Food Microbiology, 1998, **15**, 651–657 Article No. fd980207

ORIGINAL ARTICLE

Detection and identification of *Listeria monocytogenes* in food by PCR and oligonucleotide-specific capture plate hybridization

M. Manzano[1], L. Cocolin[1], C. Cantoni[2] and G. Comi[1]*

This paper reports on an ELISA-based detection method for PCR-amplified Listeria monocytogenes *iap gene fragment. During PCR, a label (digoxigenin-11-dUTP) is incorporated in the amplicon. After amplification, the product obtained is hybridized in streptavidin-coated microtitre plates prepared with biotinylated-specific oligonucleotide as a DNA probe, and then an enzyme immunoassay reveals the specifically bound complex, which permits identification of* L. monocytogenes. *A total of 48 food samples were tested to validate the method involved. The PCR-oligonucleotide specific capture plate hybridization (OSCPH) is easily applicable and much faster than traditional detection of* L. monocytogenes *in food. The hybridization in microtitre plates is also more sensitive than routine agarose gel electrophoresis.*

© 1998 Academic Press

Introduction

Increasing recognition of *Listeria monocytogenes* as a food-borne pathogen has elicited a world-wide response by food industries, health agencies, and government bodies concerned with preventing infections caused by the presence of these organisms in food (Harvey and Gilmour, 1994). In comparison with the standard procedures of isolation and biochemical identification of *L. monocytogenes* in the food, which requires at least 5 days, the recent application of polymerase chain reaction (PCR) has successfully shortened the detection time (Manzano et al. 1997, Bansal 1996, Bickley et al. 1996, Simon et al. 1996, Herman et al. 1995, Makino et al. 1995, Cooray et al. 1994). One of the problems which may be encountered in direct detection by PCR is the presence of PCR-inhibitory factors in the food (Fluit et al. 1993). Therefore a pre-enrichment method (Manzano et al. 1997, Niederhauser et al. 1992, Thomas et al. 1991), or separation of bacteria using a monoclonal antibody (Fluit et al. 1993) has been reported.

At present, amplified sequences are generally detected by gel electrophoresis, followed by Southern blot hybridization, but as this system requires DNA transfer on nylon membrane, subsequent analytical procedures

* Author for correspondence.

Received: 18 March 1998

[1] *Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, Università di Udine, Italy*
[2] *Istituto di Ispezione degli Alimenti di Origine Animale, Facoltà di Medicina Veterinaria, Università di Milano, Italy*

0740-0020/98/060651 + 07 $30·00/0

© 1998 Academic Press

are not fully compatible with automated processes suitable for diagnostic laboratories (Ferre-Aubineau et al. 1995).

This report describes the detection of *L. monocytogenes* by PCR amplification, of a 453-bp fragment of the iap gene, and oligonucleotide-specific capture plate hybridization (OSCPH) (De Beenhouwer et al. 1995). The PCR products were labelled with digoxigenin during the amplification process, and analysed by solution hybridization to a specific capture probe that was complementary to the inner part of the amplification product. The capture probe was labelled with biotin to allow immobilization of the hybrid on a streptavidin-coated microtitre plate surface. At the end, the bound hybrid was detected by an antidigoxigenin peroxidase conjugate and by using a colorimetric substrate. The simplicity and sensitivity of this system make it applicable in routine analysis to detect and identify *L. monocytogenes*.

Materials and Methods

Bacterial strains and growth media

Thirty-five strains of *L. monocytogenes* were tested for the PCR–OSCPH method. *L. monocytogenes* NCTC (National Collection of Typed Cultures, London, UK) 10527 serovar 4b, and *L. monocytogenes* CIP (Collection Istitut Pasteur, Paris, France) 78·35 serovar 3b, isolated from human cerebrospinal fluid, were obtained from the Istituto Lattiero Caseario e di Biotecnologie Agroalimentari, Thiene, Italy; *L. monocytogenes* 0·19, 0·20, 0·21, 0·22, 0·23, 0·24, 0·25 and 0·26 serovar 4b, *L. monocytogenes* 0·09 serovar 3b, *L. monocytogenes* 0·12, 0·15, 0·16 serovar 1 and 2b, *L. monocytogenes* 0·14 serovar 1 and 2c and *L. monocytogenes* 0·26 serovar 1 and 2a from human listeriosis, were obtained from the Azienda Ospedaliera, Policlinico di Modena, Italy; *L. monocytogenes* 0·47, 0·48, 0·60, 0·68 and 0·70 serovar 4b, *L. monocytogenes* 0·30, 0·43, 0·58 serovar 1 and 2b *L. monocytogenes* 0·27, 0·28, 0·40, 0·64 serovar 1 and 2c and *L. monocytogenes* 0·35, 0·36, 0·61, 0·62 serovar 1 and 2a, isolated from food (pork meat, ground beef and sausages), were obtained from the Istituto di Ispezione degli Alimenti di Origine Animale, Milan, Italy. *L. monocytogenes* ATCC (American Type Culture Collection, Rockville, Maryland, USA) 7644, *L. monocytogenes* SCOTT A and *L. monocytogenes* OHIO were obtained from the Istituto Lattiero Caseario, Lodi, Italy.

Listeria monocytogenes strains were routinely grown for 18 h at 37°C in brain–heart infusion broth (Oxoid-Unipath, Italy) and DNA extracted using proteinase K treatment as previously described (Manzano et al. 1997). After extraction, the DNA was quantified using a spectrophotometer (DMS 80 UV Visible Varian, Techtron Pty. Ltd. Australia) at 260 nm (Maniatis et al. 1982), and several dilutions were made to determine the sensitivity of the PCR-OSCPH method.

Synthesis and labelling of oligonucleotides

The oligonucleotides, Mar 1 (5′–GGGCTTTATCCATAAAATA–3′) and Mar 2 (5′–TTGGAAGAACCTTGATTA–3′) (Pat. Pend. Manzano et al. 1997), specific for *L. monocytogenes*, and the DNA probe Mar 3 (5′–GTTATTTGTTTTATTAGCATTG–3′) were synthesized with t-methoxyphosphoramidite chemistry using an applied biosystem (USA) 392-A DNA synthesizer. They were designed on the basis of the complete iap gene sequence obtained from the GeneBank (Los Alamos, New Mexico) (accession no. M80348), using 'Amplify For Analyzing PCR Experiments' molecular biology software (University of Wisconsin, Genetics, Madison, Wisconsin, USA). The DNA probe was 3′-end labelled with biotin-16 dUTP (Boehringer Mannheim, Germany) using terminal transferase (Boeringer Mannheim, Germany) as recommended by the manufacturers.

PCR assay

The PCR was performed in a volume of 50 μl containing 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 10 pg μl^{-1} of each primer, 1·25 UI of Taq-DNA polymerase

(Pharmacia Biotech, Italy) and 5 μl of diluted DNA. During PCR, a label was incorporated in the amplified product by replacing the concentration of deoxinucleoside triphosphate (dNTPs) in the PCR buffer by 0·125 mM each of dATP, dCTP and dGTP, 0·118 mM dTTP and 7 μM DIG-11-dUTP (Boehringer Mannheim, Germany). Amplification in a DNA Thermal cycler (MJ, Genenco, USA) consisted of 35 cycles at 95°C for 90 s, 46°C for 80 s and 72°C for 120 s. PCR assay results were compared with negative and positive control results. The positive control was an 'in house' standard containing approximately 10^5 cells ml^{-1} *L. monocytogenes* NCTC 10527 serovar 4b. In the negative control the bacterial DNA was replaced with sterile distilled water.

OSCPH

The DIG-labelled PCR products were detected using the PCR-ELISA, DIG-Detection Kit (Boeringher Mannheim, Germany) and duplicates of 10 μl aliquots of PCR mixtures were analysed in the microtitre plate assay. The capture probe (5 pmol ml^{-1}) was annealed to the DIG-labelled PCR product, neutralized with a solution containing NaOH, and immobilized on the streptavadin-coated surfaces of the microtitre plates by shaking for 3 h at 37°C. The hybrid was then incubated with an anti-DIG–POD conjugate at 37°C for 30 mins and detection by addition of a colorimetric substrate. Sterile distilled water was used in the reaction instead of DNA for a negative control. The optical densities at 405 nm (reference filter 492 nm) were read in an automatic reader (EL 340 microplate, BIO-TEK Instruments, Inc., USA). To determine the cut off point, mean values for the 12 PCR-negative control wells and standard deviation were calculated. Values greater than the means, plus two standard deviations, were considered positive.

Analysis procedure of food samples

Forty-eight samples were collected from local laboratories: 10 fresh ham, eight minced pork, five minced beef, seven poultry meat, seven raw milk and 11 soft cheese. Twenty-five g or ml of samples were added to 25 ml of sterile distilled water and homogenized in a stomacher (PBI, Italy). Two hundred microlitres were treated for PCR-OSCPH assay with proteinase K treatment, as previously described (Manzano et al. 1997), while 0·1 ml of the same homogenate was plated onto Oxford agar (Oxoid-Unipath, Italy) and incubated for 24 h at 37°C. Subsequently, 225 ml of Fraser broth (Oxoid-Unipath, Italy) were added to the samples and incubated at 30°C overnight. One millilitre of enrichment culture was added to 99 ml of sterile distilled water, and 200 μl were extracted for PCR assay. 0·1 ml of the enrichment culture was plated onto Oxford agar (Oxoid-Unipath) and incubated at 37°C for 24–48 h. All the presumptive colonies, that is growth on Oxford agar, were further identified according to the USDA–FSIS method (Carnevale and Johnston 1989).

Results

To determine the sensitivity of the combined PCR–OSCPH assay, several dilutions of template DNA were applied to PCR, with further analysis of amplification products by hybridization in microtitre plate wells. The extracted DNA from a suspension of 10^7 cells of *L. monocytogenes* ml^{-1} was quantified using a spectrophotometer at 260 nm and subsequently diluted to obtain different concentrations to apply to the PCR assay. After amplification, visual inspection of an ethidiumbromide-stained gel revealed a positive signal up to 360 fg of total template DNA loaded in PCR reaction (faint band), but a dilution containing 36 fg of template DNA was negative (Fig. 1). In the OSCPH assay, however, the detection limit obtained was 3·6 fg of total template DNA loaded in PCR reaction (Fig. 2). When amplicons of culture suspensions were tested, the absorbance value for positive samples was normally in the range of 1·2–2·0, after correction for the intrinsic extinction of the substrate solution. Negative samples had extinctions below 0·1.

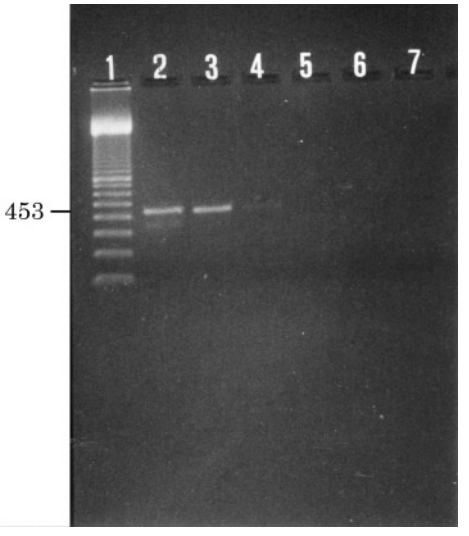

Figure 1. Sensitivity of PCR. Serial dilutions of chromosomal *L monocytogenes* DNA were subjected to PCR and a total of 10 μl of amplification reaction mixtures analysed. (1), DNA molecular weight marker (100 bp ladder, Pharmacia Biotech, Italy); (2) 36 pg of template; (3) 3·6 pg of template; (4) 360 fg of template; (5) 36 fg of template; (6) 3·6 fg of template; (7) 0·36 fg of template. The number on the left indicates the PCR product size obtained with Mar 1 and Mar 2 primers.

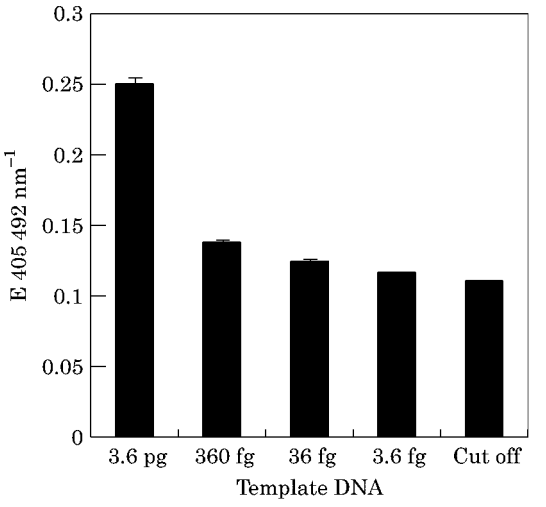

Figure 2. Hybridization sensitivity of amplification products in microtitre plates. The data presented are the means of duplicate values. Serial dilution of *L. monocytogenes* DNA was applied to PCR, and duplicate 10 μl aliquots of the amplification products were analysed in microtitre plates. The cut-off is the mean of PCR-negative controls plus two standard deviations.

The specificity of the PCR-OSCPH method utilizing Mar 1 and Mar 2 primers was evaluated by analysing the PCR products obtained from *Listeria ivanovii*, *Listeria innocua* and *Listeria*-related species. The extinctions were always below cut off. The specific detection of *L. monocytogenes* using these primers is increased, considering that the DNA probe Mar 3 is also specific for *L. monocytogenes*. In fact, DIG-labelled PCR products of non-listerial micro-organisms analysed with the OSCPH assay always gave extinctions lower than the cut off. A total of 45 food samples were tested for the presence of *L. monocytogenes* by the optimized PCR-OSCPH protocol. Table 1 shows the results of the food analysis: nine samples were positive by direct detection with the PCR-OSCPH protocol. The 18-h enrichment procedure improved the results, with 20 positive samples

Table 1. PCR-OSCPH and traditional method results obtained in food samples

Samples	OSCPH— Direct detection		OSCPH— 18 h culture enrichment		Traditional method	
	Positive samples	Negative samples	Positive samples	Negative samples	Positive samples	Negative samples
Fresh ham	2	8	4	6	4	6
Minced pork	2	6	3	5	3	5
Minced beef	0	5	2	3	2	3
Poultry meat	2	5	4	3	4	3
Raw milk	0	7	3	4	3	4
Soft cheese	3	8	4	7	4	7
Total	9	39	20	28	20	28

after this period. No difference was noted between the PCR–OSCPH method and the traditional one (Table 1); no false-negative or false-positive results were observed. The presence of a large number of non-listerial micro-organisms, especially after 18 h enrichment and consequently an excess of competing DNA, did not influence the specificity and sensitivity of the PCR-OSCPH protocol for *L. monocytogenes*.

Discussion

The aim of this work was to obtain a more sensitive and semiautomated detection protocol to detect *L. monocytogenes* in food than conventional detections of PCR products by gel electrophoresis. A considerable amount of information about PCR methods to detect *L. monocytogenes* species is available (Bansal 1996, Bickley et al. 1996, Simon et al. 1996, Herman et al. 1995, Makino et al. 1995, Niederhauser et al. 1992, Wernars et al. 1991). In order to become a routine procedure, the detection of PCR amplification products needs to be simplified with regard to saving time and processing large numbers of samples, as well as avoiding the use of radioactively labelled compounds. So far, very promising studies have been published, which take advantage of the biotin–streptavidin interaction for immobilization of amplification products either on beads (Rimstead et al. 1990, Syvanen et al. 1988, Urdea et al. 1988) or on microtitre plates (Stamminger et al. 1996, Stevens et al. 1996, De Beenhouwer et al. 1995, Boni and Schupbach 1993, Gibellini et al. 1993, He et al. 1993, Holmstrom et al. 1993, Lüneberg et al. 1993, Landgraf et al. 1991, Mantero et al. 1991).

In the present study, the PCR products were random labelled during amplification with DIG-11-dUTP, and immobilized on streptavidin-coated microtitre wells by hybridization with a biotin-labelled DNA probe. With the optimized hybridization condition, the amplified segment of the iap gene, DIG-11-dUTP labelled, is captured in a very specific way by just the species-specific probe. Incompletely matched amplicons do not bind strongly and were removed by washing. The bound complex is revealed by the use of anti DIG-POD conjugate, and a simple automated colorimetric readout provides sensitive *L. monocytogenes* detection and identification. The assay combines the advantages of simple and rapid processing of PCR amplification products, with the possibility of using oligonucleotides that can be easily synthesized and labelled on a large scale at reasonable expense. The detection of *L. monocytogenes* contamination in food, by the PCR-OSCPH method involved, is conceivable on the basis of species-specific PCR amplification and hybridization, and a positive result depends exclusively on the amount of *L. monocytogenes* DNA present in the sample, whether subjected to enrichment culture or not. Since the microtitre plate assay allows the processing of a large number of samples, this protocol is suitable for application in routine analysis.

References

Bansal, N. S. (1996) Development of a polymerase chain reaction assay for the detection of *Listeria monocytogenes* in foods. *Lett. Appl. Microbiol.* **22,** 353–356.

Bickley, J., Short, J. K., McDowell, D. G. and Parkes, H. C. (1996) Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. *Lett. Appl. Microbiol.* **22,** 153–158.

Böni, J. and Schüpbach, J. (1993) Sensitive and quantitative detection of PCR-amplified HIV-1 DNA products by an enzyme linked immunoassay following solution hybridization with two differently labelled oligonucleotide probes. *Mol. Cell. Probes* **7**, 361–371.

Carnevale, R. A. and Johnston, P. (1989) Method for the Isolation and Identification of *Listeria monocytogenes* from Meat and Poultry Products. *USDA-FSIS, Laboratory Communication No. 57*. Washington, D.C., USDA.

Cooray, K. J., Nichibori, T., Xiong, H., Matsuyama, T., Fujita, M. and Mitsuyama, M. (1994) Detection of multiple virulence-associated genes of *Listeria monocytogenes* by PCR in artificially contaminated milk samples. *Appl. Environ. Microbiol.* **60,** 3023–3026.

De Beenhouwer, H., Liang, Z., De Rijk, P., Van Eekeren, C. and Portaels, F. (1995) Detection and identification of mycobacteria by DNA amplification and oligonucleotide-specific capture plate hybridization. *J. Clin. Microbiol.* **33,** 2994–2998.

Ferre-Aubineau, V., Imbert-Marcille, B. M., Raffi, F., Besse, B., Loirat, R. and Billaudel, S. (1995) Colorimetric microtiter plate hybridization assay using monoclonal antibody for detection of an amplified hyman immunodeficiency virus target. *J. Virol. Methods* **55,** 145–151.

Fluit, A. C., Torensma, R., Visser, M. J., Aarsman, C. J., Poppelier, M. J., Keller, B. H., Klapwijk, P. and Verhoef, J. (1993) Detection of *Listeria monocytogenes* in cheese with magnetic immuno-polymerase chain reaction assay. *Appl. Environ. Microbiol.* **59,** 1289–1293.

Gibellini, D., Zerbini, M., Musiani, M., Venturoli, S., Gentilomi, G. and La Placa, M. (1993) Microplate capture hybridization of amplified parvovirus B19 DNA fragment labelled with digoxigenin. *Mol. Cell. Probes* **7**, 453–458.

Harvey, J. and Gilmour, A. (1994) Application of multilocus enzyme electrophoresis and restriction fragment length polymorphism analysis to the typing of *Listeria monocytogenes* strains isolated from raw milk, non-dairy foods, and clinical and veterinary sources. *Appl. Environ. Microbiol.* **60,** 1547–1553.

He, Y., Coutlée, F., Saint-Antoine, P., Oliver, C., Voyer, H. and Kessous-Elbaz, A. (1993) Detection of polymerase chain reaction-amplified human immunodeficiency virus type 1 proviral DNA with digoxigenin-labelled RNA probe and an enzyme-linked immunoassay. *J. Clin. Microbiol.* **31,** 1040–1047.

Herman, L. M. F., De Block, J. H. G. E. and Moermans, R. J. B. (1995) Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by two-step PCR with nested primers. *Appl. Environ. Microbiol.* **61,** 817–819.

Holmstrom, K., Rossel, L. and Rasmussen, O. F. (1993) A highly sensitive and fast non-radioactive method for detection of polymerase chain reaction products. *Anal. Biochem.* **209,** 278–283.

Landgraf, A., Reckmann, B. and Pingoud, A. (1991) Direct analysis of polymerase chain reaction products using enzyme-linked immunosorbent assay techniques. *Anal. Biochem.* **198,** 86–91.

Lüneberg, E., Jensen, J. S. and Frosch, M. (1993) Detection of *Mycoplasma pneumoniae* by polymerase chain reaction and non-radioactive hybridization in mictotiter plates. *J. Clin. Microbiol.* **31,** 1088–1094.

Makino, S. I., Okada, Y. and Maruyama, T. (1995) A new method for direct detection of *Listeria monocytogenes* from foods by PCR. *Appl. Environ. Microbiol.* **61,** 3745–3747.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Mantero, G., Zonaro, A., Albertini, A., Bertolo, P. and Primi, D. (1991) DNA enzyme immunoassay: general method for detecting products of polymerase chain reaction. *Clin. Chem.* **37,** 422–429.

Manzano, M., Cocolin, L., Ferroni, P., Cantoni, C. and Comi, G. (1997) A simple and fast PCR protocol to detect *Listeria monocytogenes* from meat. *J. Sci. Food Agric.* **73,** 25–30.

Niederhauser, C., Candrian, U., Höfelein, C., Jermini, M., Bühler, H.-P. and Lüthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Appl. Environ. Microbiol.* **58,** 1564–1568.

Rimstad, E., Hornes, E., Olsvik, O. and Hyllseth, B. (1990) Identification of a double-stranded RNA virus by using polymerase chain reaction and magnetic separation of the synthesized DNA segments. *J. Clin. Microbiol.* **28,** 2275–2278.

Simon, M. C., Gray, D. I. and Cook, N. (1996) DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. *Appl. Environ. Microbiol.* **62,** 822–824.

Stamminger, G., Przybilla, D. and Burkhard, C. (1996) Sensitive detection of lymphoma cells carrying t(14;18) by PCR ELISA. *Biochem. Inform.* **97,** 30–32.

Stevens, J., Yu, F. S., Hassoun, P. M. and Lanzillo, J. J. (1996) Quantification of polymerase chain reaction products: enzyme immunoassay based systems for digoxigenin- and biotin-labelled products that quantify either total or specific amplicons. *Mol. Cell. Probes* **10,** 31–41.

Syvänen, A.-C., Bengtström, M., Tenhunen, J. and Söderlund, H. (1988) Quantification of polymerase chain reaction products by affinity-based hybrid collection. *Nucl. Ac. Res.* **16,** 11327–11338.

Thomas, E. J. G., King, R. K., Burchak, J. and Gannon, V. P. J. (1991) Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with polymerase chain reaction. *Appl. Environ. Microbiol.* **57,** 2576–2580.

Urdea, M. S., Warner, B. D., Running, J. A., Stempien, M., Clyne, J. and Horn, T. (1988)

A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemioluminescent and enzyme labelled synthetic oligodeoxyribonucleotide probes. *Nucl. Ac. Res*. **16,** 4937–4956.

Wernars, K., Heuvelman, C. J., Chakraborty, T. and Notermans, S. H. W. (1991) Use of polymerase chain reaction for direct detection of *Listeria monocytogenes* soft cheese. *J. Appl. Bacteriol.* **70,** 121–126.