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Role of Potassium Tellurite and Brain Heart Infusion in Expression of the Hemolytic Phenotype of Listeria spp. on Agar Plates

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The influence of potassium tellurite (PT) and brain heart infusion agar (Difco), two components of modified Listeria selective agar medium (LSAMm), on the hemolytic phenotype of Listeria spp. was studied. L. monocytogenes and L. ivanovii displayed higher zones of hemolysis on brain heart infusion agar compared with Columbia agar base. The addition of PT increased the sizes of zones of hemolysis displayed by L. monocytogenes. This effect seemed to be produced by the enhancement of the cytolytic effect of listeriolysin O. PT decreased the hemolysis produced by L. ivanovii, and this effect seemed to be due to an inhibition of the sphingomyelinase C produced by this species.

The isolation of Listeria spp. from sources with low levels of contamination by these microorganisms, such as environmental or food samples, is often impaired by the naturally occurring background microflora. This makes it necessary to use highly selective media. These media have been progressively improved to enhance their selectivity and ability to detect small numbers of Listeria colonies, thus avoiding previous selective enrichment steps (2, 4). The goal of these improvements has been to provide a reliable, sensitive, and rapid isolation method for Listeria monocytogenes and other Listeria spp., and there are several selective plating media available to isolate and enumerate Listeria colonies (1–4).

The hemolytic phenotype is currently a fundamental criterion for the differentiation and identification of Listeria spp. (6), and it is also a marker of pathogenicity (16). Consequently, hemolytic activity can be used as an indicator of the presence of pathogenic listeriae in a plating medium. The only exception is Listeria seeligeri, which is weakly hemolytic but nonpathogenic. This does not substantially affect the validity of this indicator, as this species is isolated only sporadically. Therefore, it is important to easily determine the hemolytic character of Listeria isolates on plating media. In accordance with these observations, a further improvement introduced in selective Listeria isolation has been the detection of hemolytic colonies of L. monocytogenes directly on selective agar by using an erythrocyte top layer (RBCTL) technique (1). This RBCTL technique provides an easy method for the differentiation of hemolytic and nonhemolytic Listeria colonies isolated from the same sample, avoiding further subcultures to determine the hemolytic character either on blood agar or by the CAMP test, both of which are less sensitive or unreliable (1, 5, 6, 19). The performance of the RBCTL technique was higher when used with Listeria selective agar medium (modified) (LSAMm) than it was when used with other types of Listeria selective agar (1). In the work described here, we analyzed the reasons why such enhancement of hemolysis of L. monocytogenes is observed on LSAMm. We found that potassium tellurite (PT) and the basal medium are factors that influence this phenomenon.

The following Listeria strains were used: Listeria monocytogenes NCTC (National Culture Type Collection) 7973, NCTC 5105, NCTC 6348, and P-9 (animal clinical isolate); Listeria innocua SLCC (Special Listeria Culture Collection) 3379 and C-645 (from the collection of the Microbiology Department, University of Leicester, Leicester, United Kingdom); Listeria ivanovii ATCC (American Type Culture Collection) 19119 and C-659; Listeria seeligeri CIP (Collection of the Institut Pasteur, Paris, France) 100100 and NCTC 11806; and Listeria welshimeri CIP 8149 and SLCC 5334. Additionally, the following three mutant strains of L. monocytogenes were used: a Tn545-inducible nonhemolytic L. monocytogenes mutant strain (7) (a producer of phospholipases but not of listeriolysin O [LLO] (LLO−) obtained from P. Berche, Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, Paris, France), BUG 206 (15) (a producer of LLO and lecinthinase but not of phosphatidylinositol-specific phospholipase C [PI-PLC]), and LUT-12 (a Tn917-lac lecinthinase-negative mutant, a producer of LLO and PI-PLC). BUG 206 and LUT-12 were obtained from P. Cossart, Unité de Génie Microbiologique, Institut Pasteur, Paris, France.

The hemolytic activities of the Listeria strains were screened on brain heart infusion agar (BHA) (Difco) and Columbia agar base (CAB) (Oxoid). Both media were prepared and sterilized according to the manufacturer’s instructions. When these media were used to grow mutant strains, they were supplemented with the appropriate antibiotics. Hemolysis was determined by the top-layer technique proposed by Blanco et al. (1). After incubation at 37°C for 48 h, plates were chilled at 4°C for 2 h before being covered by 15 ml of an RBCTL for hemolysis detection. The composition, preparation, and storage of the top layer have been described previously (1, 5). After top-layer addition, plates were reincubated for 24 h at room temperature and screened for hemolysis.

The influence of BHA and CAB on the hemolysis of streaked Listeria spp. was determined by streaking each strain on plates of both media (four strains per plate), which were incubated at 37°C for 48 h. After incubation, RBCTL was added and hemolysis screening was performed as described above. The zone of hemolysis was measured as the

* Corresponding author.
distance between the edge of the streak of growth and the limit of the zone of hemolysis.

The influence of PT on the hemolysis and growth of streaked strains of *Listeria* was determined as follows. In a first trial, 200 mg of PT per liter (0.02% [wt/vol]) (the concentration of PT used in the formulation of LSAMm) was added to BHIA and CAB (BHIA-PT and CAB-PT). In a second trial, BHIA was supplemented with PT to reach concentrations of 0.004, 0.008, 0.01, 0.02, 0.1, 0.5, and 1%, and the effect of each mixture on the growth and hemolysis of all *Listeria* strains was determined. Simultaneously, BHIA plates without PT were used as controls. BHIA and CAB were prepared and sterilized as described above. After they had cooled to about 50°C, the appropriate amount of filter-sterilized PT was added. BHIA and CAB plates in the first trial and BHIA plates in the second trial were cultured, incubated, and screened for hemolysis as mentioned above.

To determine the hemolytic activities of *Listeria* supernatants in the presence of PT, *Listeria* strains were grown in 1,000 ml of brain heart infusion broth (Difco) at 37°C for 24 h with shaking (1,000 rpm). After incubation, cells were harvested by centrifugation (12,000 x g for 30 min), and the supernatant fluid was concentrated to 65 ml by ultrafiltration through 10-kDa-exclusion-limit membranes (Millipore Corp.) and filtered through a sterile filter (pore size, 0.22 μm). BHIA and CAB were supplemented with bacteriological agar (Difco) to a final concentration of 2%. Media were boiled, distributed in aliquots of 25 ml in glass tubes, and sterilized (121°C for 15 min). After being cooled to about 50°C, PT was added to final concentrations of 0.004, 0.008, 0.01, and 0.02%. The tubes were mixed vigorously and dispensed immediately into petri dishes. When the mixture had solidified, wells (7 mm diameter) were made in the medium by using a drill bit. The agar plugs were removed with a sterile needle. After drying (37°C for 24 h), 200 μl of the supernatant fluid was placed in wells as follows. Wells were filled with 100 μl of supernatant fluid, and plates were stored for 12 h at room temperature to allow supernatant absorption. Wells were filled again with another 100 μl of supernatant fluid, and plates were stored for another 12 h at room temperature. The nonabsorbed supernatant was removed, and RBCTL was added. Further incubation of plates to screen the hemolytic activities of the supernatants was done as described above. The hemolytic activity was measured as the diameter of the zones of hemolysis.

Selective sequestration of thiol-activated *L. ivanovii* cytolysin (ivanolysin O) was performed by the method described by Vazquez-Boland et al. (20, 21). To the concentrated and filtered supernatant of *L. ivanovii*, 200 μl of a 10-mg/ml cholesterol (Panreac, Barcelona, Spain) solution in absolute ethanol was added (final concentration of cholesterol was 35 μg/ml). After incubation (30 min at 37°C) with shaking, the resultant suspension of cholesterol and thiol-activated cytolysin complexes was pelleted by centrifugation (40,000 x g for 45 min at 4°C). The supernatant obtained, which lacked the thiol-activated cytolysin activity, was tested for hemolytic activity.

All strains of *L. monocytogenes* and *L. ivanovii* displayed bigger and clearer zones of hemolysis on BHIA than they did on CAB (Table 1). The zones of hemolysis displayed by *L. monocytogenes* (an average of 36 determinations [9 determinations per strain]) and *L. ivanovii* (an average of 18 determinations [9 determinations per strain]) were about 1 and 3.5 mm larger, respectively, on BHIA than they were on CAB. *L. seeligeri* displayed very small hemolytic zones (always smaller than 1 mm) on both BHIA and CAB.

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**TABLE 1. Effect of PT on zone of hemolysis diameters of streaked strains of *L. monocytogenes* and *L. ivanovii***

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Medium</th>
<th>Zone of hemolysis diam (mm) with PT concn (% [wt/vol]) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>NCTC 7973</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>NCTC 5101</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>P-9</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>NCTC 6384</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td>ATCC 19119</td>
<td>9.1 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>C-659</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.6 ± 2.0</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations of nine determinations.

---

**TABLE 2. Effect of PT on zone of hemolysis diameters of supernatants of *L. monocytogenes* and *L. ivanovii* strains***

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Medium</th>
<th>Zone of hemolysis diam (mm) with PT concn (% [wt/vol]) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>NCTC 7973</td>
<td>14.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>NCTC 5101</td>
<td>14.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>P-9</td>
<td>17.8 ± 2.9</td>
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<td></td>
<td></td>
<td>17.3 ± 2.0</td>
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<td></td>
<td></td>
<td>10.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.5 ± 2.8</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td>ATCC 19119</td>
<td>20.0 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>C-659</td>
<td>22.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.4 ± 2.0</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations of six determinations.
Most cases bizonal 6384 and comparatively higher for weakly hemolytic CAB.

...the with L. (E). ...of concentrations from 0.02%, to not shown). supernatants of L. strains all Listeria strains grew perfectly at PT concentrations up to 0.02%, while concentrations of PT equal to or higher than 0.1% decreased or completely inhibited their growth (data not shown). The hemolysis of both streaked strains and supernatants of L. monocytogenes was enhanced in the range of concentrations from 0.004 to 0.02% PT, compared with the same medium without PT (Table 2 and Fig. 1, panels 1 and 2 [A, B, and D]). The increase in hemolysis was comparatively higher for weakly hemolytic strains (NCTC 6384 and P-9) than for moderate or strongly hemolytic strains (NCTC 7973 and NCTC 5105); this fact was more evident with the streaked strains. The greatest increase in the zone of hemolysis was detected with 0.004% PT on both BHIA and CAB.

L. ivanovii ATCC 19119 usually displayed a characteristic bizonal hemolytic area on BHIA without PT (Fig. 1, panel 1 [E]). L. ivanovii C-659 did not display a bizonal hemolysis in most cases (Fig. 1, panel 1 [C]). In the presence of PT, the zones of hemolysis displayed by the streaked strains and supernatants of L. ivanovii decreased in parallel with the increase in the concentration of PT (Table 2). Thus, streaked strains of L. ivanovii displayed hemolytic zones similar to or even smaller than those produced by L. monocytogenes strains on BHIA supplemented with 0.02% PT (Table 1). The zones of complete hemolysis of L. ivanovii (Fig. 1, panels 2 and 3 [E]) were bigger in the presence of PT than they were in the absence of PT (Fig. 1, panel 1 [E]); nevertheless, it was impossible to recognize the external ring of incomplete hemolysis. Overall, in the presence of PT, the streaked strains of L. monocytogenes and L. ivanovii always had bigger zones of hemolysis on BHIA than on CAB (Table 1), whereas the hemolysis displayed by the supernatants of these species was similar on BHIA and CAB (Table 2). L. seeligeri did not show any detectable enhancement in its hemolysis with any of the PT concentrations used. L. innocua and L. welshimeri strains were always nonhemolytic, as expected.

Both streaked strains and supernatants of the LLO- L. monocytogenes mutant strain were always nonhemolytic on BHIA and CAB with or without PT. On the other hand, streaked cells and supernatants of L. monocytogenes mutant strains affected either in PI-PLC or in lecithinase production displayed clear zones of hemolysis which were enhanced in the presence of PT.

The cholesterol-treated supernatants of L. ivanovii did not display the inner halo of complete hemolysis. Without PT, the external ring of incomplete hemolysis on BHIA and CAB was of a size similar to that of the nontreated supernatants. Nevertheless, the zone of incomplete hemolysis was strongly inhibited in both media when PT was added.

PT was first recommended, at concentrations between 0.5 and 1%, for its use in media for isolating L. monocytogenes more than 40 years ago (4, 10). Although further studies reported that PT inhibited the growth of some strains of L.
Listeria spp. much better production of cytolysins in the other hand, the results obtained on BHIA and the was not affected by the factors. The addition of the cholesterol-treated hemolytic effect in the presence of PT, which strongly demonstrated, selective sequestration of ivanolysin BHIA and CAB, reflecting the C activity. The fact that supernatants of L. monocytogenes played increases in hemolysis in the presence of PT, and did not show increases in their hemolytic activities on BHIA and CAB, reflecting the specific activity. The fact that supernatants of L. monocytogenes showed increases in their hemolytic activities on PT-containing plates rules out a possible stimulatory effect of this compound in the production and/or secretion of the hemolysin. Whether PT acts directly on the toxin activity, affects its diffusion, or sensitizes the erythrocyte membranes remains to be determined.

L. ivanovii displays a characteristic strong hemolysis, which is often bizonal, in sheep blood agar (17, 21). A thiol-activated cytolysin of 61 kDa, termed ivanolysin O, is the cytolysin factor responsible for the inner zone of complete hemolysis, and a 27-kDa hemolytic sphingomyelinase C is the responsible for the external halo of incomplete hemolysis (21). In accordance with our results, the PT could interact with one or both cytolysins. Without PT, L. ivanovii ATCC 19119 displayed a typical bizonal hemolytic area on BHIA and CAB, reflecting the presence of both cytolysin factors. The addition of PT produced an increase in size of the zones of complete hemolysis, denoting an increase in the cytolytic effect of the ivanolysin O. As previously demonstrated, selective sequestration of ivanolysin O by cholesterol leads to a culture supernatant in which sphingomyelinase C would be the only cytolytic factor (21). As expected, the cholesterol-treated supernatants of L. ivanovii did not display zones of complete hemolysis, and the zones of incomplete hemolysis did not change. On the other hand, these supernatants displayed almost no hemolytic activity in the presence of PT, which strongly suggests an inhibition of the sphingomyelinase C by PT. However, the mechanism of this inhibition also remains to be determined.

L. seeligeri did not show any detectable increase in its hemolytic effect in the presence of PT under our experimental conditions, which could be explained by the low levels of thiol-activated hemolysin production by this species (8).

The similar hemolytic activities displayed by the supernatants of L. monocytogenes and L. ivanovii in diffusion assays in both types of media indicate that the cytolysin activity was not affected by the basal medium composition. On the other hand, the results obtained with the streaked strains of these species, which always yielded larger zones of hemolysis on BHIA than they did on CAB, suggest a higher production of cytolysins in the former medium. This is most likely due to the fact that BHIA supports the growth of Listeria spp. much better than does CAB (data not shown).

The stronger hemolysis displayed by L. monocytogenes on BHIA and the enhancement of its hemolytic effect in the presence of PT could explain the better recognition and differentiation of colonies of L. monocytogenes grown on LSAmM (formulated with BHIA as the basal medium and 200 mg of PT per liter) compared with other selective plating media, which, although they have similar compositions, use different basal media and do not include PT in their formulations (5). The addition of PT to Listeria selective plating media for direct isolation and enumeration of hemolytic Listeria spp. could replace the use of other hemolysis-enhancing substances (e.g., culture supernatants of Staphylococcus aureus or Rhodococcus equi) (6, 18), the use of which is more difficult, less easily standardized, and more time consuming. According to our results, the performance of the RBCTL technique could be improved by decreasing the concentration of PT in LSAmM to 40 mg/liter (0.004%), as the hemolysis of L. monocytogenes and L. ivanovii is slightly higher at this concentration and the selectivity of LSAmM is not affected (unpublished results).

In summary, the combination of BHIA with PT provides a reliable manner for differentiating and enumerating hemolytic Listeria spp. in Listeria selective plating media.

We thank P. Cossart (Unité de Gène Microbiologique, Institut Pasteur, Paris, France) for providing us with the PI-PLC and the lecinthin-deficient mutants of L. monocytogenes and P. Berche (Laboratoire de Microbiologie, Faculté de Médecine Necker-Enfants Malades, Paris, France) for the LLO-1 L. monocytogenes mutant strain. The technical assistance provided by F. Fernández is gratefully acknowledged.

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