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Development of Polymerase Chain Reaction Assays for Detection of *Listeria monocytogenes* in Clinical Cerebrospinal Fluid Samples

K. JATON,[1] R. SAHLI,[2] AND J. BILLE[1]*

Centre National des Listeria[1] *and Department of Virology,*[2] *Institute of Microbiology, University Hospital, 1011 Lausanne, Switzerland*

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In order to improve the diagnosis of *Listeria* meningitis or meningoencephalitis, especially in patients who have received antibiotics before their cerebrospinal fluid (CSF) has been examined, two assays for the detection of *Listeria monocytogenes* based on the polymerase chain reaction (PCR) were evaluated. After a standard PCR, the amplified DNA was detected either by a second round of PCR with internal primers followed by gel electrophoresis and ethidium bromide staining (nested PCR) or by dot blot hybridization to an internal digoxigenin-labeled probe (PCR-dot blot). For PCR, two sets of primers within the invasion-associated protein gene (*iap* gene) were chosen. They allowed for the highly specific detection of all *L. monocytogenes* reference strains tested (serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 7). These primers did not detect amplification products from various other gram-positive or gram-negative bacterial DNAs or human DNA. The sensitivities of both assays were assessed on sterile CSF samples that were artificially seeded with serial dilutions of *L. monocytogenes* serotype 4b cells. By both methods the limit of detection was <10 cells in the initial reaction. Since the nested PCR is more prone to contamination because of manipulation of the amplified products, a standard PCR assay followed by dot blot hybridization was applied to 52 CSF samples in a retrospective study. Of 28 CSF samples which were sterile or positive for bacteria other than *Listeria* species, 24 were PCR negative. In contrast, from 17 patients with culture-proven *Listeria* meningitis, 14 of 17 initial CSF samples were PCR positive, as were 3 of 7 culture-negative follow-up CSF samples taken after patients received antibiotics. These results support the usefulness of this approach in the diagnosis of *Listeria* meningitis, in particular, when antibiotic administration precedes culture of CSF.

The increase in the incidence of human listeriosis in developed countries and the recent association of severe *Listeria monocytogenes* infections with food-borne outbreaks have stimulated research on new diagnostic procedures. In humans, *L. monocytogenes* mainly causes meningitis, encephalitis, or septicemia in nonpregnant adults. When it infects the central nervous system, *L. monocytogenes* leads to severe disease with a high mortality rate (20 to 50%) and neurological sequelae among the survivors. Therefore, this infection requires prompt recognition and therapy (21).

The diagnosis of *Listeria* meningitis, meningoencephalitis, or septicemia is based on the culture of cerebrospinal fluid (CSF) and blood. The direct examination of Gram-stained clinical specimens is generally of little value, because *L. monocytogenes* is often present in low numbers in some samples, particularly in CSF, at the time of presentation. Furthermore, in some cases, antibiotics have already been administered to the patient before the CSF can be cultured, thus hampering the diagnosis.

Use of polymerase chain reaction (PCR) technologies for the detection of low numbers of *L. monocytogenes* in food samples has been reported (1, 3, 5, 8, 10, 22). To our knowledge, no PCR has been tested with human clinical samples. Several genes specific for *Listeria* species, which could serve as targets in a PCR, have been sequenced (6, 7, 9, 15). One of them, the *iap* gene, encodes the invasion-associated protein p60 (11), which may be involved in the invasion process (12). Köhler et al. (11) showed that synthetic probes previously described as specific for the detection of the presumptive β-hemolysin gene of *L. monocytogenes* by colony hybridization (4) are in fact part of the *iap* gene. Therefore, the *iap* gene seems an appropriate target for the detection of *L. monocytogenes* by PCR.

We report here a description and comparison of two PCR assays for the detection of *L. monocytogenes* DNA in human CSF samples. The first assay is a two-step PCR followed by gel electrophoresis and ethidium bromide (EtBr) staining (nested PCR), and the second one is a standard PCR followed by dot blot hybridization with a digoxigenin-labeled probe (PCR-dot blot). We found that the latter methodology is sensitive enough to detect fewer than 10 cells. Its application was suitable for the retrospective diagnosis of *L. monocytogenes* in 52 clinical CSF samples collected between 1983 and 1991.

MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used in this study: (i) 18 reference or type strains representing all known *Listeria* species and all *L. monocytogenes* serotypes (H. P. R. Seeliger, University of Würzburg, Würzburg, Germany) except *L. monocytogenes* serotype 4ab (Table 1); (ii) 16 *L. monocytogenes* serotype 4b strains isolated from different patients during the outbreak of listeriosis in Canton de Vaud (Switzerland) (2); one of them (strain K27) was used for the serial dilution assays; (iii) 15 strains of gram-positive bacteria other than *Listeria* species (Table 2); (iv) 7 strains of *Haemophilus influenzae* isolated from CSF samples in the

* Corresponding author.

TABLE 1. Species, serotypes, and source of the *Listeria* strains used in this study

Species	Serotype	Source[a]
L. monocytogenes	1/2a	NCTC 7973
L. monocytogenes	1/2b	SLCC 2755
L. monocytogenes	1/2c	NCTC 5348
L. monocytogenes	3a	NCTC 5105
L. monocytogenes	3b	SLCC 2540
L. monocytogenes	3c	SLCC 2479
L. monocytogenes	4a	NCTC 5214
L. monocytogenes	4b	NCTC 10527
L. monocytogenes	4c	NCTC 4883
L. monocytogenes	4d	ATCC 10888
L. monocytogenes	4e	ATCC 19118
L. monocytogenes	7	SLCC 2482
L. innocua	6a	NCTC 11288
L. ivanovii	5	ATCC 19119
L. welshimeri	6a	SLCC 5334
L. seeligeri	1/2b	CIP 100100
L. murrayi		ATCC 25403
L. grayi		ATCC 19120
L. monocytogenes (16 strains including K27)[b]	4b	Human clinical isolates

[a] NCTC, National Culture Type Collection, Central Public Health Laboratory, London, England; SLCC, Special *Listeria* Culture Collection, Würzburg, Germany; ATCC, American Type Culture Collection, Rockville, Md.; CIP, Institut Pasteur Collection, Paris, France.

[b] The 16 strains were wild-type strains. All other strains listed here were reference or type strains.

Centre Hospitalier Universitaire Vaudois and 2 strains of *Neisseria meningitidis*.

All strains were kept in skim milk at −80°C. They were streaked onto 5% human blood Columbia agar (Oxoid, Unipath Ltd., Basingstoke, England). *H. influenzae* and *N. meningitidis* strains were streaked onto chocolate Columbia blood agar (Oxoid). All the plates were incubated for 24 h at 37°C. One colony was then inoculated in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and was incubated overnight at 37°C before DNA preparation.

Human CSF samples. A total of 52 CSF samples from 44 patients were tested (see Table 4), as follows: (i) 14 sterile CSF samples from 13 patients with nonbacterial meningitis or no CSF infection; half of them were collected during the Swiss outbreak of listeriosis (1983 to 1987) (2); (ii) 14 CSF samples positive for microorganisms other than *Listeria* species, as follows: *N. meningitidis* ($n = 2$), *Staphylococcus aureus* ($n = 1$), *Staphylococcus epidermidis* ($n = 2$), *Streptococcus agalactiae* ($n = 1$), *Streptococcus pneumoniae* ($n = 2$), *H. influenzae* ($n = 5$), and *Cryptococcus neoformans* ($n = 1$); 10 of the samples were collected during 1983 to 1987, two were collected in 1988, and two were collected in 1991; (iii) 24 CSF samples from 17 patients with culture-proven *Listeria* meningitis; 17 samples were positive for *L. monocytogenes* by culture at the time of presentation; the other 7 samples were negative by culture and were follow-up samples taken between 2 and 15 days after the patient had started antibiotic treatment. All CSF samples except two were collected during the Swiss outbreak of listeriosis (1983 to 1987) (2).

TABLE 2. Genus, species, and source of bacteria other than *Listeria* species used in this study

Genus and species	Source[a]
Brochothrix thermosphacta	CIP 69.6
Cellulomonas turbata	CIP 100331 T
Corynebacterium xerosis	NCTC 9755
Enterococcus avium	ATCC 14025
Enterococcus durans	ATCC 19432
Enterococcus faecalis	ATCC 29212
Erysipelothrix rhusopathiae	BTCC 65
Haemophilus influenzae	Wild-type strains
Jonesia denitrificans	ATCC 14870
Kurthia zopfii	NCTC 10597
Lactobacillus casei	ATCC 393
Neisseria meningitidis	Wild-type strains
Staphylococcus aureus	Wild-type strain
Staphylococcus epidermidis	Wild-type strain
Streptococcus agalactiae	Wild-type strain
Streptococcus pneumoniae	Wild-type strains

[a] CIP, Institut Pasteur Collection, Paris, France; NCTC, National Culture Type Collection, Central Public Health Laboratory, London, England; ATCC, American Type Culture Collection, Rockville, Md.; BTCC, Bulgarian Type Culture Collection, Sofia, Bulgaria.

All specimens were divided into aliquots and were stored at −20°C without any particular precautions, as would now be required for studies involving PCR (13, 18).

DNA preparation. (i) DNA extraction. DNA was purified from bacteria by the method of Le Febvre et al. (14), with some modifications (16). Cells from a 1.5-ml overnight culture or from chocolate agar plates incubated overnight were harvested and washed in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl. After centrifugation the pellets were resuspended in 25 mM Tris-HCl (pH 8.0)–10 mM EDTA–50 mM glucose. Cells were then treated with lysozyme (Sigma Chemical Co., St. Louis, Mo.) at 10 mg/ml and RNase A (Boehringer, GmbH, Mannheim, Germany) at 200 μg/ml at 37°C for 30 min. Proteinase K (Sigma) and sodium dodecyl sulfate were then added at 200 μg/ml and 1% (wt/vol), respectively, and the samples were incubated at 50°C for 60 min. DNA was extracted three times with 1 volume of phenol-chloroform-isoamyl alcohol (25/24/1). The aqueous phase was recovered and adjusted to 0.3 M sodium acetate, and nucleic acids were precipitated with 0.6 volume of isopropanol at room temperature for 5 min. The DNA was centrifuged, washed with 70% ethanol, and air dried. The pellets were resuspended in 40 μl of 10 mM Tris-HCl–0.1 mM EDTA (pH 7.5). For quantification, DNA was electrophoresed through a 0.8% agarose gel containing 0.5 μg of EtBr per ml. The DNA was then revealed under UV light, and the fluorescence of the bacterial band was compared with that of a known amount of *Hin*dIII-digested bacteriophage λ DNA.

(ii) Crude lysates of bacterial cells. Cells of *L. monocytogenes* serotype 4b (human isolate K27) from an overnight culture were serially diluted in 500 μl of sterile 0.9% NaCl. After centrifugation for 10 min in an Eppendorf centrifuge, the pellet from each dilution was resuspended in 1 ml of sterile water and 10 μl of each suspension was plated onto blood agar for CFU counts. The suspension was then left for 1 h at room temperature prior to DNA release by boiling for 10 min. PCR was done with 10 μl of the lysate.

(iii) Artificially seeded CSF samples. *L. monocytogenes* serotype 4b (human isolate K27) from an overnight culture was serially diluted in 200 μl of pooled sterile human CSF containing between 50 and 500 × 10^6 leukocytes per liter. After centrifugation for 10 min, the pellet of each dilution was suspended in 100 μl of sterile water and 10 μl of each suspension was plated onto blood agar for CFU counts. Each dilution was then left at room temperature for 1 h. Since bacteria were in a small volume, their DNA was

TABLE 3. Sequences of the oligonucleotide primers and their locations in the *iap* gene

Name	Sequence (5′ to 3′)	Location	Product (bp)
External			
iap-A	CAAAGGTGGAT[a]CC[a]AAAGTAACTGT	760–783	468
iap-B	TGGAGCTTCCGA[a]A[a]TTCACTTCTG	1228–1206	
Internal			
iap-1	CGAATCTAACGGCTGGCACA	793–812	287
iap-3	GCCCAAATAGTGTCACCGCT	1080–1061	

[a] One mismatch was introduced at these positions for cloning purposes.

released by freezing at −80°C and boiling five times for 30 s each time rather then boiling once for 10 min. PCR was done with 10 μl of the lysate.

(iv) Human CSF samples. Two hundred microliters of CSF was centrifuged for 10 min in an Eppendorf centrifuge. The pellets were suspended in 100 μl of sterile water, and DNA was released by freezing and boiling five times as described above. PCR was done with 10 μl of the lysate. All samples were tested at least twice.

PCR. (i) Oligonucleotide characteristics. Primers were chosen from the invasion-associated protein gene (*iap* gene) on the basis of the sequence reported by Köhler et al. (11). Specific data on the two pairs of oligonucleotide primers are given in Table 3. The external primers *iap-A* and *iap-B* delimit a 468-bp segment of the *iap* gene of *L. monocytogenes*. The internal primers *iap-1* and *iap-3* delimit a 287-bp segment within the 468-bp product. *iap-3* was used as a probe in the dot blot assay to identify the PCR product obtained with the external primers (*iap-A* and *iap-B*).

(ii) Standard PCR. PCR (19) amplification was performed with 10 μl of DNA preparation in a 50-μl reaction containing 50 mM Tris-HCl (pH 8.8)–1.5 mM $MgCl_2$–50 mM KCl–0.01% gelatin–0.1% Triton X-100, 200 μM (each) four deoxynucleotide triphosphates, and primers *iap-A* and *iap-B* or *iap-1* and *iap-3* at 0.8 or 1 μM, respectively. The samples were overlaid with 50 μl of paraffin oil. The PCR was done with a Techne PHC 2 thermal cycler. Positive (0.05 ng of *L. monocytogenes* serotype 4b DNA) and negative (no DNA) controls were included in each set of PCRs.

(iii) Cycling. The precycle consisted of 97°C for 5 min and 74°C for 1 min, 1 U of *Taq* polymerase (Cetus) was added, annealing was done at 50°C for 1 min, and extension was done at 74°C for 2 min. For cycles 1 to 30, denaturation was done at 94°C for 30 s, primer annealing was done at 50°C for 1 min, and extension was done at 74°C for 1 min, with an additional extension step at 74°C for 5 min at the end of the last cycle.

(iv) Nested PCR. A new PCR was performed by using 10 μl of the standard PCR as a template. Buffer, deoxynucleotide triphosphates, internal primers *iap-1* and *iap-3* at 1 μM, and *Taq* polymerase were added. The reactions were then subjected to an additional 30 cycles of amplification as described above.

(v) Negative controls. At the time of DNA preparation, every fourth or fifth sample was an internal negative control, in which water replaced DNA. All the clinical CSF samples were given a code and assayed by one of the investigators (K.J.), who was unaware of the culture results. The samples that were negative by the standard PCR were tested for inhibitors. For this, human placental DNA (1 ng) was added to the CSF sample together with human leukocyte antigen DQα primers (20), and PCR was done by using the same conditions as those for the standard PCR. The target of these primers was the human leukocyte antigen locus. An amplified product of 227 bp was expected if no inhibitors of the amplification were present in the sample.

Detection of the amplification products. (i) Electrophoresis and ethidium bromide staining. Ten microliters of each reaction was electrophoresed for approximately 30 min at 10 V/cm through a 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, Maine) containing 0.5 μg of EtBr per ml. DNA was revealed under UV light.

(ii) Dot blot hybridization. Three microliters of the amplified product of the standard PCR was applied to a nylon membrane (Boehringer). DNA was denatured in 0.5 N NaOH–1.5 M NaCl for 5 min and was then neutralized with 1 M Tris-HCl (pH 7.5)–1.5 M NaCl three times for 2 min each time. The membrane was then rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 min and was baked at 120°C for 30 min. Prehybridization was performed at 52°C for more than 1 h in 3 M tetramethylammoniumchloride (TMAC)–50 mM Tris-HCl (pH 8.0)–1% blocking reagent (Boehringer)–0.02% sodium dodecyl sulfate–0.1% *N*-lauryl sarcoside–50 μg of salmon sperm DNA per ml. The prehybridization solution was then replaced with an identical solution containing the *iap-3* probe at 1 nM, and hybridization was allowed to proceed for more than 2 h at 52°C. The probe was prepared by labeling the 3′ end of the internal primer *iap-3* with digoxigenin-dUTP by using terminal transferase as recommended by the manufacturer (Boehringer). After the hybridization, the membrane was washed twice at 62°C for 15 min in 3M TMAC–50 mM Tris-HCl (pH 8.0). Immunoenzymatic detection of the hybridized probe was performed with an anti-digoxigenin antibody coupled to alkaline phosphatase by using 3-(2′-spiroadamantane)-4-methoxy-4-(3′′-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD; Boehringer) as a substrate, as recommended by the manufacturer. The chemiluminescence reaction was revealed by exposure of the membrane to X-ray film for 2 h.

RESULTS

We used the PCR methodology to improve the diagnosis of *L. monocytogenes* meningitis. Initially, 30 cycles of PCR (standard PCR) were performed with 10-fold dilutions of *L. monocytogenes* DNA (from 5 to 0.05 ng, corresponding to 10^6 to 10^4 cells, respectively) by using the external primers *iap-A* and *iap-B* or the internal primers *iap-1* and *iap-3*. The amplification products were clearly detectable after agarose gel electrophoresis and EtBr staining (Fig. 1). To assess whether these primers were specific for *L. monocytogenes*, 0.05 ng of DNA from 34 *Listeria* strains and 24 non-*Listeria* strains was used as template for the PCR. DNA from strains representing all serotypes of *L. monocytogenes* gave a single amplified product of the expected size. For *L. ivanovii* ATCC 19119 and *L. welshimeri* SLCC 5334, DNA could be amplified with the external primers, whereas no product was found with the internal primers. This suggests that *iap-A* and *iap-B* are not specific for *L. monocytogenes*, whereas *iap-1* and *iap-3* are. Therefore, the specificity for *L. monocytogenes* detection is provided by the *iap-1* and *iap-3* primers. No amplification products were detectable with DNA from 7 *H. influenzae* strains, 2 *N. meningitidis* strains, or 15 gram-positive bacteria other than *Listeria* species or with human DNA.

To determine the level of sensitivity of the standard PCR

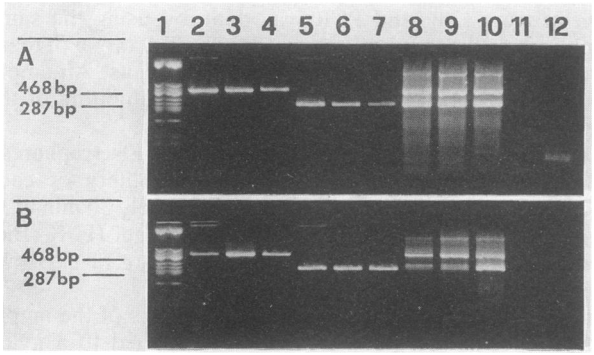

FIG. 1. Agarose gel electrophoresis of PCR-amplified products from 10-fold dilutions of lysates of *L. monocytogenes* serotype 4b cells (strain K27) ranging from 10^6 to 10^4 cells (A) and DNA extracted from *L. monocytogenes* ranging from 5 to 0.05 ng (B). Lane 1, molecular size markers; lanes 2 to 4, standard PCR with the external primers *iap-A* and *iap-B*; lanes 5 to 7, standard PCR with the internal primers *iap-1* and *iap-3*; lanes 8 to 10, nested PCR; lane 11, negative control of the standard PCR; lane 12, negative control of the nested PCR.

assay, serial dilutions of DNA of a human *L. monocytogenes* serotype 4b strain (K27) were used as templates with either the external primers (Fig. 2A) or the internal primers (Fig. 2B). After 30 cycles of amplification, the specific products were still detectable by EtBr staining when 5×10^{-3} ng of DNA (corresponding to 10^3 cells) served as template. Since this level of sensitivity was not satisfactory, we improved it using the nested primer methodology (nested PCR).

L. monocytogenes DNA from an aliquot of the reaction done with the external primers was reamplified for an additional 30 cycles by using the internal primers *iap-1* and *iap-3*. An aliquot of the reaction was then submitted to gel electrophoresis, and the DNA was stained with EtBr. The specific 287-bp amplification product was still detected when DNA corresponding to 5×10^{-5} ng (equivalent to 10 cells) was used as a template in the reaction (Fig. 2C). The level of detection was thus enhanced at least 100-fold up to the theoretical detection limit with this nested-PCR procedure

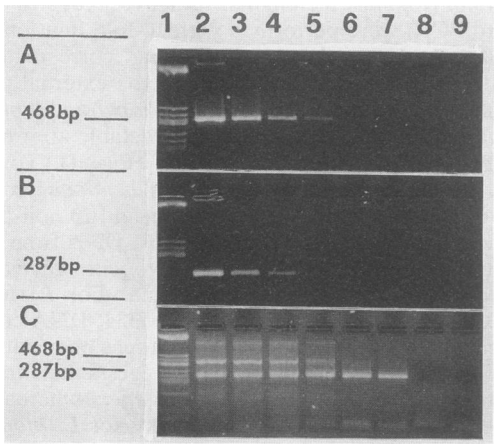

FIG. 2. Agarose gel electrophoresis of PCR-amplified products from 10-fold dilutions of purified *L. monocytogenes* DNA. (A) Standard PCR with *iap-A* and *iap-B* primers giving a 468-bp product. (B) Standard PCR with *iap-1* and *iap-3* primers giving a 287-bp product. (C) Nested PCR. Lane 1, molecular size markers; lanes 2 to 8, dilutions ranging from 5 to 5×10^{-6} ng; lane 9, negative controls.

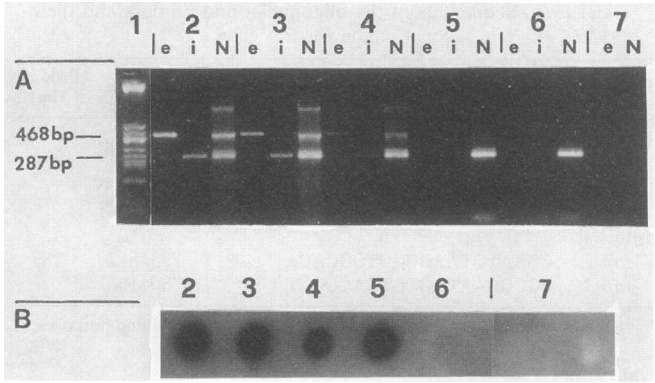

FIG. 3. (A) Agarose gel electrophoresis of PCR-amplified products from CSF artificially seeded with 10-fold dilutions of *L. monocytogenes* cells. Lane 1, molecular size markers; lanes 2 to 6, dilutions of *L. monocytogenes* cells ranging from 4×10^4 to 4 cells; lane 7, negative controls of the standard PCR and the nested PCR; lanes e, standard PCR with the external primers *iap-A* and *iap-B*; lanes i, standard PCR with the internal primers *iap-1* and *iap-3*; lanes N, nested PCR. (B) Dot blot analysis of the amplified products from the standard PCR. Lanes 2 to 7 are the same as those described above for panel A.

compared with that of the standard PCR. When dilutions of crude *L. monocytogenes* cell lysates instead of purified DNA were used in the PCR, the sensitivity level was the same (data not shown).

Detection of *L. monocytogenes* in artificially seeded CSF samples. In order to mimic a clinical situation, sterile CSF samples were artificially seeded with serial dilutions of *L. monocytogenes* serotype 4b (strain K27). Four hundred bacteria corresponding to 2×10^4 cells per ml of CSF were detectable by the standard PCR assay after electrophoresis and EtBr staining (Fig. 3, lanes e and i). By the nested PCR approach, as few as four bacteria, corresponding to 200 cells per ml of CSF, were detectable, again increasing the sensitivity to its theoretical limit (Fig. 3, lanes N). Since this procedure involves handling of amplified DNA, it is more prone to contamination and may not be suitable. Therefore, to increase the sensitivity without increasing the risk of contamination, a more sensitive method was used to detect the amplification product of the standard PCR. For this, the internal primer *iap-3* was labeled with digoxigenin and was used as a probe for the detection of the 468-bp amplified product in a dot blot assay (PCR-dot blot). With this nonradioactive probe, 4 cells (equivalent to 200 cells per ml of CSF) were detected (Fig. 3B). This level of sensitivity was the same as that for the nested PCR procedure.

Application of the PCR and dot blot methodology to clinical CSF samples. The PCR-dot blot methodology was used for the detection of *L. monocytogenes* in 52 CSF samples (Table 4 and Fig. 4). All 14 sterile CSF samples from patients without meningitis were PCR-dot blot negative (Table 4).

Of 14 CSF samples that were positive for microorganisms other than *L. monocytogenes*, 10 were negative by PCR-dot blot, whereas 4 were positive (Table 4). All four were culture positive for *H. influenzae* and represented false-positive specimens under our experimental conditions. The DNAs extracted from the corresponding four *H. influenzae* strains were tested by PCR, dot blot, and Southern blot analyses. None of the DNAs could be detected, thus excluding nonspecific amplification with the *iap-A* and *iap-B* primers and nonspecific hybridization of the *iap-3* probe (data not

TABLE 4. Detection of *L. monocytogenes* in clinical CSF samples by comparison of conventional culture and PCR-dot blot

CSF samples (no. of samples; no. of patients)	No. of samples (Total n = 52)	Culture	PCR-dot blot
Sterile CSF (14; 14)	14	−	−
CSF positive for organisms other than *L. monocytogenes* (14; 13)	10	−	−
	4	−	+
CSF from patients with culture-confirmed *Listeria* meningitis (24; 17)	14	+	+
	4	−	−
	3	+	−
	3	−	+

shown). In addition, all PCR-negative controls (in two independent PCR runs) were negative, thus reasonably excluding contamination at the time of the PCR assay. These CSF samples may have been contaminated previously during storage and/or manipulation.

Of 24 CSF samples collected from 17 patients with culture-confirmed *Listeria* meningitis, 14 were PCR-dot blot hybridization and culture positive and four were negative by both techniques (Table 4).

Three other CSF samples which were culture positive for *L. monocytogenes* remained PCR-dot blot negative. One was a supernatant from the original CSF sample. The second was positive in one broth only after 72 h. The third PCR-dot blot false-negative CSF sample probably contained very few bacteria because none of two solid media and one of two broth culture media only was positive at the time of presentation. No PCR inhibitors could be found in these three samples or in any other negative samples. DNA from the *L. monocytogenes* strains in two of these CSF samples was extracted, and 0.5 ng of this DNA was submitted to the PCR-dot blot methodology. A strong positive signal was obtained, suggesting that these false-negative results were not due to a lack of specificity of our primers. These negative results by PCR-dot blot probably reflect the low concentrations of *L. monocytogenes* in these CSF samples (fewer than 400 cells per ml). Finally, three other samples negative by culture were positive by PCR-dot blot. All three samples were follow-up CSF samples (2, 2, and 15 days, respectively) from patients with a previous CSF sample that was positive for *L. monocytogenes* by culture and who were already treated with antibiotics. These data suggest that nonviable cells can be detected by PCR-dot blot.

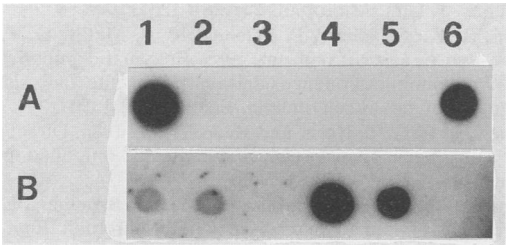

FIG. 4. Dot blot analysis of amplified CSF samples probed with digoxigenin-labeled *iap-3* oligonucleotide. (A) Lane 1, positive control; lane 2, *H. influenzae* culture-positive CSF (false-positive result); lane 3, *C. neoformans* culture-positive CSF; lane 4, *N. meningitidis* culture-positive CSF; lane 5, sterile CSF; lane 6, positive control. (B) Lane 1, *L. monocytogenes* serotype 4b culture-positive CSF; lane 2, *L. monocytogenes* serotype 4b culture-positive CSF; lane 3, *L. monocytogenes* serotype 4b culture-negative sample (follow-up of a positive CSF); lane 4, *L. monocytogenes* serotype 4b culture-positive CSF; lane 5, *L. monocytogenes* serotype 4b culture-positive CSF; lane 6, negative control. A2 and B3 were well seen on the original X-ray film.

DISCUSSION

In order to improve the diagnosis of *Listeria* meningitis, especially after antibiotic administration, we developed and compared two PCR-based procedures. A nested PCR assay followed by gel electrophoresis and EtBr staining was compared with a standard PCR assay followed by dot blot hybridization. Two sets of primers were selected within the *iap* gene (11). The selected oligonucleotides allowed the detection of all *L. monocytogenes* reference strains tested. The external primers (*iap-A* and *iap-B*) were not specific for *L. monocytogenes*, because they allowed the amplification by PCR of DNA from two non-*L. monocytogenes* reference strains. However, the internal primers *iap-1* and *iap-3* provided specificity for the detection of *L. monocytogenes* by the nested PCR, with *iap-3* providing the specificity for the dot blot hybridization assay. This difference between both pairs of primers was not surprising. It is known that the specificity of a PCR highly depends on the 3′-end sequence of the primers and that one mismatch in this region may result in no amplification.

We, like others, have observed that purification of the bacterial DNA is not necessary for PCR (1, 3, 17). Thus, the use freeze-thaw lysates of CSF reduces the need for manipulation of the clinical samples and could diminish the risk of false-positive results because of contamination.

Under our experimental conditions, the standard PCR assay for the direct detection of *L. monocytogenes* had a sensitivity of 1,000 to 400 viable cells. Because the theoretical limit of detection by PCR is on the order of one copy of the target sequence initially in the reaction, we assessed whether this limit could be obtained. Indeed, this was achieved by increasing the number of amplification cycles in the nested methodology or by increasing the sensitivity of PCR product detection in the hybridization procedure. The nested PCR methodology can be completed in 8 h. However, its routine application in a standard microbiological setting may be difficult because of the high risk of contamination (13, 18). Although the dot blot methodology is more time-consuming (more than 24 h to complete), it is less susceptible to contamination. Thus, it may be more useful in a diagnostic laboratory.

For the reasons mentioned above, we chose the PCR-dot blot methodology to examine 52 CSF samples collected between 1983 and 1991. All four false-positive results obtained by PCR-dot blot were obtained with CSF samples which were positive by culture for *H. influenzae*. This was very intriguing but could not be explained by a lack of specificity of the reaction. When DNA from each of the four *H. influenzae* strains was used as a template in the PCR, no amplification product could be detected by Southern blotting or dot blotting with the *iap-3* probe (data not shown). All the experiments were done at least twice, with negative controls performed at each step. Physical separation of the pre- and postamplification procedures, separate sets of supplies and pipetting devices for setting up reactions, and positive-displacement pipettes were used to avoid contamination,

according to current standards for performing DNA amplification (13, 18). Contamination during the performance of the assay could thus be reasonably excluded. These false-positive results might result from a contamination of the samples during collection, storage, and subsequent manipulations. They were all collected during the period of the Swiss outbreak of listeriosis (1983 to 1987) (2) and were manipulated in the same laboratory where the true-positive specimens and all the strains of *L. monocytogenes* related to the epidemic were investigated. At that time, the possibility of doing DNA amplification was not considered, and appropriate care was not necessarily applied to the sampling of the specimens. Our results stress the fact that clinical samples must be manipulated with special caution when a nucleic acid amplification assay is to be used.

A positive signal could be obtained with 14 of 17 CSF samples positive for *L. monocytogenes* by culture and tested after 1 to 7 years of storage. Three additional specimens that were culture positive for *L. monocytogenes* were PCR-dot blot negative. No PCR inhibitors were found in these three false-negative specimens. The amount of *L. monocytogenes* could have been minimal in these samples, reaching the limit of sensitivity of the method.

Three other CSF samples collected from patients with *Listeria* meningitis were culture negative and PCR-dot blot positive. All three samples were follow-up CSF samples taken from patients with a previous CSF culture that was positive for *L. monocytogenes*, and who had already been treated for 2 or more days with antibiotics. In that situation, PCR-dot blot may be more sensitive than conventional culture because it can detect cells even though they are dead. For this reason, these results were not considered false positive. They suggest that standard PCR followed by dot blot hybridization is an encouraging approach for the diagnosis of *Listeria* meningitis, in particular, when antibiotic administration precedes culture of CSF.

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