Identification of *Listeria monocytogenes* from Unpasteurized Apple Juice Using Rapid Test Kits

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**ABSTRACT**

A microbiological survey of 50 retail juices was conducted in the fall of 1996. These juices were analyzed for *Listeria monocytogenes*, *Escherichia coli* 0157:H7, *Salmonella*, coliforms, fecal coliforms, and pH. Two unpasteurized juices were positive for *L. monocytogenes*: an apple juice and an apple raspberry blend with a pH of 3.78 and 3.75, respectively. Three *L. monocytogenes* isolates were characterized. The colonies were typical for *Listeria* sp. on Oxford and lithium chloride–phenylethanol–moxalactam agars and were β-hemolytic on sheep blood agar. The isolates required 5 days of incubation at 35°C to produce a positive rhamnose reaction in a phenol red carbohydrate broth. This slow rhamnose utilization resulted in these isolates not being identified using the Micro-ID test strip (Organon Technika). However, the isolates were positive for *L. monocytogenes* using the API *Listeria* strip (BioMerieux) and a multiplex polymerase chain reaction for detection of the hemolysis (*hyla*) and invasion-associated protein (*iap*) genes.

*L. monocytogenes* has been isolated from a wide variety of foods including dairy products, eggs, meat products, vegetables, and seafood (7, 20). Several large, foodborne listeriosis outbreaks have been associated with coleslaw (17), a Mexican style soft cheese (13), chocolate milk (4), paté (14), and recently, smoked trout (6).

Pasteurization of juices eliminates pathogenic bacteria such as *L. monocytogenes*. However foods with a pH of less than 4.6 are generally considered to be safe in the U.S. Food and Drug Administration (FDA) Food Code (19). Some juice manufacturers use low pH and refrigeration as a means of controlling pathogenic bacteria in unpasteurized juices. However, pathogens such as *Escherichia coli* 0157:H7 have been detected in unpasteurized apple juice and have caused several deaths or illnesses (2, 8).

Several studies have been performed to determine the effect of pH on growth and survival of *L. monocytogenes* in various products. For example, the effects of four variables, different acids, pH, incubation time, and temperature, were compared in four strains of *L. monocytogenes* (18). The latter report showed that *L. monocytogenes* could grow and survive at pH 4.4 within the temperature range 10 to 35°C (18). *L. monocytogenes* has demonstrated its ability to grow at pH 4.39 and 5.23 at 4 and 30°C, respectively (10). However in juice products there may be other factors present that inactivate *L. monocytogenes* at lower pH values. A pH of <4.8 in cabbage juice was sufficient to inactivate *L. monocytogenes* (3). Another investigation demonstrated that the lethal and inhibitory effects of carrot juice on *L. monocytogenes* was greatest within a pH range of about 5.0 to 6.4 (1). *L. monocytogenes* grew and survived in filtered, concentrated frozen orange juice at a minimum pH of 4.8 (16). Thus, the principle conclusion from these investigations is that growth and survival of *L. monocytogenes* is impacted by acidic pH values in some products.

In this investigation, a survey of 50 juices was conducted and *Listeria* species were isolated from two unpasteurized juices, an apple and an apple raspberry blend. These isolates were unidentifiable by a commercial rapid identification kit. Additional biochemical tests, a second commercial identification kit, and polymerase chain reaction (PCR) analysis for the *L. monocytogenes* *iap* and *hyla* genes were used to further characterize and speciate these isolates.

**MATERIALS AND METHODS**

Juice samples were analyzed as described in the FDA Bacteriological Analytical Manual (BAM) (11). Briefly, 25 g from each juice was weighed into 225 ml of modified *Listeria* enrichment broth (EB) (Difco, Detroit, Mich.). EB was streaked onto lithium chloride–phenylethanol–moxalactum (LPM) and Oxford agars (OXA) after 24 to 48 h incubation at 30°C and 35°C, respectively. Typical colonies were picked from both LPM and OXA plates, streaked for purity to tryptic soy agar–yeast extract (TSA–YE) plates, and incubated overnight at 30°C. Colonies from this agar were used to examine cells microscopically for tumbling...
motility in wet mounts, catalase activity, oxidase test, and hemo-
lytic activity on 5% sheep blood agar plates. Phenol red carbohy-
drate broth (substituted for purple carbohydrate broth) contained
rhamnose (0.5% wt/vol), and these tubes were incubated at 35°C
for 7 days (11). The isolates were serotyped with commercial
somatic sera (Difco). Suspensions of the above isolates were
inoculated into Micro-ID Listeria kits (Remel, Durham, N.C.) and
API Listeria strips (BioMerieux, Marcy-l’Etoile, France), incub-
ated, and results interpreted according to the respective manufac-
turers’ instructions. A multiplex PCR method for detection of the
listeriolysin O gene (hyla) and invasion-associated protein (iap)
genes was performed as described previously. Overnight tryptic
soy broth–yeast extract (TSB-YE) cultures (1 ml) from each isolate
were washed twice in physiological saline, resuspended in 1 ml
sterile distilled water, and boiled 10 min to prepare PCR templates.
Amplification reactions (100 μl) consisted of 10 mM Tris–HCl pH
8.3, 50 mM KCl, 2 mM MgCl2, 200 μM each deoxynucleoside, 2
units Taq polymerase (Perkin Elmer, Branchburg, NJ), 0.25 μM
each primer (iapF, ACAAAGCTGCACCTGTTGCAG; iapR,
TGACAGCGTGTGTAGTATCA; hyla-F, CGGAGGTTCG-
CAAAAGATG; and hyla-R, CCTCCAGAGTGATCGATGT;
primers synthesized by Geneosys Biotechnologies, Inc., The Wood-
lands, Tex.), and 2 μl of the prepared PCR template. The PCR cycle
conditions consisted of an initial 10 min denaturation at 95°C, 40
cycles of 30 s at 95°C, 1 min at 55°C, 1 min 72°C, followed by a
final extension for 10 min at 72°C. Amplification procedures were
analyzed on a 1.5% agarose gel. Positive reactions were indicated
by the presence of a 234-base pair (bp) fragment from the
hyla gene and 130 bp for the iap gene.

RESULTS AND DISCUSSION

Following the detection of E. coli O157:H7 in unpasteur-
ized apple juice in the fall of 1996 (2), a microbiological
study was conducted on a random sampling of retail juice
products marketed in the State of Washington. L. monocytogenes
was included in this survey because it is a potential
human pathogen that has demonstrated its ability to survive
at low pH conditions (18). Apple juice or apple cider
comprised 31 of the 50 juices analyzed. Ten of the 50 juices
were blends of apple juice and other fruit juices. Five other
fruit juices consisted of blackberry, strawberry, and other
citrus juices. The last four juices were vegetable juices and
blends of vegetable juices. L. monocytogenes was detected
in two unpasteurized juices (apple and apple raspberry
juice). The pHs for these two juices were 3.78 and 3.75,
respectively. Although L. monocytogenes may not grow at
<pH 4.3 (10), these organisms were able to survive at pH
3.75 in these unpasteurized juice samples.

The Listeria genus consists of several species that are
distinguished by only a few biochemical differences. The
FDA BAM Listeria method (11)_differentiates the Listeria
spp. based primarily on the hemolysin reaction, CAMP test,
and ability to utilize various carbohydrates, specifically
rhamnose and xylose within a 7-day incubation period.
L. monocytogenes isolates should produce β-hemolysis and
enhanced hemolysin production by Staphylococcus aureus
but not Rhodococcus equi (CAMP test). In addition
L. monocytogenes utilize rhamnose but not xylose. One
isolate (644-1) was characterized from the positive apple
juice sample. Two isolates (647-1 and 647-2) were character-
ized from the apple raspberry blend. All three juice isolates
were identified as L. monocytogenes by the above criteria,
although it was noted that the rhamnose reaction was slow
relative to typical L. monocytogenes isolates, taking 5 days
to develop.

The Micro-ID test strip is a series of miniaturized
biochemical tests with hemolysin and CAMP tests run
concurrently and within 24 h. The species identifications are
based primarily on the same criteria as the FDA BAM
procedure. The results of the Micro-ID test strip are listed in
Table 1. Due to the delayed rhamnose utilization by these
test isolates, the rhamnose reaction was interpreted as
negative. This resulted in a code for which no species
identification could be made for these isolates. Results were
the same with two separate lots of Micro-ID strips.

The API Listeria test strip also uses a series of
miniaturized biochemical tests to allow a species identifica-
tion within 24 h. An offline hemolysis test can be run
concurrently for final species determinations in some cases.
The results from the API Listeria test strip for these juice
isolates are listed in Table 2. The API Listeria test strip
determinations for Listeria species are based in part on a
unique biochemical test referred to as DIM. All L. mono-
cytogenes isolates are negative for the DIM test while strains of

### Table 1. Results from Micro-ID test strip analysis of three Listeria monocytogenes isolates from unpasteurized juice samples

| Isolate  | VP | N  | PD | H2S | I  | OD | LD | M   | U   | E  | ONPG | XYL | RHA | MANN | SORB | ST  | RE | β-  | Hemolysis |
|----------|----|----|----|-----|----|----|----|-----|-----|----|-----|-----|-----|------|------|-----|----|------|
| 644-1    | +  | -  | +  | +   | -  | -  | -  | -   | -   | +  | -   | -   | -   | -    | -    | -   | +  | -    | +       |
| 647-1    | +  | -  | +  | +   | -  | -  | -  | -   | -   | +  | +   | -   | -   | -    | -    | -   | +  | -    | +       |
| 647-2    | +  | -  | +  | +   | +  | +  | -  | -   | -   | +  | -   | -   | -   | -    | -    | -   | +  | -    | +       |
| L. monocytogenes<sup>a</sup> | +  | -  | +  | -   | -  | +  | -  | -   | -   | -  | -   | -   | -   | -    | -    | -   | +  | -    | +       |
| L. innocua<sup>c</sup> | +  | -  | +  | -   | -  | -  | -  | -   | +   | +  | -   | -   | -   | -    | -    | -   | +  | -    | -       |

### Notes

- **VP**: Voges Proskauer; **N**: nitrate reductase; **PD**: phenylalanine deaminase; **H2S**: hydrogen sulfite; **I**: indole; **OD**: ornithine decarboxylase; **LD**: lysine decarboxylase; **M**: malonate utilization; **U**: urease; **E**: esculin; **ONPG**: β-galactosidase; **XYL**: xylose fermentation; **RHA**: rhamnose fermentation; **MANN**: mannitol fermentation; **SORB**: sorbitol formation; **ST**: Staphylococcus aureus ATCC 25923; **RE**: Rhodococcus equi SEA 6281.

<sup>a</sup>L. monocytogenes Scott A.
<sup>b</sup>L. monocytogenes Scott A.
<sup>c</sup>Listeria innocua SEA 6273.
TABLE 2. Results from API Listeria strip analysis of three L. monocytogenes isolates from unpasteurized juice samples

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<th>Isolate</th>
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* DIM, differentiation L. innocua/L. monocytogenes; ESC, esculin hydrolysis; αMAN, α-mannosidase; DARL, D-arabitol acidification; XYL, D-xylose acidification; RHA, rhamnose acidification; MDG, α-methyl-D-glucoside acidification; RIB, ribose acidification; GIP, glucose-1-phosphate acidification; TAG, D-tagatose acidification.

most other Listeria spp. are positive. The rhamnose utilization test was interpreted as negative for the juice isolates. However, based on the negative DIM test and β-hemolysin procedures by these three juice isolates, the API Listeria test strip results led to a species determination of L. monocytogenes.

The multiplex PCR protocol described by Furrer et al. (9) is directed at the detection of two recognized L. monocytogenes virulence genes. The listeriolysin O virulence factor is encoded on the hyla gene (15). The invasion-associated protein is another virulence factor encoded by the iap gene (12), previously referred to as a hemolysin gene (5, 9). Both of these L. monocytogenes-specific genes were detected in these three juice isolates (Fig. 1).

The three juice isolates (644-1, 647-1, and 647-2) all appear to be slow rhamnose-utilizing L. monocytogenes strains, based on the hemolysin reaction, CAMP test, API Listeria test strip DIM test, positive hyla and iap PCR amplification products, and positive rhamnose reaction when a conventional rhamnose biochemical test was used. Thus, the short incubation times associated with the miniaturized biochemical test format used for rapid identification method kits may exhibit negative results for specific strains. These same strains may be positive when analyzed by a conventional method, which has a longer incubation time, and therefore analysts and other investigators should exercise caution when interpreting Listeria results from rapid microbiological test procedures.

REFERENCES


