Separation of Pathogenic from Apathogenic *Listeria monocytogenes* by Three In Vitro Reactions

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One hundred-twelve isolants of *Listeria monocytogenes* cultured from clinical and nonclinical sources were examined for hemolytic activity by means of the CAMP phenomenon and tested for acidification of xylose and rhamnose. The reactions of the isolants were noted and correlated with the pathogenicity of the organisms. All of the CAMP-negative (nonhemolytic) isolants were apathogenic, and all but one of the CAMP-positive (hemolytic) isolants were pathogenic. All xylose-acidifying isolants were apathogenic, but not all apathogenic isolants were xylose acidifiers. All pathogenic isolants but one were rhamnose acidifiers, but not all rhamnose acidifiers were pathogenic. The pattern of hemolysis, rhamnose acidification, and non-acidification of xylose is associated with pathogenic *L. monocytogenes*; departure from this pattern is associated with nonpathogenic *Listeria*, which otherwise share the properties of *L. monocytogenes*. These in vitro tests should prove useful in screening the apathogenic isolants whose clinical and epidemiological significance should be carefully assessed.

From the time of its discovery in 1923 through the 1950s, *Listeria monocytogenes* was considered a pathogen, often producing fatal illness in man and animals. Pathogenicity for laboratory animals, the production of beta hemolysis on sheep or horse blood agar, and the stimulation of monocytosis in animals were regarded as species characteristics of the organism, which was isolated from humans or animals with clinically active infections. In the 1960s, apathogenic nonhemolytic strains of *Listeria* were isolated. Bojsen-Møller in 1967 (2) reported that 12% of the strains that he isolated from the feces of asymptomatic humans were nonhemolytic. Since that time, many other investigators have isolated apathogenic, nonhemolytic *Listeria* from inanimate sources such as soil, vegetation, sewage, and silage as well as from carcasses, healthy humans, and animals (4, 10-12, 14, 16, 17, 19, 20). Because of the increasing number of isolations of the apathogenic, nonhemolytic organisms, some workers have expressed concern that the specific designation of these *Listeria* as *L. monocytogenes* may be misleading. As stated by Seeliger (16), this may result in the erroneous interpretation of their roles in human and veterinary medicine and epidemiology as well as in the tendency to minimize the potential danger of *L. monocytogenes* to human and animal health.

It would be desirable to have at hand simple and rapid procedures that could be employed to

differentiate the classical *L. monocytogenes* from the apathogenic strains without inoculating animals. Many workers have observed that pathogenic strains of *L. monocytogenes* are hemolytic, whereas apathogenic strains are nonhemolytic (1, 8, 10, 11, 13, 15, 20, 21); however, hemolysis frequently is weak or questionable and leads to subjective interpretation. The CAMP phenomenon (6, 7), which has been found to potentiate the hemolytic activity of *L. monocytogenes* (1, 5), was employed in the present study to eliminate the subjective interpretation of questionable hemolysis.

Bojsen-Møller (3) suggested that a definite sugar that would be fermented rapidly or not at all could be used for subdividing *L. monocytogenes* strains. Larsen (see reference 3) and Bojsen-Møller (3) observed 4 of 160 and 5 of 122 strains, respectively, that acidified xylose but did not acidify rhamnose. All of these nine strains were isolated from feces. From aquatic animals, Botzler et al. (4) isolated 65 strains of *L. monocytogenes*, which fell into two fermentation groups. One group, all apathogenic, acidified xylose in 24 h but did not acidify rhamnose in 14 days, whereas the other group, all pathogenic, acidified rhamnose in 24 h but did not acidify xylose in 14 days. In their examination of *L. monocytogenes* isolated from diseased animals and humans in Hungary, Durst and Berencsi (8) mentioned that virulent strains of *L. monocytogenes* were xylose negative, whereas the majority of the avirulent strains were xy-

lose positive.

The purpose of this study was to examine *L. monocytogenes* strains isolated from a variety of sources and note the relationship between xylose reactions, rhamnose reactions, and hemolytic activity and pathogenicity of the organisms.

MATERIALS AND METHODS

***Listeria monocytogenes* strains.** One hundred twelve strains of *L. monocytogenes* were used in this study, of which 49 (43.7%) were isolated from human patients with bacteriologically confirmed, clinically active listeriosis; 33 strains (28.6%) were isolated from vegetation or soil; and 31 strains (27.7%) were isolated from animals. Eleven of the animal strains were isolated from the brains of sheep or cattle with fatal listeriosis, and 20 strains were isolated from the feces of apparently healthy elk. The elk strains were isolated by R. G. Botzler of Humboldt State College at Arcata, Calif., and graciously supplied by W. C. Eveland of the University of Michigan at Ann Arbor. All strains were preserved by lyophilization and maintained at 4°C on slants of tryptose blood agar base (Difco) to which 1% glucose had been added. The stock cultures were transferred at intervals of 6 weeks, incubated at 34°C for 18 to 24 h, and then held at 4°C.

Carbohydrate media. Purple broth base (Difco), adjusted to pH 7.5, was sterilized by autoclaving, and rhamnose or xylose was aseptically added (1% final concentration) after passage through a 0.45- μ m membrane filter (Millipore Corp.). Broths were incubated at 34°C. Positive reactions (acidification) occurred within 24 to 72 h, although all tubes were held for 14 days.

CAMP phenomenon. The procedure of Christie et al. (6) as modified by Darling (7) was used in this study. Trypticase soy agar (BBL) (15 to 20 ml) containing 5% sterile defibrinated sheep blood was poured into sterile petri plates. With an inoculating needle, *Staphylococcus aureus* strain A was streaked in a straight line across the center of the plate. This staphylococcus was found to produce a high level of staphylococcal beta toxin. Strains of *L. monocytogenes* to be tested were streaked in a straight line 2 to 3 cm in length at right angles to the line of deposited staphylococci, taking care not to touch the staphylococcus streak. Plates were incubated for 24 to 48 h at 34°C in candle jars. A positive CAMP reaction was recognized by the production of a rectangular zone of complete hemolysis surrounding the *Listeria* streak within the weak secondary lytic zone produced by the staphylococcal beta toxin. Negative cAMP reactions showed no response within the secondary lytic zone. As a control to verify the suitability of the sheep blood used in each experiment as well as a general control on all experimental conditions, a known Lancefield group B streptococcus was inoculated on each plate in a manner similar to that for the *L. monocytogenes* strains. The production of the typical "arrowhead" pattern of hemolysis around the streptococcal streak within the secondary lytic zone of the staphylococcal toxin,

noted by Brzin and Seeliger (5) and Darling (7), proved the suitability of the sheep blood and experimental conditions. We have confirmed the observations of Brzin and Seeliger (5) and Darling (7) that the blood of some sheep fails to give a positive reaction with the streptococcus and is unsuitable.

Pathogenicity testing. All strains of *L. monocytogenes* isolated from bacteriologically confirmed, clinically active cases of listeriosis in humans and animals were considered pathogenic. To identify the pathogenic and apathogenic strains among the remaining isolants, animal inoculation studies were performed as described by Gray and Killinger (9). Suspensions of these strains were inoculated intraperitoneally into Swiss-Webster mice weighing 16 to 18 g and observed for 2 weeks. Those strains failing to kill sets of three mice given a dose of approximately 2×10^9 organisms were considered apathogenic. At that dose, the mice either died within 5 days or survived the observation period. The dead mice were autopsied, and Gram-stained smears of spleen, liver, and heart blood were examined. An abundance of typical small, gram-positive rods was observed in all preparations from the dead mice.

RESULTS

As indicated in Table 1, there was excellent correlation between xylose-positive isolants, rhamnose-negative isolants, and CAMP-negative isolants and non-pathogenicity of these isolants. All xylose-positive isolants were apathogenic, as were all CAMP-negative isolants and 95.7% of the rhamnose-negative isolants. There was also excellent correlation between CAMP-positive isolants and pathogenicity. Of CAMP-positive isolants, 98.7% were pathogenic, with the remaining 1.3% representing only one apathogenic strain of the 77 CAMP-positive isolants. Of xylose-negative isolants, 88.4% were pathogenic, whereas 84.3% of rhamnose-positive isolants were pathogenic. We observed other correlations between the pathogenicity and the three reactions (Tables 2 through 4).

Table 2 correlates rhamnose reactions, CAMP reactions, and pathogenicity or non-pathogenicity with the xylose reaction. All pathogenic isolants were xylose negative, whereas 98.7% of CAMP-positive isolants and 94.4% of rhamnose-positive isolants were also xylose negative. On the other hand, 91.3% of rhamnose-negative isolants were xylose-positive, whereas 71.4% of CAMP-negative isolants and 72.2% of apathogenic isolants were xylose positive.

In Table 3 it can be seen that the correlations were high between xylose-negative isolants, CAMP-positive isolants, and pathogenic isolants and the rhamnose-positive reactions of these isolants. Conversely, there were only fair correlations between xylose-positive isolants, CAMP-negative isolants, and apathogenic isolants and rhamnose-negative reactions of these

TABLE 1. Association of xylose reaction, rhamnose reaction, and CAMP reaction with pathogenicity of *L. monocytogenes* isolants

Pathogenicity of isolants	Reaction (%)					
	Xylose		Rhamnose		CAMP	
	Positive (26) ^a	Negative (86)	Positive (89)	Negative (23)	Positive (77)	Negative (36)
Pathogenic	0	88.4 (76)	84.3 (75)	4.3 (1)	98.7 (76)	0
Apathogenic	100 (26)	11.6 (10)	15.7 (14)	95.7 (22)	1.3 (1)	100 (36)

^a The numbers in parentheses indicate the number of isolants exhibiting a particular reaction.

TABLE 2. Association of rhamnose reaction, CAMP reaction, and pathogenicity with xylose reaction of *L. monocytogenes* isolants

Xylose reaction	Reaction (%)				Pathogenicity (%)	
	Rhamnose		CAMP		Positive (76)	Negative (36)
	Positive (89) ^a	Negative (23)	Positive (77)	Negative (35)		
Positive	5.6 (5)	91.3 (21)	1.3 (1)	71.4 (25)	0	72.2 (26)
Negative	94.4 (84)	8.7 (2)	98.7 (76)	28.6 (10)	100 (76)	27.8 (10)

^a The numbers in parentheses indicate the number of isolants exhibiting a particular reaction.

TABLE 3. Association of xylose reaction, CAMP reaction, and pathogenicity with rhamnose reaction of *L. monocytogenes* isolants

Rhamnose reaction	Reaction (%)				Pathogenicity (%)	
	Xylose		CAMP		Positive (76)	Negative (36)
	Positive (26) ^a	Negative (86)	Positive (77)	Negative (35)		
Positive	19.2 (5)	97.7 (84)	97.4 (75)	40.0 (14)	98.7 (75)	38.9 (14)
Negative	80.8 (21)	2.3 (2)	2.6 (2)	60.0 (21)	1.3 (1)	61.1 (22)

^a Numbers in parentheses indicate the number of isolants exhibiting a particular reaction.

TABLE 4. Association of xylose reaction, rhamnose reaction, and pathogenicity with CAMP reaction of *L. monocytogenes* isolants

CAMP reaction	Reaction (%)				Pathogenicity (%)	
	Xylose		Rhamnose		Positive (76)	Negative (36)
	Positive (26) ^a	Negative (86)	Positive (89)	Negative (23)		
Positive	3.8 (1)	88.4 (76)	84.3 (75)	8.7 (2)	100 (76)	2.8 (1)
Negative	96.2 (25)	11.6 (10)	15.7 (14)	91.3 (21)	0	97.2 (35)

^a The numbers in parentheses indicate the number of isolants exhibiting a particular reaction.

isolants.

Correlations between xylose reactions, rhamnose reactions, and pathogenicity or non-pathogenicity of isolants and their CAMP reactions are shown in Table 4. Of the apathogenic isolants, 97.2% were CAMP negative (with the remaining 2.8% representing only one isolant), whereas 96.2% of the xylose-positive isolants and 91.3% of the rhamnose-negative isolants were also CAMP negative. Conversely, all

pathogenic isolants were CAMP positive, but 88.4% of the xylose-negative isolants and 84.3% of the rhamnose-positive isolants were also CAMP positive.

In consideration of the reactions of the organisms with relation to their origin, of the 49 pathogenic isolants from patients with active listeriosis, all were CAMP positive and xylose negative and, with the exception of one strain, they were all rhamnose positive.

TABLE 5. Summary of CAMP, rhamnose, and xylose reactions by pathogenic and apathogenic *L. monocytogenes* isolants

Response	No. of isolants giving CRX ^a response						
	++- ^b	+-	---	++	+-	++	---
Pathogenic	75	1	0	0	0	0	0
Apathogenic	0	0	20	9	1	5	1

^a CRX, CAMP, rhamnose, and xylose.^b Reaction corresponding with each component of CRX, respectively.

The group of 31 isolants from animal tissues and elk feces exhibited two sets of characteristics, and every isolant was associated with one or the other. All of the pathogenic isolants were CAMP positive, xylose negative, and rhamnose positive, whereas apathogenic isolants were CAMP negative, xylose positive, and rhamnose negative, with one exception, which was xylose negative and rhamnose positive. Seven of the 20 isolants from elk feces were pathogenic and gave the typical xylose-negative, rhamnose-positive, CAMP-positive reactions, and the 13 remaining apathogenic isolants were xylose-positive, rhamnose-negative, and CAMP-negative reactors.

The pathogenic organisms isolated from soil, silage, and vegetation were all CAMP positive, xylose negative, and rhamnose positive. The apathogenic isolants were CAMP negative, more diversity in their pattern of reactions than did the other sources. All but one of the apathogenic isolants were CAMP negative, whereas 38.1% were rhamnose negative and 57.2% were xylose positive. Of the apathogenic isolants, 22.7% were both xylose positive and rhamnose positive. All of the xylose-positive isolants were apathogenic, but only 61.5% of the xylose-positive isolants were rhamnose negative.

DISCUSSION

Assuming other characteristics are compatible with those ascribed to *L. monocytogenes*, we note that pathogenicity, with rare exception, is associated with a combination of three responses: (i) beta hemolysis or positive CAMP reaction on sheep blood agar; (ii) acidification of rhamnose; and (iii) nonreactivity on xylose (Table 5). Those organisms that are nonhemolytic or CAMP negative and acidify xylose are apathogenic. Although xylose-positive organisms are apathogenic and pathogenic organisms are rhamnose positive, apathogenic organisms may be xylose negative and rhamnose positive.

With the increasing number of isolations of nonhemolytic, nonpathogenic *L. monocytogenes* strains in recent years, there is concern

that these isolants are designated as *L. monocytogenes*. The true taxonomic position of these strains has been recently questioned (18, 21), and some workers feel that the species designation *L. monocytogenes* should be restricted only to hemolytic, pathogenic strains. The separation of hemolytic strains from nonhemolytic strains by the numerical taxonomy studies of Wilkinson (22) strengthens the argument for limiting the species *L. monocytogenes* to only hemolytic, pathogenic strains.

The advent of greatly improved selective and enrichment media (14) for the isolation of *L. monocytogenes* has resulted in the isolation of *Listeria* from specimens abounding in mixed flora such as feces of apparently healthy humans and animals. In assessing the clinical and epidemiological significance of *Listeria* isolated from such specimens, attention should be directed toward distinguishing the "classical" pathogenic *L. monocytogenes* from the apathogenic *Listeria* to avoid attaching undue significance to the apathogenic *Listeria* or deemphasizing the role of *L. monocytogenes* in disease. The above in vitro tests should be helpful for recognition and separation of the two categories of *Listeria*, whatever the subsequent taxonomic assignments may be.

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