hlyA* sequences and removal of primer-complementary DNA by *Hin*dIII digestion of the amplicon in order to preclude the possibility of the formation of RNA-DNA hybrids on the plate due to the occurrence of "primer-dimer" during the PCR stage of the test. An alternative approach could involve amplification of internal *hlyA* sequences with a different set of priming oligonucleotides. With this strategy, probe DNA for virtually any nucleotide sequence can be readily prepared. The principle of transcriptional enhancement of the PCR and immunoenzymatic assay of the product with an anti-RNA-DNA hybrid antibody should be widely applicable to the detection of food pathogens as well as other bacteria and viruses of clinical or economic interest.

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