A Research Note

Comparison of Selective Direct Plating Media for Enumeration and Recovery of *L. monocytogenes* from Cold-process (smoked) Fish

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ABSTRACT

Three selective media were evaluated for direct plating recovery and enumeration of *Listeria monocytogenes* in the presence of high levels of a variety of microorganisms occurring on cold-process (smoked) salmon products. Sliced salmon was brined to contain either no added salt, 3, or 5% water-phase NaCl, or 3 or 5% NaCl plus 140 ppm NaN₃. The slices were packaged in oxygen-permeable film or sealed under vacuum in oxygen-impermeable film, and stored at 10°C or 5°C until total microbial loads reached 10⁶ to 10⁹ CFU/g. Oxford formulation of Listeria selective agar and Lee’s modification of Listeria selective agar achieved quantitative recovery of 10⁴ cells per ml of *L. monocytogenes* strain Scott A in the presence of diluted slurries of these fish containing 10⁴ to 10⁸ CFU/ml of background organisms. A modification of lithium chloride-phenylethanol-moxalactam agar containing an iron-esculin indicator system sometimes failed because of interfering growth by the background microflora.

Following recognition of *Listeria monocytogenes* as a major cause of foodborne illness in the 1980’s (2,9,13,24), regulatory agencies in the United States adopted a zero tolerance policy toward this organism in ready-to-eat foods. In the United States, *L. monocytogenes* has been found in a variety of raw and ready-to-eat fishery products including cold- and hot-smoked fish, crab meat, shrimp, lobster meat, and unpasteurized imitation crab legs (3,20,28). Inoculated pack studies have been undertaken at this laboratory to identify hazards and critical control points for processing of smoked fish products in relation to incidence, survival, and growth of *L. monocytogenes* and to develop process parameters to increase safety. Since these experiments require not only isolation of the organism, but quantitative recovery and enumeration, the objective of the research reported here was to develop suitable methodology for these particular products.

Many recent investigations have focused on direct plating methods for detection and enumeration of *L. monocytogenes* from foods (3-5,10-12,25) using media with selective agents to suppress organisms other than *Listeria*. The suitability of the media varies, however, depending upon the type of food and the relative levels of *L. monocytogenes* and the type and level of background organisms (3-5,10,11).

Some media (6,15,26) have incorporated a useful indicator system, developed by Dominguez Rodriguez et al. (8), which capitalizes on the ability of *L. monocytogenes* to hydrolyze esculin and the reaction of the hydrolytic product with ferric ammonium citrate to form a black color around the colonies. Two media employing esculin hydrolysis, Listeria selective agar (OX) and Lee’s modification of OX (LMO), have been incorporated into procedures by the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA), respectively, for selective isolation of *Listeria* from foods (23). Although these media have been used for isolation of *L. monocytogenes* from seafoods, there have been no comparative studies to show their efficacy for quantitative recovery and enumeration of *L. monocytogenes* from these products.

The present study is an evaluation of three plating media with the iron-esculin system, OX, LMO, and LiCl-phenylethanol-moxalactam agar containing iron-esculin indicator (LPM,FE), for quantitative recovery and enumeration of relatively low numbers of *L. monocytogenes* in the presence of large populations of background organisms in cold-process (smoked) salmon. The brined salmon slices contained different levels of sodium chloride and sodium nitrite and were stored for various times at 5 and 10°C in both oxygen-permeable and impermeable packages so that diverse populations of background organisms grew.

MATERIALS AND METHODS

Plating media

Selective plating media included: a) LiCl-phenylethanol-moxalactam agar (LPM) consisting of LPM base (BBL 12336;
BBL, Cockeysville, MD) with 20 mg/L moxalactam (Sigma M-1900) added after sterilization and cooling to 47°C (16); b) Oxford formulation, Listeria selective medium (OX) (Oxoid Ltd., Basingstoke, Hampshire, England), based on the formulation by Curtis (6,7) and consisting of Listeria selective agar base (CMS86) and Listeria selective supplement (SR140); c) A modification of OX (LMO) suggested by Lee (15). LMO contains as a base Oxoid’s Oxford formulation of Listeria selective agar base with the addition of 10 mg/L Collistin methane sulfonate (Sigma C-1511), and 20 mg/L moxalactam (Sigma M-1900) added after sterilization and cooling to 47°C; d) LPM,FE consisting of LPM agar (16) modified by addition of 0.5 g/L ferric ammonium citrate and 1 g/L esculin.

Four plates rather than spread plates were used because they were easier to count and did not have problems with spreading colonies that sometimes occur with surface inoculated plates. The plates prepared from the four differential media were counted after 24 and 48 h incubation at 30°C. 

Preparation of inoculum

*L. monocytogenes* strain Scott A (serotype 4b), a clinical isolate from a listeriosis outbreak from pasteurized milk (9), was used. For the experimental inocula, an overnight trypticase soy broth culture incubated at 30°C was diluted in fresh media to 0.3 absorbance units at 525A., which represented a population of approximately 5.0 x 10⁶ organisms per ml. A portion was diluted further in 0.1% peptone to yield the desired concentration for inoculation. The inoculum was enumerated by plating appropriate dilutions in triplicate, using trypticase soy agar (BBL 11046) with 0.6% yeast extract (TSA, YE) and 0.2% glucose.

Preparation of salmon product

Raw fillets of chum salmon (*Oncorhynchus keta*) were thinly sliced (7 mm) and brined in NaCl or NaCl plus NaN0₂, at 1°C for 4 h with a 1:4 fish to brine ratio, and equilibrated overnight (18 h) at 1°C to allow uniform penetration of NaCl and NaN0₂. The brines contained brown sugar equal to the weight of salt. NaCl, NaN0₂, and H₂O concentrations in the fish after brining were determined by methods previously described (22). Smoke was not applied to the samples because its contribution to inhibition would tend to obscure the effects of the other substrate variables.

Packaging and storage

Salmon slices were individually packaged in either oxygen-permeable film (1.5-mil polyethylene: Oxygen transmission 7195 cc/sq m/24 h at 760 mm Hg, 23°C, and 0% RH; CO₂ transmission 22,858 cc/sq m/24 h) or under vacuum in oxygen-impermeable film (2-mil polyester: Oxygen transmission 108 cc/sq m/24 h; CO₂ transmission 526 cc/sq m/24 h). The oxygen-impermeable bags (Kapak Corp., Bloomington, MN) were evacuated to 640 mm of mercury using a Multivac Model A 300/16 vacuum sealer (Koch Supplies Inc., Kansas City, MO). The packaged samples were stored at 5 or 10°C in biological oxygen demand incubators equipped with fans to prevent temperature stratification.

Preparation and inoculation of fish slurries

Packaged salmon slices containing either no added NaCl or 3 or 5% water-phase NaCl, or 3 or 5% water-phase NaN0₂ with 140 ppm NaN0₂, were stored until the total microbial load reached the range of 10⁶ to 10⁹ CFU/g. Samples were diluted with 4 parts 0.1% sterile peptone per 1 part of fish and pumped in a Stomacher (Tekmar Co., Cincinnati, OH) for 3-5 min or until thoroughly comminuted. The slurries were plated on OX to ensure absence of endogenous contamination by *L. monocytogenes.*

A 1/50 dilution of fish slurry was then inoculated with 10⁵ cells of Scott A per ml. Six plates of each selective media were inoculated with 1 ml of the suspension per plate to compare recovery of *L. monocytogenes.* Serial dilutions were plated in triplicate on TSA, YE with 0.2% glucose to determine the total microbial load in the suspension. Scott A cells diluted to 10⁷/ml, with no added slurry, served as controls.

Confirmation and identification of organisms

For each test, representative colonies that were presumptively *Listeria* from each of the direct plating media were confirmed as *L. monocytogenes* by following identification schemes described by Lovett (18) and McClain and Lee (19). Organisms other than *L. monocytogenes* were identified according to methods described by Pelroy and Eklund (21) and Lee and Pfeifer (14).

Statistical analysis

*L. monocytogenes* counts from the different media and treatments were analyzed by analysis of variance at the 0.05 level of significance. Mean counts were separated using Student-Newman-Keuls’ multiple comparison procedure. The analyses were conducted using superANOVA software (1).

RESULTS

Enumeration of *L. monocytogenes* from fish slurries containing high numbers of background organisms

Enumeration of 10⁶ Scott A cells per ml with OX, LMO, and LPM,FE was evaluated in the presence or absence of the various fish slurries containing total microbial loads of 10⁶ to 10⁹ CFU/ml in six different trials (Table 1). OX showed the least variation, with only one sample, No. 3 in Trial 1, in which the *L. monocytogenes* count in the presence of fish slurry was significantly different from the control. The fish in Trial 1 were not brined with NaCl or NaN0₂, and undoubtedly contained the largest variety of background microorganisms.

Visualization and recovery of colonies were easiest with OX. There was no growth of background flora at 24 h and very little after 48 h. *L. monocytogenes* colonies with black zones were readily countable after 24 h at 30°C. After 48 h, some occasional darkening of the background made counting more difficult.

*L. monocytogenes* colonies on LMO were larger than on OX, but the LMO plates were slightly more difficult to count because LMO had a greater tendency than OX to develop large black areas. Occasionally, some of the background microorganisms appeared as tiny white colonies on LMO.

LPM,FE showed significant variation in 12 out of the 16 samples tested in the first five trials. In 10 of these samples, the variation was from interfering growth of the background microflora. In some instances the background organisms formed black colonies on LPM,FE. On many of the plates, the iron-esculin reaction was so strong that the entire plate became black, even after 1 d of incubation, making differentiation almost impossible.

Background organisms capable of growth on selective media

Further tests were done to determine the response of background organisms that had overgrown the *L. monocytogenes* on LPM,FE plates (Table 1). Portions of undiluted slurries from three of these samples, 13, 14, and 15, that had been reserved and not inoculated with *L. monocytogenes*...
TABLE 1. Comparison of three selective media for recovery of *L. monocytogenes* in the presence of competing microflora in slurries prepared from cold-smoked salmon stored under a variety of conditions.

<table>
<thead>
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<th>Trial/Sample number</th>
<th>Storage temperature °C</th>
<th>Packaging film</th>
<th>%NaCl water-phase</th>
<th>ppm NaNO₂</th>
<th>Medium</th>
<th>APC³ (CFU/ml)</th>
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<td>OX</td>
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<td>I</td>
<td>5</td>
<td>150</td>
<td>132.4ª</td>
<td>119.0ª</td>
</tr>
</tbody>
</table>

¹ Each value from selective media is the mean CFU from six plates.


³ Aerobic plate count of test sample. Values are the mean of three plates from an appropriate dilution.

⁴ Controls were inoculated with *L. monocytogenes* cells but no fish slurry.

⁵ Storage times for samples 1-6, 7-12, 13-16, and 17-20, were 6, 12, 20, and 27 d.

⁶ = Plates were overgrown with colonies other than *L. monocytogenes*.

abed Values within same trial followed by same letter are not significantly different (P < 0.05).

*genes*, were plated on OX, LMO, LPM, FE, and TSA.YE. Total CFU on TSA.YE from the slurries ranged from 10⁸ to 10⁹/ml. Of the slurries plated, only one produced a single black colony with OX pour plates. Some of the samples produced numerous tiny white colonies with LMO, and a total of 14 black colonies were also found. Black colonies developed with LPM.FE yielding 7.7 x 10³, 2.0 x 10³, 3.4 x 10³, and <1.0 x 10² CFU/g for samples 13, 14, 15, and 16, respectively.

The black colonies from OX and LMO and the small white colonies from LMO were lactic acid bacteria. Of 546 representative black colonies picked from LPM.FE, 510 were lactic acid bacteria, 34 were *Pseudomonas* spp., and two were *Enterobacteriaceae* (gram-negative, oxidase-negative rods). When 20 lactic acid bacterial cultures capable of producing black colonies on LPM.FE were also tested on LPM, six showed slight growth and 14 did not grow.

**Enumeration of *L. monocytogenes* from pure culture controls**

For pure culture controls, OX, LMO, LPM, LPM.FE, and TSA.YE were equivalent (Table 2). No significant
differences were found in the counts from the five media in the first three trials. In the fourth trial, the counts from LMO and TSA.YE were significantly different from each other, but not from the other three media (P < 0.05). When the data were analyzed at the P < 0.01 level, the counts from these two media were not significantly different from each other.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Trial No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>OX</td>
<td>SEM</td>
<td>145.8</td>
<td>97.7</td>
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<td>SEM</td>
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<td>TSA.YE</td>
<td>SEM</td>
<td>138.7</td>
<td>104.7</td>
<td>146.8</td>
<td>132.0</td>
</tr>
</tbody>
</table>

1 Data expressed as mean ± standard error of the mean (SEM) CFU/ml. Means based on six plates (n = 6).

2 Means in the same column with the same superscript are not significantly different (P < 0.05).

**DISCUSSION**

OX and LMO performed satisfactorily for direct plating recovery and enumeration of *L. monocytogenes* from artificially contaminated cold-process salmon products. Quantitative recovery was accomplished in the presence of high numbers of background microorganisms from samples treated with different NaCl and NaN3 concentrations and stored under a variety of packaging and storage conditions. Occasional appearance of tiny white background colonies on LMO may indicate that LMO could be slightly less selective than OX for use with cold-process products. LPM.FE, although equivalent to the other media for quantifying growth of pure cultures of Scott A, was not suitable for recovery of *L. monocytogenes* from the salmon slurries. It was less selective and subject to more variation in counts because of interfering growth by the background organisms. Since some of these contaminants were not able to grow when they were replated on LPM, it may be that the iron and esculin modification reduces the selectivity of LPM. Lee and McClain (16), however, had originally reported encountering poorly growing enterococci and occasional *Pseudomonas* on LPM. In addition, Loessner and others (17), in testing recovery of *L. monocytogenes* from milk, found small colonies of lactic acid bacteria on LPM. They did not interfere with recognition of *L. monocytogenes*, however.

Both OX and LMO have been adopted into the isolation protocols of the FDA and USDA, respectively, and are very acceptable for detecting *L. monocytogenes* in meat, poultry, and seafood products. In recent comparative studies by 11 laboratories in Canada, modifications of both the FDA and USDA methods, which incorporate these media, were not significantly different (P < 0.05) in ability to isolate *L. monocytogenes* from over 512 environmental and food samples, including fish, shellfish, and shrimp (27). In the Canadian study, OX was marginally better than LPM and significantly better (P < 0.05) than LMO for isolation of *L. monocytogenes*. Their procedures, as well as those of the FDA and USDA, employ an enrichment step, however, and direct plating is not used as a method of enumeration.

There is little information on methods for direct plating enumeration of *L. monocytogenes* from seafoods. LPM, but not OX and LMO, has been compared with other selective media in investigations with scallops (17) and oysters (4), where the researchers found that media that were best for isolation of *L. monocytogenes* were not necessarily the best for enumeration. Cassiday et al. (4), for example, found that selective direct plating could be used for isolation and enumeration of *L. monocytogenes* in hams, but not for oysters.

Since there are inherent differences in natural microflora found in various foods and also seafoods, it is unlikely that any one particular media will be found to be suitable for maximal recovery of *L. monocytogenes* from all types of foods. Nevertheless, the need for methodology for direct, quantitative recovery of *L. monocytogenes* from various foods is becoming increasingly important.

**ACKNOWLEDGMENTS**

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**REFERENCES**

DIRECT PLATING MEDIA FOR L. MONOCYTOGENES

909


