A Research Note

Selective and Differential Medium for Isolation of Listeria monocytogenes from Foods

FRANK T. POYSKY, ROHINEE N. PARANJPYE, LAURA C. LASHBROOK, MARK E. PETERSON, GRETCHEN A. PELROY, AND MEL W. EKLUND*

Northwest Fisheries Science Center, NMFS, NOAA, Utilization Research Division, U.S. Department of Commerce,
2725 Montlake Boulevard East, Seattle, Washington 98112

(Received for publication August 10, 1992)

ABSTRACT

Hemolytic ceftazidime lithium chloride agar (HCLA) has been developed as a selective and differential plating medium for the isolation of Listeria monocytogenes from fishery products. Selectivity is based upon lithium chloride, colistin methane sulfonate, and ceftazidime. When horse blood was incorporated in the agar overlay, L. monocytogenes colonies were easily distinguished by their characteristic blue-gray color surrounded by narrow zones of B-hemolysis. Listeria innocua, a species commonly present in foods, does not produce hemolysis on the medium. Therefore, one or more colonies of L. monocytogenes were easily distinguished from large populations of L. innocua. When used in combination with Food and Drug Administration and U.S. Department of Agriculture enrichment methodology, HCLA was effective in inhibiting competitive organisms, differentiating colonies of L. monocytogenes by their B-hemolysis, and shortening the incubation time at 35°C for presumptive identification to 17-24 h.

Listeria monocytogenes has been recognized as a major foodborne pathogen in the past decade with outbreaks of listeriosis linked to a variety of contaminated foods such as coleslaw, Mexican-style soft cheese, pasteurized milk, and turkey franks. L. monocytogenes is a gram-positive psychrotrophic bacterium that is ubiquitous in nature. The bacterium has been isolated from fresh produce, dairy products, processed meats, and seafood products. The isolation of L. monocytogenes from mixtures of competitive microorganisms present in many foods is often difficult. In earlier studies, cold enrichment was the only method used for isolating Listeria spp. Current Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) methods for the enrichment and isolation of Listeria spp. have markedly reduced the 3 months needed for cold enrichment but are still costly and time consuming.

The need for rapid and accurate confirmation of the presence of L. monocytogenes in a product and the suppression of background flora has encouraged the use of chemicals and antibiotics in an array of selective media. Recently, blood agar overlay techniques were described by Blanco et al. (4) and Cox et al. (10) which utilize the hemolytic properties of L. monocytogenes for differentiation. Blanco’s medium requires 64 h of total incubation with an overlay of blood agar at 48 h. The medium of Cox et al. (10) utilizes sphingomyelinase to enhance the lysis of ovine erythrocytes. This medium requires 48 h of incubation.

Our investigations of the incidence of Listeria spp. in fishery products and the need for a more rapid presumptive test for L. monocytogenes have prompted the formulation of a new selective and differential medium. Hemolytic ceftazidime lithium chloride agar (HCLA) medium relies on the defined B-hemolytic characteristics of L. monocytogenes and the bacterium’s resistance to the antibiotic ceftazidime, lithium chloride, and colistin. The characteristic B-hemolysis is readily recognized within 17 to 24 h incubation, and L. monocytogenes colonies can be differentiated from numerous colonies of Listeria innocua. This paper describes the medium and experiments leading to the development of optimum conditions for hemolysis, and over two years of experience using HCLA medium for isolating L. monocytogenes from fishery products.

MATERIALS AND METHODS

Cultures

The following strains used in this study were isolated at this laboratory from fish, shrimp, crab meat, cheese, and milk. L. monocytogenes: 1455, 4-121, 1522, 5313, 3105, 531. L. innocua: 4-4, 1532, 326-D, 362, V72-1, 1538, 2107, 2714. Listeria seeligeri: 803, 806, 818, 836, 847, 874. In addition, the following species were tested: L. innocua 2966, Listeria ivanovii 19119, and L. seeligeri 35967 (Dr. H. Kinde, California Department of Food and Agriculture); Listeria welshimeri 3388, L. seeligeri 3110, 3517,
Identification of Listeria

Listeria wet mounts), Gram stain, CAMP test, catalase, and carbohydrate biochemical tests were performed from the broth: motility (Sims, Slide Test method (Difco). Identification was performed on random isolates using the Rapid (26).

Serological fermentations following the procedures of Lovett (30).

Food samples included cold-smoked fish products, skin and flesh from fresh salmon, cooked crab meat, individually quick-frozen shrimp, unpasteurized milk, and cheese. Seafood samples were collected from different geographical areas in the United States. All samples were transported to the laboratory on gel ice and incubated at 30°C. The following biochemical tests were performed from the broth: motility (Sims, wet mounts), Gram stain, CAMP test, catalase, and carbohydrate fermentations following the procedures of Lovett (26). Serological identification was performed on random isolates using the Rapid Slide Test method (Difco).

RESULTS AND DISCUSSION

Initially, pure cultures of L. monocytogenes, L. innocua, L. seeligeri, L. ivanovii, and L. welshimeri were used in the development of HCLA medium. The HCLA formulation was based upon the growth, and production of β-hemolysis by L. monocytogenes, L. ivanovii, and L. seeligeri. When additional strains of L. monocytogenes isolated from fishery and milk products failed to produce distinct zones of hemolysis and competitive microorganisms became a problem, modifications were made based upon the results of the following experiments.

Effects of esculin and ferric ammonium citrate

Esculin and ferric ammonium citrate have been added to various media (12,36) to differentiate Listeria from other microorganisms. Listeria spp. hydrolyze esculin to glucose and aglycone aesculetin, which forms a complex with the iron from ferric ammonium citrate, resulting in black colonies with haloes (10).

When ferric ammonium citrate was added to HCLA, it enhanced both growth and hemolysis. The addition of esculin by itself, however, inhibited β-hemolysis. When the combination of esculin and ferric ammonium citrate was tested, the blackened colony and halo masked the detection of any possible hemolysis.

Lithium chloride concentration

McBride and Girard (29) used lithium chloride (LiCl) at a concentration of 0.5 g/L as one of the selective agents in their original plating medium. Since then, LiCl has been used in numerous other selective media at concentrations ranging from 5 to 15 g/L (1,8,10,11,22,24). In the first formulation of HCLA medium, pure cultures of L. monocytogenes grew well and produced distinct β-hemolysis on medium containing 15 g LiCl per L. When these experiments were expanded to the isolation of Listeria from fishery and dairy products, some strains produced hemolysis on HL plates but failed to exhibit hemolysis on HCLA agar. In subsequent experiments, hemolysis on HCLA agar was shown to be dependent upon lithium chloride concentration. Lithium chloride concentrations varying from 0 to 15 g/L in increments of 2.5 g were tested in HCLA. The optimum concentration of LiCl was 7.5 g/L. At this concentration, the hemolysis of the colonies was stronger, the colonies grew larger, and the blue luster of the colonies decreased. Below the 5 g/L level, other background microorganisms were not inhibited.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Difco-Bacto Columbia blood agar base</td>
<td>39 g/L</td>
</tr>
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<td>5 g/L</td>
</tr>
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<td>Sigma L0505 Lithium chloride anhydrous</td>
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Final pH 7.0

Base layer 10 ml No horse blood

Overlay 5 ml 4% horse blood

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TABLE 2. Examples of comparative recovery of *L. monocytogenes* from enrichments of fishery products on HCLA, OX, and LMO agar.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>HCLA</th>
<th>OX</th>
<th>LMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>697</td>
<td>3/3</td>
<td>0/16</td>
<td>1/16</td>
</tr>
<tr>
<td>699</td>
<td>16/16 (75)</td>
<td>0/16 (40)</td>
<td>1/16 (54)</td>
</tr>
<tr>
<td>709</td>
<td>16/16 (60)</td>
<td>2/16 (35)</td>
<td>0/16 (55)</td>
</tr>
<tr>
<td>6601</td>
<td>16/16 (19)</td>
<td>0/16 (13)</td>
<td>0/16 (19)</td>
</tr>
<tr>
<td>6602</td>
<td>16/16 (27)</td>
<td>1/16 (25)</td>
<td>2/16 (36)</td>
</tr>
<tr>
<td>6604</td>
<td>16/16 (21)</td>
<td>3/16 (19)</td>
<td>2/16 (31)</td>
</tr>
<tr>
<td>6605</td>
<td>16/16 (27)</td>
<td>0/16 (21)</td>
<td>2/16 (20)</td>
</tr>
<tr>
<td>6606</td>
<td>11/11 (11)</td>
<td>0/16 (9)</td>
<td>1/16 (12)</td>
</tr>
</tbody>
</table>

*Results are expressed as number of colonies of *L. monocytogenes* per number of colonies tested. All hemolytic colonies from HCLA were tested when there were 16 or less per plate. When there were more than 16 hemolytic colonies, only 16 were tested. Sixteen black colonies were selected from all OX and LMO plates.

*Number in parentheses represents number of colonies that were hemolytic and identified as *L. monocytogenes* when all the colonies from selective media were replicated onto HL plates.

In the present study, certain *Bacillus* spp. were resistant to the effect of LiCl even at 7.5 g/L. The *Bacillus* colony morphology and type of hemolysis, however, were easily distinguished from *L. monocytogenes*. When large numbers of β-hemolytic *Bacillus* spp. were resistant to the primary enrichments and HCLA agar, inoculation into a second selective UVM-2 (30) usually eliminated the problem.

**Effect of acriflavin and moxalactam**

 Attempts were made to increase inhibition of *Bacillus* spp. by adding acriflavin or moxalactam to the medium at concentrations of 10, 25, and 50 mg/L, and 5, 10, 15, and 20 mg/L, respectively. Unfortunately, neither compound consistently inhibited all of the *Bacillus* spp., and acriflavin also inhibited the hemolytic activity of *L. monocytogenes* on HCLA.

**Ceftazidime and agar concentrations**

Ceftazidime has been used in various *Listeria* selective plating media at a concentration of 50 mg/L (1,3,22). In HCLA medium, ceftazidime was evaluated at concentrations of 20, 30, 40, and 50 mg/L. A concentration of 20 mg/L was selected because it was as effective as 50 mg in inhibiting background microorganisms.

When the concentration of agar was 1.5%, *L. monocytogenes* often overgrew the hemolytic zones. Distinct zones of hemolysis were always observed when the agar content was increased to 2.0%.

**Comparison of horse and sheep blood**

B-hemolysis was compared on HCLA medium containing 2, 3, 4, and 5% horse and sheep blood. The blood was incorporated into an overlay poured over a base of HCLA without blood. Characteristic B-hemolytic zones around the colonies were most distinctive with 4% horse blood. Sheep blood was not as effective as horse blood but in most cases could be used if the concentration did not exceed 2%. At greater concentrations, the β-hemolysis on sheep blood was not evident.

**Incubation temperature**

Hemolytic activity by HCLA medium was compared at 20, 25, 30, and 35°C. Colonies did not exhibit consistent hemolysis when grown at 20, 25, or 30°C. Colonies always produced distinct β-hemolytic zones within 17-24 h at 35°C.

**Comparison of HCLA, OX, and LMO media**

*L. monocytogenes* formed blue-gray and occasionally white colonies surrounded by distinct zones of hemolysis on HCLA within 17-24 h of incubation. The colony characteristics were readily observed with transmitted light, without the aid of any oblique lighting systems or magnifying lenses. An individual colony of *L. monocytogenes* was easily distinguishable from hundreds of colonies of *L. innocua*, which were always nonhemolytic on HCLA medium. In contrast, *L. monocytogenes* could not be distinguished from other *Listeria* species on OX or LMO because all species produce black colonies on those media.

HCLA was compared with LMO and OX agar for recovery of *L. monocytogenes* from UVM or LEB enrichments of fishery products, by inoculating 0.1 ml from each enrichment onto the surface of each medium and incubating for 24 h at 35°C. All hemolytic colonies from HCLA were selected and identified when there were less than 16 per plate. When there were larger populations, 16 hemolytic colonies were randomly selected. Sixteen black colonies were randomly selected from all OX and LMO plates. Typical results are shown in Table 2. All of the hemolytic colonies from HCLA were identified as *L. monocytogenes*. In comparison, the inability to differentiate *Listeria* species on OX and LMO resulted in *L. monocytogenes* being identified only a small percentage of the time, particularly in samples where large populations of *L. innocua* were present.

To determine how many colonies of *L. monocytogenes* were actually present on the three different media, the colonies were replicated onto HL agar following procedures of Cassiday et al. (9). No additional colonies produced hemolysis on HL agar when they were transferred from HCLA medium. Numerous hemolytic colonies, undetected during random selection from OX and LMO, were observed and identified as *L. monocytogenes*, when the entire population was replicated onto HL medium (Table 2).

Over a 2-year period, HCLA was compared with OX media for recovery of *L. monocytogenes* from over 500 enrichment samples of fishery products. In no instance was *L. monocytogenes* recovered on OX, without also being recovered on HCLA.

Although testing was not extensive, the ease of using HCLA to detect *L. monocytogenes* in dairy products was also examined. Eight samples of unpasteurized milk and six samples of cheese, known to be contaminated with *L. monocytogenes* (Dr. Kinde, University of California), were...
enriched in UVM and LEB. L. monocytogenes was readily recognized on HCLA plates from all of the samples. L. monocytogenes colonies from these products were the most sensitive to variation in LiCl and hemolysis was inhibited when concentrations exceeded 7.5 g/L.

Reactions of other Listeria species

L. ivanovii grew well on HCLA and produced β-hemolysis within 17-24 h at 35°C. The zones of hemolysis, however, were larger than those observed with L. monocytogenes. Growth of L. seeligeri on HCLA was slower and growth of L. welshimeri on HCLA was slower and older compared to the other species. Not one of the hemolytic isolates proved to be other than L. monocytogenes. Samples positive for L. monocytogenes produced characteristic blue-gray hemolytic colonies. Not horse blood into the agar overlay easily differentiates the media.

HCLA offers a reliable method for shortening the presumptive isolation of L. monocytogenes from contaminated food products to 17-24 h of incubation. LiCl, colistin methane sulfonate, and ceftazidime acid pentahydrate proved to be presumptive isolation of L. monocytogenes. From contaminated samples, 86.3% of the L. monocytogenes isolates were recovered on HCLA plates. HCLA also exhibited black colonies on OX and LMO media.

HCLA offers a reliable method for shortening the presumptive isolation of L. monocytogenes from contaminated food products to 17-24 h of incubation. LiCl, colistin methane sulfonate, and ceftazidime acid pentahydrate proved to be a selective medium for isolating L. monocytogenes from heavily contaminated material. Environ. Microbiol. 54:165-167.

REFERENCES

bacteria (7). Results of experiments to determine whether or not MA caused stressing of E. coli O157:H7 revealed no obvious stressing of the bacterium under the conditions tested (Fig. 4).

Based on our results, it appears that E. coli O157:H7 is unlikely to grow well but will survive during refrigeration storage. In addition, the gas compositions within the tolerable range for vegetables have no inhibitory effect on E. coli O157:H7. Therefore, the possibility exists that E. coli may survive in fresh and minimally processed vegetables long enough to infect a consumer.

Eklund et al., cont. from p. 329
