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Simple Color Tests Based on an Alanyl Peptidase Reaction Which Differentiate *Listeria monocytogenes* from Other *Listeria* Species

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The hydrolysis of DL-alanine-β-naphthylamide and D-alanine-*p*-nitroanilide for identification of *Listeria* spp. has been studied with 227 cultures. All species of *Listeria*, except *L. monocytogenes*, hydrolyzed these substrates. The reactions were detected by simple chromogenic reactions and could substitute for the CAMP test.

Prior to the early 1980s, human listeriosis was considered of relatively minor concern. However, in the 1980s, the incidence of individual cases of listeriosis increased in several countries, and this, together with a series of foodborne outbreaks, caused *Listeria monocytogenes* to become of significant public health importance (5). *Listeria* species are ubiquitous in the environment, and within the genus, *L. monocytogenes* is the major human pathogen (5). Therefore, it is important that *Listeria* species be correctly identified. For the identification of species within the genus, phenotopic characters, which rely on the fermentation of sugars, and hemolytic reactions are generally used. This paper reports on two color-based amidase tests, carried out with microtiter plates, which differentiate *L. monocytogenes* from the remainder of the genus.

In this study, 141 cultures of *Listeria* were obtained from culture collections which comprised the following: The Department of Veterinary Medicine and Immunology, University of Guelph, Guelph, Ontario, Canada; National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands; Instituto Cantonale Bacteriologico, Lugano, Switzerland; University of Madison, Madison, Wis.; U.S. Department of Agriculture, Minneapolis, Minn.; USDA Agricultural Research Service, Philadelphia, Pa; Bureau of Microbial Hazards HPB, Ottawa, Canada; Centre National de Reference des Listeria, Lausanne, Switzerland; PHLS, Colindale, United Kingdom; American Type Culture Collection, Rockville, Md.; and National Collection of Type Cultures, Colindale, United Kingdom. For this study, there were 96 *L. monocytogenes*, 26 *L. innocua*, 4 *L. ivanovii*, 8 *L. welshimeri*, 5 *L. seeligeri*, and 2 *L. grayi* cultures. Nine different serotypes had been established for 85 of the *L. monocytogenes* cultures. Among the *L. monocytogenes* cultures, 26 were from human listeriosis infections, 5 were from animal infections, 52 were from food, and 13 were from other sources. All of the remaining *Listeria* species in the collections were from food or other environmental sources. Six strains of *L. innocua* were rhamnose negative in standard tests.

The identity of all cultures was reconfirmed with either the API Listeria (biomérieux, Marcy-L'etoile, France) identification test kit or standard biochemical tests as described elsewhere (1, 2), which included the following: catalase; motility tests; acid formation from D-mannitol, D-xylose, and L-rhamnose after 7 days of incubation at 35°C; hemolysis on horse blood plates; and the CAMP test for *Rhodococcus equi* (ATCC 6939) and *Staphylococcus aureus* (ATCC 25923).

A further 67 *Listeria* isolates obtained in Toronto during a study of retail red meats were identified by standard methods (described above): 17 were *L. monocytogenes*, 16 were *L. innocua*, and 34 were *L. welshimeri.*

Nine cultures of *L. monocytogenes*, with altered hemolytic properties due to mutations or transposon insertion (8), plus three isolates of *L. monocytogenes* with weak or absent hemolytic reactions (Murray B and two variants of NCTC 5105 from the culture collections) were among the isolates tested. A further 10 rhamnose-negative *L. monocytogenes* cultures were also tested.

Listeria isolates were propagated in Trypticase soy broth without glucose (TSB; BBL, Becton Dickinson, Cockeysville, Md.), Trypticase soy agar (TSA; BBL, Becton Dickinson) with 0.6% (wt/vol) (Difco, Detroit, Mich.) yeast extract, or 5% (vol/vol) defibrinated sheep blood in a TSA base.

For the alanyl peptide hydrolysis test, cultures were grown on TSA or blood agar for 18 to 24 h at 35°C, and the majority of the growth was harvested with a sterile cotton swab into 1.0 ml of sterile saline, and of this, 25-μl aliquots were added to 96-well microtiter plates (Nunc-Immuno Plate; Poly Sorp. Intermed). A 20 mM DL-alanine-β-naphthylamide HCl (DLABN; catalog no. A 2503; Sigma, St. Louis, Mo.) solution was prepared in sterile 0.05 M Tris-HCl buffer (pH 7.0) and filter sterilized, and aliquots were frozen at −40°C. A 20 mM D-alanine-*p*-nitroanilide (DAPN) solution (L1080; BACHEM Biosciences, Philadelphia, Pa.) was similarly prepared, but stored at 4°C for up to 1 month in the dark. Fast violet B solution (FVB) was prepared by dissolving a 12.0-mg capsule (Sigma catalog no. 85-1) in 5 ml of sterile distilled water. This reagent was stored at 5°C in the dark and was stable for 1 week.

Just prior to the test, the DLABN or the DAPN stock was diluted in 0.05 M Tris-HCl (pH 7.0) to give a 15 mM solution, and 50 μl was added to 25 μl of the bacterial suspensions in the wells of the sterile 96-well microtiter plate. Negative controls contained 25 μl of saline in place of the bacterial suspension. The plate was covered with stretch plastic and incubated in the dark at 37°C for 4 h for DLABN or overnight for DAPN. The cover was removed, and 50 μl of FVB solution was added to the colorless DLABN tests. Free β-naphthylamine was detected by observing the deep yellow-orange complex, which developed within 5 min. Hydrolysis of the DAPN was seen by the liberation of the intense yellow *p*-nitrophenol.

Table 1 summarizes the data for the 227 cultures tested for hydrolysis of DLABN and DAPN. No *L. monocytogenes* cultures (including those containing strains with weak or absent

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TABLE 1. Hydrolysis of DLABN and DAPN by 227 *Listeria* cultures

Species	No. of cultures tested	No. of cultures hydrolyzing:	
		DLABN	DAPN
L. monocytogenes	132	0	0
L. innocua	42	42	42
L. ivanovii	4	4	4
L. welshimeri	42	42	42
L. seeligeri	5	5	5
L. grayi	2	2	2

hemolytic reactions) hydrolyzed either substrate, whereas all other *Listeria* species gave an easily detected hydrolysis of both substrates.

Although the use of nucleic acid probes has been reported for the detection of *L. monocytogenes* in foods (4) and commercial kits are available, conventional techniques based on hemolysis and sugar utilization reactions are almost exclusively used to identify species of *Listeria*. These conventional patterns of sugar fermentation and hemolytic reactions, especially the CAMP test (9, 10), can be problematic for the identification of *Listeria*. In addition, occasional cultures give atypical or equivocal identifications with the API Listeria (biomérieux) test kit (3).

Kampfer et al. (6) described a physiological characterization of 136 strains of *Listeria* with over 300 miniaturized tests. All strains of *L. monocytogenes* could be differentiated from *L. innocua* by the hydrolysis of DAPN, although some of the *L. innocua* strains gave slow reactions. These were possibly due to a lower cell concentration than we used, together with a prolonged, 7-day incubation and possible toxicity of *p*-nitroaniline. No such problems with *L. innocua*, even the rhamnose-negative strains, were encountered in the present study. Kampfer later reported (7) that a similar arylamidase reaction could be demonstrated fluorometrically with D-alanine-β-naphthylamide.

The observations reported here confirm that a single test based on the hydrolysis of substituted alanine compounds differentiates *L. monocytogenes* from other species of *Listeria*. The fact that poorly hemolytic and nonhemolytic strains of *L. monocytogenes* (which could be misidentified as *L. innocua*) were correctly identified as *L. monocytogenes* underlies the utility of this test. The more conveniently obtained substrate for the test is DL-alanine-β-naphthylamide, which also has the advantage of giving a 4-h test when FVB is used to detect the free β-naphthylamine. There are, however, potential hazards from the carcinogenic properties of the free β-naphthylamide, which are not a problem with the nitrophenol substrate.

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