JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 179–183 Vol. 35, No. 1
0095-1137/97/$04.00+0

Isolation of Catalase-Negative *Listeria monocytogenes* Strains from Listeriosis Patients and Their Rapid Identification by Anti-p60 Antibodies and/or PCR

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Received 3 May 1996/Returned for modification 9 July 1996/Accepted 14 October 1996

Two catalase-negative *Listeria monocytogenes* serovar 1/2b strains were isolated from listeriosis patients in 1995 in Germany. The infections appeared in individuals from different cities at different seasons and were caused by *L. monocytogenes* strains of different clonal types. In particular, the catalase reaction of one strain isolated from blood was consistently negative, whereas this reaction was only reversibly blocked when the strain was freshly isolated from ascitic fluid. After subculturing, the catalase-positive reaction was restored. Initially, identification of these isolates was difficult to achieve not only because of the lack of a catalase reaction, which generally distinguishes *L. monocytogenes* from other morphologically similar pathogenic gram-positive bacteria, but also because other routinely used biochemical tests such as CAMP and the commercial API test gave unclear results. However, rapid and unequivocal identification of these strains was possible by analyzing secretions of the p60 protein in culture supernatants by enzyme-linked immunosorbent assay or Western blot (immunoblot) analysis with our recently developed *Listeria*- and *L. monocytogenes*-specific anti-p60 antibodies. Additionally, the identifications were confirmed by *Listeria*- and *L. monocytogenes*-specific PCR analyses with primers derived from the *iap*, *hly*, and *prfA* genes. Immunoanalyses also allowed for the differentiation of these two strains, whereas no differentiation was possible by PCR when the internal, variable repetitive *iap* gene portion was analyzed. However, size variations of the PCR products comprising these gene portions which were obtained from a number of *L. monocytogenes* strains belonging to the same serotypes indicated that this type of PCR is not only useful for specific identifications but may be used in parallel as an additional marker for epidemiological studies. In conclusion, the data suggest that catalase production should not be taken as a strict criterion for the identification of *listeriae*. Furthermore, at least the infection caused by the stably catalase-negative strain supports the notion that catalase does not seem to be necessary for the intracellular growth of *L. monocytogenes*.

Listeria monocytogenes is considered an ubiquitous foodborne pathogen which can lead to serious infections, especially in newborns and in elderly, pregnant, and immunocompromised people. Listeriosis mainly manifests itself as sepsis, meningitis, and encephalitis, but occasionally, endocarditis, peritonitis, and hepatitis can also occur (20). *L. monocytogenes* is a facultative intracellular gram-positive bacterium that invades, replicates, and multiplies in a variety of mammalian cells. A number of genes and gene products necessary for the intracellular survival of this pathogen were identified, and these have recently been reviewed (13). The incidence of human listeriosis has been growing in the last few years, whereby most cases are sporadic but some are occasionally also epidemic (20).

Standard identification of listeriae from samples contaminated with multiple species (e.g., samples of food and feces or from the environment) relies on selective enrichments and subsequent biochemical analyses and can be difficult, laborious, and time-consuming (20). In contrast, identification of *L. monocytogenes* from clinical samples (e.g., blood, spinal fluid, or other normally sterile sites) is thought to be easier to perform, although the sometimes weak hemolytic production of freshly isolated clinical strains on blood agar plates, which is an important classification marker, may lead to some confusion (20).

Here we report on the isolation of two unusual *L. monocytogenes* strains from the blood and ascites of listeriosis patients, respectively, in Germany in 1995. These bacteria could not be positively identified by routinely used biochemical methods when they were freshly isolated because they were catalase negative. However, after subculturing of the isolate obtained from ascitic fluid, it became catalase positive, whereas the other strain remained stably negative. The catalase-positive reaction is common to all *Listeria* species and has been described as a routine method of distinguishing *L. monocytogenes* from other clinically important gram-positive bacteria (21). Furthermore, catalase, together with superoxide dismutase, has been suggested to play a potential role in the intracellular survival of *L. monocytogenes* (24). On the other hand, it has been shown that the virulence of transposon-induced catalase-negative mutants was not significantly affected (16). In addition, the commercial API *Listeria* assay used for the differentiation of *Listeria* species, which is based mainly on the

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differential fermentation of various sugars, together with the CAMP test, gave conflicting results. The CAMP test enhances the lysis of sheep erythrocytes on blood agar by a synergistic effect between a phospholipase and listeriolysin of *L. monocytogenes* and a sphingomyelinase of *Staphylococcus aureus* and is used for the differentiation of apathogenic *Listeria* species (17).

Recently, we developed antibodies which recognize the surface-associated major extracellular p60 protein of *L. monocytogenes* (2, 3, 11, 18). This protein seems to be involved in adherence of this organism to certain eukaryotic cells (2, 12). A p60 protein which is encoded by the *iap* (invasion-associated protein) gene (11) is secreted by each *Listeria* species and acts primarily as a murein hydrolase, which is essential in a late step of cell division (2, 26). The comparison of the whole listerial protein family indicated there were conserved and species-specific variable portions (2) which allowed for the generation of two different antibodies specific for the *L. monocytogenes* p60 protein (3). By using a set of genus- and species-specific antibodies and primers derived from the *iap* gene in enzyme-linked immunosorbent assay (ELISA) and PCR analyses (3, 5), respectively, both strains could be rapidly and unequivocally identified as *L. monocytogenes*. Immunoanalyses also enabled the differentiation of the two isolates, which both belonged to serovar 1/2b. Furthermore, the pathogenic behavior of at least one of these strains in humans supports the previous notions that catalase is not necessary for the intracellular survival of *L. monocytogenes* (16, 23) and thus does not seem to be a major virulence factor.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The stably catalase-negative strain *L. monocytogenes* AB123 was isolated from the blood of a patient with bacteremia. The reversibly catalase-negative strain *L. monocytogenes* AB78 was isolated from the ascites of a patient with spontaneous peritonitis. The strains *L. monocytogenes* EGD serovar 1/2a, *Listeria innocua* NCTC 11288 serovar 6a, *Listeria ivanovii*, *Listeria seeligeri* and *Enterococcus faecalis* were taken from the culture collection of the Theodor-Boveri-Institut für Biowissenschaften. All other *L. monocytogenes* strains were obtained from E. Bannerman, Centre National des Listeria, Lausanne, Switzerland. Bacteria were routinely cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C.

Clinical information. *L. monocytogenes* AB123 was isolated from the blood of a 69-year-old man who was taken into hospital in Aachen, Germany, in August 1995 because of acute kidney failure. It was known that this man also suffered from hepatitis C because he was hepatitis C virus RNA positive. Immediately after admission, he developed a septic fever (>39°C). From three independent blood samples, gram-positive, catalase-negative, rod-shaped bacteria were isolated. At this time, the patient was treated intravenously with a ceftriaxone-tobramycin antibiotic combination. The patient's temperature then dropped, and the following blood cultures were negative. However, the patient displayed a general vasculitis and the beginning of multiorgan failure. Approximately 2 weeks later the man died due to multiorgan failure. In conclusion, the patient developed a primary *Listeria* bacteremia, which may have been caused by diminished resistance due to the hepatitis C virus infection, along with vasculitis.

L. monocytogenes AB73 was isolated from the ascites of a 61-year-old man who was taken into hospital in Kandel, Germany, in January 1995. The patient, who already had a long-lasting liver cirrhosis because of alcohol toxicity, presented with fever, a rapid increase in abdominal volume, and abdominal pains; these symptoms were due to a spontaneous bacterial peritonitis. From ascites, gram-positive, rod-shaped bacteria with a catalase-negative phenotype could be isolated. Additionally, an increased number of granulocytes could be determined. Following determination of the antibiogram of the isolate, the patient was treated intravenously with penicillin G for 4 weeks and concurrently with gentamicin for 3 weeks. Two days after the start of the treatment his temperature dropped. During and after the antibiotic therapy, ascitic fluid was sterile and the number of granulocytes decreased. The patient left the hospital 6 weeks after admission.

Biochemical and serological methods. Catalase production was determined by applying a drop of 30% H_2O_2 to the colonies and observing the occurrence of O_2 bubbles, as described elsewhere (9). The CAMP test was performed by standard procedures by streaking out bacteria along with *S. aureus* on blood agar plates containing 5% sheep blood and observing zones of hemolysis. The commercial API *Listeria*, API Rapid ID 32 Strep, and API Coryne tests (bioMerieux, Marcy l'Etoile, France) were used according to the manufacturer's description. Serotyping of *Listeria* strains was performed according to the antigenic scheme of Seeliger and Höhne (22).

SDS-PAGE, immunoblotting, and ELISA. Proteins were harvested from the supernatants of 1-ml bacterial cultures grown overnight by precipitation with trichloroacetic acid (final concentration, 7%) on ice for 1 h. After centrifugation, the proteins were washed with acetone, dissolved in Laemmli (15) sample buffer, and heated at 95°C for 5 min. Protein separation was achieved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels as described by Laemmli (15). Transfer to nitrocellulose membranes (Hybond-C; Amersham-Buchler, Braunschweig, Germany) was performed by semidry electroblotting in a graphite chamber (14). For *Listeria* genus-specific and *L. monocytogenes* species-specific detections by Western blotting (immunoblotting), rabbit antisera raised against the whole p60 and p60-derived synthetic peptides A or D were used, respectively. The generation of these antisera has been described earlier (4, 11). All antibodies were diluted 1:500 in blocking buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin and 0.05% Tween 20). Blots were developed by using horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Hamburg, Germany) diluted 1:1,000 and 4-chloro-1-naphthol (Sigma, St. Louis, Mo.). For reprobing, nitrocellulose filters were incubated for 30 min in blocking buffer after the reaction had developed. Then, the procedure was continued as described above.

For ELISA, 100-μl samples of cell-free supernatants from overnight cultures were incubated in 96-well microplates for 2 h at 35°C. After the coated proteins were washed with PBS, the wells were blocked for 1 h with blocking buffer. The immunoglobulin G fractions of the antisera were used at a concentration of 15 μg/ml. For colorimetric reactions, horseradish peroxidase-conjugated sheep anti-rabbit antibodies (ICN, Costa Mesa, Calif.) diluted 1:500 in PBS and the substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (Pierce, Oud Beijerland, The Netherlands) were used. The optical density was measured at 405 nm in an ELISA reader apparatus (Dynatech, Torrance, Calif.).

PCR. For *Listeria* genus-specific and *L. monocytogenes* species-specific identifications by PCR, two different primer combinations derived from the *iap* gene were used. Primer pair UnilisA (5′-GCTACAGCTGGGATTGCGGT-3′) and Lis1B (5′-TTATACGCGACCGAAGCCAA-3′) is specific for all *Listeria* species, primer pair MonoA (5′-CAAACTGCTAACACAGCTACT-3′) and MonoB (5′-GCACTTGAATTGCTGTTATTG-3′) is specific for all *L. monocytogenes* serotypes (5). In addition, two primer pairs derived from the *L. monocytogenes hly* (5′-CGGAGGTTCCGCAAAAGATG-3′ and 5′-CCTCCAGAGT GATCGATGTT-3′) and *prfA* (5′-ACCAATGGGATCCACAAGA-3′ and 5′-C AGCTGAGCTATGTGCGAT-3′) genes were used. All primers were purchased from Pharmacia, Freiburg, Germany. Chromosomal DNA was prepared as follows. A total of 10^6 cells from agar plates were resuspended in 50 μl of 1× PCR buffer containing 2.5 μg of lysozyme (Sigma, St. Louis, Mo.), and the mixture was incubated for 15 min at 37°C. Then, 1 μl of proteinase K (12 mg/ml) was added, and the mixture was further incubated for 15 min at 56°C. Finally, the bacteria were lysed for 5 min at 110°C. PCRs were carried out in a Thermocycler 60-2 (biomed, Theres, Germany) in a 100-μl reaction volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 0.5 μg of each primer, and 1 U of Goldstar DNA Polymerase (Eurogentec, Seraing, Belgium). Amplifications started with an initial denaturation step at 94°C for 3 min, followed by 30 cycles, each at 94°C for 1 min, 56°C for 45 s, and 72°C for 45 s. The PCR products were separated in a 1.2% Tris-borate-agarose gel (Tris-borate is 90 mM Tris base, 90 mM H_3BO_3, and 2 mM EDTA [pH 8.0]) or in a 5% acrylamide gel and were stained with ethidium bromide.

RESULTS

Biochemical characterization of two catalase-negative clinical isolates. Three independent cultures of blood obtained from the patient with bacteremia resulted in the isolation of gram-positive and catalase-negative, rod-shaped bacteria which exhibited a negative to very weak hemolysis in the CAMP test. However, after several subcultures, hemolysis of this strain was as strong as that of the *L. monocytogenes* reference strain. The commercial biochemical API Rapid ID 32 Strep test that we used identified this strain as a *Listeria* sp. with an excellent identification value (99%). In this test, the catalase reaction is not included. The API Coryne test, which was also used, indicated a doubtful profile for *Listeria* sp. (xylose negative) because of the lack of a catalase reaction.

Cultures of ascitic fluid from the patient with peritonitis resulted in the isolation of gram-positive, catalase-negative, coccoid or rod-shaped bacteria. This strain also displayed no clear hemolysis zone when the CAMP test was used. However, after several subcultures both hemolysis phenotype and catalase production were as strong as those for the *L. monocytogenes* reference strain. The API *Listeria* test that we used did not identify the organism as *L. monocytogenes* but identified it

TABLE 1. Reactivities of the anti-p60 antibodies with proteins in supernatants from cultures of different bacterial strains by ELISA[a]

Organism	A_{405}[a]		
	Anti-p60	Anti-PepA	Anti-PepD
L. monocytogenes EGD	>2.7	2.52	1.31
L. monocytogenes AB 78	>2.7	>2.7	2.42
L. monocytogenes AB 123	>2.7	>2.7	0.27
L. innocua	>2.7	0.14	0.094
E. faecalis	0.158	0.118	0.108

[a] The antibodies were used at a concentration of 20 μg/ml. Optical density values greater than 1 optical density unit were considered a positive result.

as *L. innocua* because of a positive DIM reaction. In contrast to the variable hemolysis and catalase tests, the DIM reaction obtained with this strain remained consistently positive. Thus, identification of both strains as *L. monocytogenes* was not reliable by the standard methods due to the lack of basic classification marker reactions.

ELISA and Western blot analyses of the proteins of the clinical isolates in culture supernatants. As reported above, we generated different types of antisera which recognize the p60 protein secreted by *L. monocytogenes*. One antiserum has been raised against the whole p60 protein and has been shown to specifically cross-react with the p60-related proteins of most other *Listeria* species except that of *L. grayi* and its subspecies *murrayi* (2). PepA and PepD antisera both specifically recognize the *L. monocytogenes* p60 protein because they were raised against synthetic peptides (peptides A and D) which are derived from specific portions of this protein (4).

Initially, all three antisera were used to investigate p60 secretion of the clinical isolates by ELISA. As indicated in Table 1, the anti-whole p60 antiserum reacted efficiently with the supernatants of all strains tested so far, indicating that the clinical isolates do secrete a p60 protein. These findings imply that the analyzed bacteria are indeed *Listeria* species. Furthermore, the ELISA analyses with the anti-peptide A antibodies also gave clear positive results, indicating the secretion of an *L. monocytogenes* p60 protein. In contrast, anti-peptide D antibodies did not lead to a positive reaction with the p60 protein of the consistently catalase-negative strain *L. monocytogenes* AB123. Since this was the first example of an *L. monocytogenes* p60 protein which could not be recognized by anti-PepD antibodies, we next analyzed the reactivities in more detail by Western blotting. The reason for a lack of an ELISA reaction may have been due to different foldings of this native p60. The proteins of all tested strains found in the supernatant were denatured and separated by SDS-PAGE. As indicated in Fig. 1A, again, the anti-whole p60 antiserum strongly reacted with the 60-kDa protein comprising the p60 protein produced by all

FIG. 1. Genus-specific (A) and species-specific (B and C) identifications of the clinical *L. monocytogenes* isolates by Western blot analyses with precipitated proteins from 1 ml of supernatants of overnight cultures. Proteins were separated by SDS-PAGE, and the blot assays were performed with anti-whole p60 antibodies (A), anti-PepD antibodies (B), and anti-PepA antibodies (C). Lanes: 1, *L. monocytogenes* EGD; 2, *L. innocua*; 3, *L. monocytogenes* AB78; 4, *L. monocytogenes* AB123; 5, *E. faecalis*.

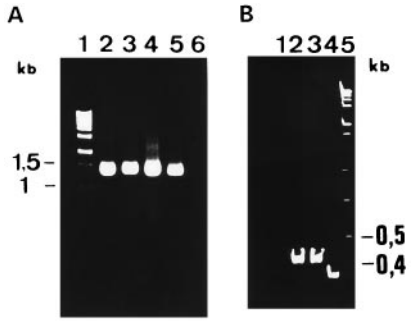

FIG. 2. Genus-specific identification of *Listeria* species (A) and *L. monocytogenes*-specific identification (B) by PCR with the primer pairs UnilisA-Lis1B and MonoA-MonoB, respectively. The genus- and species-specific PCR products were separated on a 1.2% agarose gel and a 5% acrylamide gel, respectively, and were stained with ethidium bromide. (A) Lanes: 1, molecular size marker; 2, *L. monocytogenes* EGD; 3, *L. monocytogenes* AB123; 4, *L. monocytogenes* AB78; 5, *L. innocua*; 6, *E. faecalis*. (B) Lanes: 1, *L. innocua*; 2, *L. monocytogenes* AB123; 3, *L. monocytogenes* AB78; 4, *L. monocytogenes* serovar 4b; 5, molecular size standard.

Listeria strains analyzed, whereas anti-PepD antibodies did not recognize the p60 protein from strain AB123. However, when reprobing the same filter, the use of anti-PepA antibodies led to strong reactions with this p60 protein, as expected from the ELISA results.

Identification of the two clinical isolates by PCR. To confirm the identification data obtained by ELISA and Western blotting, both clinical isolates were analyzed by PCR with different primer combinations. Primer pair UnilisA-Lis1B is derived from conserved portions of the *iap* gene and had been shown to allow for the specific amplification of the *iap* gene of all *Listeria* species (5). In contrast, primer pair MonoA-MonoB is derived from *L. monocytogenes*-specific *iap* gene portions and allows for the specific detection of all serotypes of this species. Furthermore, this combination amplifies a region which had been shown to display a length polymorphism in the *iap* gene of different *L. monocytogenes* serotypes (5).

As shown in Fig. 2A, the *Listeria* genus-specific amplification product of 1.4 kb could be obtained from both isolates, as expected. Also, the *L. monocytogenes*-specific reaction, which resulted in the amplification of an approximately 400-bp DNA fragment, identified both isolates as *L. monocytogenes* (Fig. 2B). Since these products were separated in a 5% acrylamide gel, it was possible to compare the repeat lengths in their *iap* genes. As a control reaction, we used a clinical *L. monocytogenes* serotype 4b isolate. As estimated from the gel, the repeat lengths for both isolates were identical but were larger than that for the control strain.

To validate the described species identifications, we performed additional PCR analyses using two other primer combinations that were derived from the virulence-associated genes *prfA* and *hly*. These genes are present on the chromosome in the so-called virulence gene cluster of *L. monocytogenes* (13) and have frequently been used for the specific identification of *L. monocytogenes* strains isolated from different sources (7, 8, 25). As shown in Fig. 3, *L. monocytogenes*-specific PCR products of the expected sizes (467 bp with *prfA* primers and 234 bp with *hly* primers) could be obtained from the two clinical isolates, which further clearly demonstrated that these isolates belong to *L. monocytogenes.*

Length polymorphism in the *iap* gene of *L. monocytogenes* strains of the same serotype. We also analyzed whether it is, in general, possible to distinguish *L. monocytogenes* isolates belonging to the same serotype by measuring the internal repeat

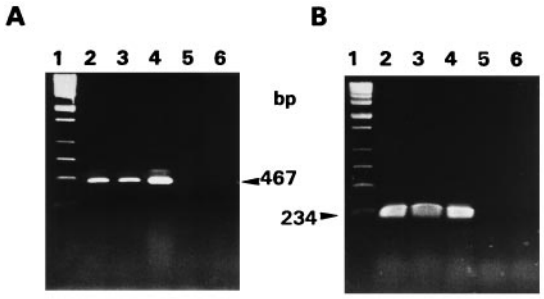

FIG. 3. Species-specific identification of clinical *L. monocytogenes* strains by PCR with primers derived from the *prfA* (A) and *hly* (B) genes. PCR products were separated in 1.2% agarose gels and were stained with ethidium bromide. Lanes: 1, molecular size markers; 2, *L. monocytogenes* EGD; 3, *L. monocytogenes* AB123; 4, *L. monocytogenes* AB78; 5, *L. ivanovii*; 6, *L. seeligeri.*

length in their *iap* genes. This may be an interesting additional method for epidemiological studies. We therefore compared the PCR products obtained from several *L. monocytogenes* strains of serotypes 1/2a and 4b by using the primer pair MonoA-MonoB. Together with serotype 1/2b, the vast majority of cases of *Listeria* disease in humans are caused by these three serotypes (20). In addition, variations of the repeat region in the *iap* gene of the less pathogenic serotypes 4a and 4c were investigated. As shown in Fig. 4, the repeat lengths, especially of serotypes 1/2a and 4b, varied in size, whereas only a few differences could be observed with serotypes 4a and 4c. This result indicated a hypervariable *iap* gene region. The smaller band of 250 bp seems to represent a truncated *iap* gene portion which may be due to intramolecular recombination events of the highly repetitive DNA sequence from a subpopulation of *L. monocytogenes* strains. Molecular analysis of this phenomenon is in progress.

DISCUSSION

Although humans frequently seem to be exposed to *L. monocytogenes* through contaminated foodstuffs, this pathogen only sporadically causes disease. Nevertheless, confirmation of presumptively positive colonies as *L. monocytogenes* by conventional methods requires 2 to 7 days and is problematic when a rapid answer is required (20).

We have reported on the isolation and identification of two *L. monocytogenes* serotype 1/2b strains from listeriosis patients which were very difficult to type because the criterion for identification of the genus *Listeria*, the catalase-positive reaction, was negative when we analyzed the strains. This reaction is recommended by Seeliger and Jones (21) for differentiating *L. monocytogenes* from other pathogenic gram-positive bacteria. In cerebrospinal fluid, *Listeria* can be mistaken as corynebacteria or streptococci because the morphology of *L. monocytogenes* cells when they are freshly isolated from tissues or blood may not be present in their usual form as rods (10). It has been observed that coccoid or even long filamentous forms with a rough phenotype similar to that of lactobacilli or filamentous forms with a smooth phenotype can appear, in the latter case under the influence of suboptimal antibiotic concentrations (10). However, naturally catalase-negative *Listeria* isolates are very rare in nature, and it was assumed previously that the reaction was absent because of nutrient deficiencies in the medium (21). In 1991, however, Swartz et al. (23) isolated and analyzed a catalase-negative *L. monocytogenes* strain from an elderly meningitis patient and mentioned in the same publication the isolation of another catalase-negative strain in the United States. The biochemical behavior of the analyzed strain was typical in all respects, and the strain expressed full virulence in an immunocompetent person (23).

To our knowledge, this has been the only description of a catalase-negative *L. monocytogenes* isolate from a patient. Four years later, two *L. monocytogenes* strains with catalase-negative phenotypes were isolated from listeriosis patients in Germany. In contrast to isolate *L. monocytogenes* AB123, which consistently remained negative, catalase production of *L. monocytogenes* AB78 was only reversibly blocked. The reason for this phenomenon is unknown, but as a speculation, it may be influenced by unusual iron access at the time of typing. Most catalases are enzymes which include a cofactor with an iron center. In addition, a DNA sequence motif upstream of the *L. seeligeri* catalase gene resembling a *fur* box could be found; thus, catalase expression in listeriae may be regulated at the transcriptional level by the available iron (9).

However, since catalase production is the prominent feature and the lack of this reaction generally rules out *Listeria*, identification of these isolates was difficult and laborious. Additional confusions arose when the CAMP test was used, because the weak hemolysis produced by both of these freshly isolated colonies could not be increased, which in general may represent a more serious problem with their identification (17). Furthermore, the positivity of the API *Listeria* DIM reaction with *L. monocytogenes* AB78, which consistently remained positive after several subculturings, indicated that it was the apathogenic species *L. innocua*, which is the species most closely related to *L. monocytogenes*. This test is based on the presence or absence of the enzyme arylamidase and had been confirmed with more than 250 *L. monocytogenes* and 170 *L. innocua* isolates (1).

The unequivocal identification of both strains could be simply and rapidly determined by investigating their proteins in crude culture supernatants by ELISA or Western blotting analyses with our *L. monocytogenes*-specific antibodies which recognize the secreted p60 protein. These recently developed antibodies are raised against synthetic peptides (PepA and PepD) specific for the *L. monocytogenes* p60 protein and were designed to allow for a more practicable immunological detection of these bacteria from foodstuffs (3, 4). For this purpose, the hydrophilic and highly immunogenic p60 protein, which

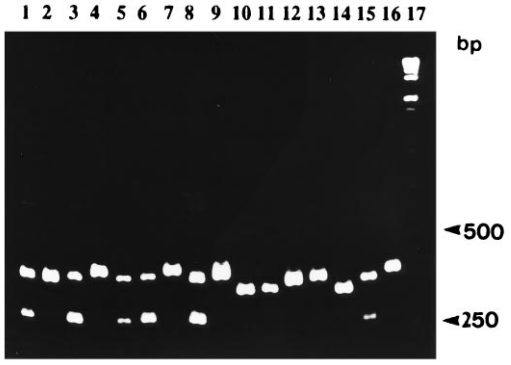

FIG. 4. Size variations of the PCR products obtained from various *L. monocytogenes* strains belonging to the same phenotype by using the primer combination MonoA-MonoB. PCR products were separated in a 5% acrylamide gel and were stained with ethidium bromide. Lanes: 1, strain VD 8200 serovar 4c; 2, NV 7609 serovar 4c; 3, VD 7065 serovar 4c; 4, NV 4755 serovar 4a; 5, NV 4702 serovar 4a; 6, NV 4700 serovar 4a; 7, SLCC 2374 serovar 4a; 8, SLCC 2375 serovar 4b; 9, LL 185 serovar 4b; 10, LL 184 serovar 4b; 11, LL 147 serovar 4b; 12, LL 142 serovar 4b; 13, SLCC 2371 serovar 1/2a; 14, LL 365 serovar 1/2a; 15, LL 141 serovar 1/2a; 16, LL 93 serovar 1/2a; 17, molecular size standard.

represents a murein hydrolase (2, 8, 26), is considered an ideal target for the detection of *L. monocytogenes* because it is essential for viability and is secreted relative stably in large amounts at all bacterial growth phases (26). The antibodies could also be of clinical interest not only because of the convenience of the ELISA but also because they have been shown to detect the p60 molecule of each *L. monocytogenes* serotype. In principle, all serotypes can lead to listeriosis, although serotypes 1/2a, 1/2b, and 4b are predominant (20).

Whereas the isolate *L. monocytogenes* AB78 could easily be detected with both anti-PepA and anti-PepD antibodies, isolate AB123 behaved unusually in this respect. Among the hundreds of *L. monocytogenes* strains analyzed so far (19), this is the first report of a misidentification obtained with anti-PepD antibodies. In contrast, detection was clearly possible with anti-PepA antibodies, which also demonstrated that both isolates must be of different clonal types. However, these data indicate that, when analyzing *L. monocytogenes* strains, both antibodies are needed for a safe identification by this immunological method.

The PCR primers that we used for the specific identification of the two clinical *L. monocytogenes* strains were derived from the *hly*, *prfA*, and *iap* genes. These genes have been widely used as specific targets for the unequivocal identification of *L. monocytogenes* strains in a number of studies (6, 7, 25). In contrast to the *hly* and *prfA* gene primers, which always lead to PCR products of identical sizes because they do not carry internal repetitive sequences, the *iap* gene primers amplify an internal portion that codes for the repetitive amino acid sequence Thr-Asn in the p60 protein. We have shown earlier that the size variations of the products obtained by this PCR reflect the various numbers of repeat units, which varied from 11 to 19 among the 13 different *L. monocytogenes* serotypes (5). By comparing the PCR products obtained from isolates belonging to the same serotype, as shown in Fig. 4 for serotypes 1/2a, 4b, and 4a, this portion is found to be hypervariable in length. Although the two described clinical isolates, which are both of serotype 1/2b, could not be differentiated by this method (Fig. 2B), this amplification reaction may be useful as an additional marker for epidemiological studies because it offers the opportunity to specifically identify and differentiate *L. monocytogenes* strains by a single PCR. In summary, we have described the isolation and the simple unequivocal identification of two different, biochemically unusual *L. monocytogenes* strains from listeriosis patients which expressed full virulence. The lack of catalase production at the time of analysis supports the notion that the prevalence of catalase-negative *L. monocytogenes* strains may be underestimated because of underrecognition (23). We agree with the opinion that catalase production should not be a strict criterion for the identification of *Listeria* (23). Furthermore, the data also indicate, at least for isolate AB123, that catalase production does not seem to be necessary for the intracellular survival of *L. monocytogenes.*

ACKNOWLEDGMENTS

We thank E. Bannerman (Lausanne, Switzerland) for kindly providing the different *L. monocytogenes* serotypes. We thank J. Daniels for carefully reading the manuscript and Simone Schmitt for excellent technical assistance.

This work was supported by grant BMFT KI00859 from the Bundesministerium für Forschung und Technologie, grant SFB 165-B4 from the Deutsche Forschungsgemeinschaft, the Universitätsbund Würzburg, and E. Merck, Darmstadt, Germany.

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