

## Hemolytic Activity Reevaluation of Putative Nonpathogenic *Listeria monocytogenes* Strains

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Identification of 12 strains originally characterized as nonpathogenic *Listeria monocytogenes* was reassessed following the evaluation of their hemolytic capability with a newly developed horse blood agar plate. Seven of the strains were observed consistently to be hemolytic and confirmed as *L. monocytogenes* with the use of two commercial systems: the Gene-Trak *L. monocytogenes*-specific colorimetric DNA hybridization assay and the API Listeria system. Except for one strain that formed typical smooth colonies, these hemolytic strains formed rough colonies on a selective medium, lithium chloride-ceftazidime agar. The rest of the strains were nonhemolytic and did not hybridize with the DNA probe; they were identified as *Listeria innocua* on the basis of their API Listeria system biochemical profile. All but one of these nonhemolytic strains formed smooth colonies on lithium chloride-ceftazidime agar.

Ever since the realization that listeriosis is a food-transmissible disease, there has been considerable interest in the virulence mechanism and characteristics of *Listeria monocytogenes* that would facilitate its detection and identification after recovery from foods (4, 17, 18). A number of factors have been shown to be associated with the virulence of *L. monocytogenes* (17), one of which is a hemolysin designated specifically as listeriolysin O. This cold-tolerant pathogen has been shown to be rendered avirulent with the loss of hemolytic activity by transposon mutagenesis. However, there does not seem to be a strict correlation between the amount of hemolysin produced and the virulence of the organism (11, 20). Hemolysis is also considered a key test in the identification of *L. monocytogenes*, the only *Listeria* species that is a significant human and animal pathogen. Until recently, hemolysis was the only phenotypic marker that distinguished *L. monocytogenes* from *Listeria innocua*. Unfortunately, difficulties in demonstrating this property have been reported, especially with low-producing strains. In 1989, Conner et al. (2) identified 15 nonpathogenic strains as *L. monocytogenes* that had varying levels of hemolytic activity on a conventional sheep blood agar plate (BAP). With some strains, weak hemolysis was discernable only when potentiated with a commercially available disc containing an extract of *Staphylococcus aureus* sphingomyelinase (CAMP test). In a subsequent report (5), demonstration of hemolysis was variable with four of five strains on a conventional horse BAP. Johnson and Lattuada (10) reported that large numbers of *L. monocytogenes* strains exhibited ambiguous hemolysis-CAMP reactions with conventional sheep BAP. However, Fujisawa and Mori (7) noted that the type of medium and the source of erythrocytes are critical factors in the bilayer BAP of McClain and Lee (15) to observe hemolytic activity consistently, especially among low-producing strains of *L. monocytogenes*.

Our laboratory has focused on developing methods that accelerate in a cost-effective fashion the detection and identification of *L. monocytogenes* in foods. In 1990, a rapid agar-based identification scheme (12) was described that included the determination of hemolytic activity of *L. monocytogenes* in 6 h subsequent to recovery on a selective medium, lithium chloride-ceftazidime agar (LCA), in 40 h (13). This was achieved by transferring a heavy inoculum from a suspect col-

ony to a very small area of the BAP and potentiating it with a commercially available disc containing an extract of *S. aureus* sphingomyelinase. The drawback of the scheme was the use of a conventional sheep BAP that may preclude the observation of hemolysis among low-hemolysin-producing *L. monocytogenes* strains (7).

In this study, a newly developed BAP designed to provide a clear-cut reading of hemolytic activity was used to reassess the hemolytic activity of strains previously identified as nonpathogenic *L. monocytogenes* strains (2, 5). The identification of these strains was subsequently verified with the use of two new commercial diagnostic kits.

Twelve of the 15 strains described by Conner et al. (2) as nonpathogenic *L. monocytogenes* were obtained from the National Food Processors Association in Washington, D.C. (see Table 1). Stock cultures in agar slants of heart infusion (Difco) were stored at 6°C and were used to propagate cultures overnight at 35°C in brain heart infusion broth (Difco). The broth cultures were serially diluted for inoculation of LCA to obtain 10 to 50 colonies per plate. Morphology of colonies was noted after 24 and 40 h at 35°C.

A thin layer of blood agar plate (mBAP) was used to improve the detection of hemolytic activity of listeriae. This involved adding about 8 ml of molten hydrated Bacto Columbia blood agar base EH medium (Difco) containing 5% defibrinated horse blood. The mBAPs were stored in a plastic bag at 6°C for ≤10 days. Bright red mBAPs were prewarmed at 35°C and inoculated on a small area with a heavy cell mass from a colony on an LCA plate. Observations of a narrow zone of hemolysis around each colony were made after 6 h at 35°C.

A colorimetric, *L. monocytogenes*-specific DNA hybridization assay kit (Gene-Trak Systems, Hopkinton, Mass.) was used to verify that the 12 nonpathogenic listeriae were indeed *L. monocytogenes*. The assay was designed and subsequently demonstrated (19) to specifically detect *L. monocytogenes* in foods at low levels after enrichments in broth and agar media. Cells obtained from an isolated colony on a 40-h LCA plate were suspended in 1.0 ml of phosphate-buffered saline. The hybridization process was done according to the recommendation of the manufacturer, and a photometric optical density value exceeding 0.1 U was considered positive for *L. monocytogenes*.

TABLE 1. Characteristics of strains previously identified as *Listeria monocytogenes*

Strain	Hemolysis <sup>a</sup>	DNA probe <sup>b</sup>	API Listeria system <sup>c</sup>	Colony morphology <sup>d</sup>	
				Size	Texture
ATCC 15313	+	+	<i>L. monocytogenes</i>	Small	Rough
ATCC 19113	+	+	<i>L. monocytogenes</i>	Small	Rough
ATCC 43248	+	+	<i>L. monocytogenes</i>	Small	Rough
N 7023	+	+	<i>L. monocytogenes</i>	Small	Rough
N 7160	+	+	<i>L. monocytogenes</i>	Medium	Rough
N 7196	+	+	<i>L. monocytogenes</i>	Small	Rough
N 7256	+	+	<i>L. monocytogenes</i>	Medium	Smooth
N 7083	-	-	<i>L. innocua</i>	Medium	Rough
N 7086	-	-	<i>L. innocua</i>	Medium	Smooth
N 7142	-	-	<i>L. innocua</i>	Medium	Smooth
N 7162	-	-	<i>L. innocua</i>	Medium	Smooth
N 7173	-	-	<i>L. innocua</i>	Medium	Smooth

<sup>a</sup> Narrow zones of hemolytic activity around colonies on a modified blood agar plate in 6 h at 35°C.

<sup>b</sup> Gene-Trak colorimetric DNA hybridization assay for *L. monocytogenes*.

<sup>c</sup> API Listeria system kit that employs 10 biochemical test reactions on a strip to identify *Listeria* species.

<sup>d</sup> Colony size in 40 h at 35°C (medium, >2.0 mm in diameter; small, <2.0 mm) and texture.

Use of the API Listeria system (BioMerieux Vitek, Inc., Hazelwood, Mo.) consisted of suspending cells from an overnight growth on mBAP to an approximate 0.5 McFarland standard for use in the inoculation of the 10-test strip. In accordance with the manufacturer's recommendation, reagent addition and reaction interpretation were carried out after an overnight incubation at 35°C.

As summarized in Table 1, 7 of the 12 putative *L. monocytogenes* strains on mBAPs exhibited hemolytic activity in 6 h. In contrast, use of a conventional BAP (about 15 ml of Columbia blood agar base containing 7% sheep blood) indicated all but two strains (N 7160 and N 7256) to be nonhemolytic. Two of the seven hemolytic strains (ATCC 15313 and ATCC 19113) were likewise reported by Johnson and Lattuada (10) as nonhemolytic. Presumably, the three hemolysis procedures employed may be deficient since Fujisawa and Mori (7) had no difficulty in observing the hemolytic activity of both strains with the bilayer BAP of McClain and Lee (15). Hybridization with the DNA probe confirmed previous studies that these seven strains were indeed *L. monocytogenes*. Moreover, they fit perfectly the API Listeria system profile for *L. monocytogenes*. With one exception (N 7196), they were observed by Conner et al. (2) to hybridize with a putative *L. monocytogenes* hemolysin gene probe of Datta et al. (3), the sequence of which has since been shown to code for a major secreted protein (6).

A minor deviation from the biochemical profile for *L. monocytogenes* was the fermentation of glucose-6-phosphate by four strains (ATCC 15313, ATCC 19113, N 7023, and N 7196). Fujisawa and Mori (7) also reported that 2 of their 24 *L. monocytogenes* strains were also glucose-6-phosphate fermenters. Five of the hemolytic strains grew slowly (colonies of <2.0 mm in diameter in 40 h) and formed rough colonies on LCA; one strain also formed rough but larger colonies (>2.0 mm) while another formed typical, smooth colonies. These six rough strains were observed under light microscopy to be filamentous, reminiscent of the isolation of a spontaneous rough mutant of *L. monocytogenes* with reduced virulence (8). Further studies are needed to determine if this deviation in colony morphology on LCA among the avirulent *L. monocytogenes* strains reflects an impairment of a virulence factor.

The other five strains were nonhemolytic. They were often observed to exhibit the so-called contact hemolysis beneath their colonies, a phenomenon that should not be confused with true hemolysis (16). They did not hybridize with the DNA probe, indicating that they were misidentified as *L. monocytogenes*. Conner et al. (2) reported that none hybridized with the putative hemolysin gene probe with the exception of N 7173. Their API Listeria biochemical profile indicated that they were typical strains of *L. innocua*. All grew well on LCA and formed smooth colonies with the exception of N 7083. Recognizing these strains as *L. innocua* provides a simple explanation of why they were reported to be nonpathogenic (2, 5).

The development of mBAP emerged from efforts to improve the hemolysis test portion of the RapID 6-h test for identifying colonies of *L. monocytogenes* on LCA (12). The goal was to facilitate the observation of low levels of hemolysin among unusual strains of *L. monocytogenes* that may occasionally be found in foods. Preliminary studies with the bilayer BAP of McClain and Lee (15) provided good results consistent with the report of Fujisawa and Mori (7) that confirmed the importance of incorporating 5% horse blood into the top thin layer of Columbia blood agar base for consistent observation of hemolysis. Further improvement in the clarity of hemolysis reading was achieved when the bilayer BAP was modified into a single thin layer. Moreover, Bacto Columbia blood agar base EH, advertised as an enhancer of beta-hemolytic zones of streptococci, was substituted for the plain Columbia blood agar base to further enhance the clarity of hemolysis reading.

Similar observations of enhanced clarity of hemolysis reading with the use of EH agar base version were reported recently by Heisick et al. (9). In examination of 59 strains of listeriae from a previous study (14), mBAP provided unequivocal observation of a narrow zone of hemolysis around colonies of *L. monocytogenes* and *Listeria seeligeri* and a wider zone around colonies of *Listeria ivanovii* (data not shown). The sensitivity and the specificity of mBAP were demonstrated by the unequivocal observation of hemolysis among *L. monocytogenes* strains that were described as variably hemolytic (2, 5) and its absence among strains identified in this study as *L. innocua*. The clarity of the zones of hemolysis on mBAP was such that no special lighting system was necessary (15); a bacterial colony counter was adequate provided that the light source was filtered with a white piece of paper or wiper.

The API Listeria system was easy to use, providing another useful marker to identify *L. monocytogenes* especially for the occasional nonhemolytic strains. Among the 10 biochemical test reactions on the API strip (arylamidase or DIM, esculin hydrolysis,  $\alpha$ -mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose,  $\alpha$ -methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-tagatose), DIM was the only characteristic that unequivocally distinguished *L. monocytogenes* from *L. innocua*, with the former being negative for DIM. The basis of the test is the hydrolysis of an unspecified naphthylamide substrate by an aminopeptidase that is produced by all *Listeria* species except *L. monocytogenes*. The system's observed specificity and sensitivity are consistent with other reports (1, 7) although one study reported the failure of the system to identify 3 of 31 *L. monocytogenes* strains examined (10). Its drawback is the length of time required (2 days) to identify a suspect colony on LCA following recovery from a food sample. In contrast, identification is obtained in about 3 h with the Gene-Trak DNA probe system and in about 7 h with the Natick RapID test (12). In conclusion, the study demonstrated that the 12 strains of listeriae originally identified as *L. monocytogenes* actually consist of two species, *L. monocytogenes* and *L. innocua*, on the basis of two commercial identification sys-

fems. The use of mBAP for demonstration of hemolytic activity provided the initial indication that the strains consisted of two species. mBAP optimized the detection of hemolytic activity of *L. monocytogenes* in a rapid and convenient fashion at minimal cost.

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