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PDX-LIB, an Improvement on Selective Enrichment Media for Detection of Environmental *Listeria spp*.

Alan Olstein[1*] **and Joellen Feirtag**[2]

[1]*Paradigm Diagnostics, Inc, 800 Transfer Road Suite 12, St. Paul, MN 55114, USA*

[2]*Department of Food Science & Nutrition, University of Minnesota, 1354 Eckles Avenue, St. Paul, MN 55108, USA*

[*]**Corresponding author:** Alan Olstein, Paradigm Diagnostics, Inc, 800 Transfer Road Suite 12, St. Paul, MN 55114, USA; Tel: 651-295-7768; Fax: 651-405-9490; E-mail: alan.olstein@PDX-inc.com

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Abstract

PDX-LIB is a selective enrichment indicator broth developed for use as a diagnostic screening test for environmental *Listeria* spp. contamination. The test method was developed to provide a single step selective enrichment media incorporating a colorimetric indicator to identify presumptive Listeria-positive samples. The method permits high throughput screening of the sample population streamlining the number of steps to confirmatory testing. In the original AOAC Research Institute guided performance test method the PDX-LIB selection media was shown to be significantly more sensitive for detection of *Listeria* spp. on selected surfaces than the USDA method, which utilizes UVM media as its primary selection step. The present study has shown that the PDX-LIB selection media permits substantially greater numbers of recoverable *Listeria monocytogenes*- positive samples in the AOAC surface study protocol than comparable samples enriched with UVM media. The data presented demonstrate that the PDX-LIB method is a more sensitive method than procedures utilizing UVM enrichment media as the primary step particularly with regard to detection of *Listeria* sp.

Keywords: *Listeria* Detection; Selective enrichment; Environmental screening

Introduction

Listeriosis is the disease caused by consuming food contaminated with *L. monocytogenes.* It is one of the leading causes of death not because it occurs often but because of the extremely high mortality rate [1]. In the United States alone, there are over 2,500 reported cases of Listeriosis every year; of these there are approximately 500 deaths. Individuals who are immuno-compromised, young, old, or pregnant should be careful not to consume adulterated products. Symptoms may include fever, muscle aches, and diarrhea. If the nervous system becomes infected symptoms may include headache, stiff neck, confusion, loss of balance and convulsions. In pregnant women, listeriosis can produce flu like illness and can lead to miscarriage or stillbirth of the fetus [2]. Because of the seriousness of the illness caused by *Listeria* contamination, government agencies have taken steps to reduce incidence of *Listeria* outbreaks including a "zero-tolerance" policy for *Listeria* in 25 g samples of food product. Environmental screening for *Listeria* spp. as an indicator organism was mandated for all USDA regulated establishments producing high risk ready to eat food products as a more efficient means of mitigating the microbiological hazards of *Listeria* contamination in the production of these food products [3].

Detection and isolation of *Listeria* species from food product matrices are well described in publications of both the USDA [4] and the FDA [5]; although the procedures for analysis of environmental samples are detailed in the FSIS publication explicitly. Many methods rely on the use of UVM media, buffered-*Listeria* enrichment broth and/or Fraser broth. All of these enrichment medias employ combinations of lithium chloride, acriflavin and nalidixic acid as the principal selective agents. *Listeria* enrichment broth and its derivative media compositions were described by Lovett [6]. Procedures for selective enrichment of *Listeria monocytogenes* in dairy products were proposed by Lovett while at the FDA in the 1980's [7]. This author cited general acceptance of the acriflavin, nalidixic acid containing Listeria enrichment media employed by European laboratories as a rationale for its use in the United States [8]. McClain and Lee at the USDA/FSIS developed a procedure for isolation of *Listeria monocytogenes* using a similar media compositions for meat products [9].

During the 1990's reports of adverse effects of acriflavin containing enrichment media on the growth rate and recovery of *Listeria monocytogenes* from food products surfaced. Curiale and Lewus reported on the suppression of *L. monocytogenes* when grown in the presence of *L. innocua* [10]. Beumer et al. documented the increase in the lag phase of *L. monocytogenes* in the presence of acriflavin was dose dependent. Their paper pointed out the potential for background microflora out-competing *L. monocytoegnes* in acriflavin-containing selection media resulting in loss of sensitive detection for the pathogen [11].

PDX-LIB was developed as diagnostic screening test for identification of presumptively positive environmental samples from food processing facilities. The media was designed to optimize recovery of sub-lethally injured *Listeria* spp. from sanitized surfaces by incorporating recovery agents. The screening test was shown to be more sensitive than the USDA method in the AOAC Research Institute guided study [12]. PDX-LIB was developed to take advantage of alternative selective agents to acriflavin, which significantly suppressed the recovery of several *Listeria* spp, in the enrichment media [13]. In contrast to the USDA method the PDX-LIB method permits identification of presumptive positives in one step within 48

hours incubation while the USDA cultural method requires multiple inoculations and platings, which may require three days [14].

The proposed study was conducted to examine the quantitative recovery of *Listeria monocytogenes* on various environmental surfaces utilizing PDX-LIB, an acriflavin-free proprietary selective enrichment media, compared to a widely employed selective enrichment media, UVM, the primary selective enrichment media detailed in the USDA method for detection of *Listeria* spp in environmental samples [4].

Material and Methods

AOAC International has devised an excellent protocol to study the performance of different microbiological methods at the limit of sensitivity of these methods. The AOAC guidelines have detailed these methods [15]. We utilized the same methodology to examine the sensitivity of several strains of *Listeria monocytogenes* to dry injury prior to sample collection and analysis.

The pure cultures were obtained from R-Tech Labs, Arden Hills, MN, American Typed Culture Collection (ATCC), Manassas, VA, Veterinary Medical Diagnostics Labs at University of Minnesota, St. Paul, MN, and Microbiology Labs of Dr. Diez and Dr. Feirtag at the University of Minnesota, St. Paul, MN. Original pure cultures were grown overnight in sterile Brain Heart Infusion Broth (BHI) at 32-37°C. In order to have reproducible cultures, 50% glycerol stock solutions of cultures were prepared and stored in a freezer. Fifty percent glycerol stock cultures were prepared by diluting 500 µl of pure culture grown overnight with sterile 500 µl glycerol. Then 200 µl portions were filled into sterile 2 ml centrifuge tubes, capped and kept in a freezer at -15°C until the day before use. On the day before use, a loop-full of freezer-stored stock cultures were inoculated into 5 ml of sterile BHI, the same loop was streaked onto tryptic soy agar (TSA) containing ferric ammonium citrate and esculin (TSAIE). The TSA and TSAIE were incubated overnight at 37°C. TSAIE plates were checked for contamination, based on -glucosidase activity and colony morphology. If the TSAIE plates suggested there was no apparent contamination, logarithmic level dilutions were made into sterile peptone solutions (0.1% peptone in distilled water). Aliquots containing 100 µL of 7 and 8 log dilutions from overnight cultures were plated onto TSAIE for estimating the cell concentration (CFU/ml) and checked for contamination.

Li on ceramic tiles

Ceramic tiles at 4 × 4 inches in dimensions were bought from a local Home Depot. They were wrapped in aluminum foil and autoclaved for 15 min at 121°C. All tiles were kept wrapped at room temperature (RT) until the time of inoculation. The inoculation of surfaces were done at 0.250 ml/surface from the log-4 (high) and log-5 (medium) dilutions, corresponding approximately to 4.67 log CFU and 3.67 log CFU respectively, twenty replicates for each level, per method. Inoculations on surfaces were spread within the corresponding 4" × 4" zone with the help of sterile spreaders. Surfaces were left to dry for at least 18 hours at RT.

After the samples were dried on the surfaces for a minimum of 18 hours, they were removed by swabbing using the Tecra-Enviroswabs. Both the test method (PDX-LIB) and the reference method (USDA) [16] was done in twenty replicates per level (high and low) of inoculations.

In order to confirm the presence-absence of Listeria in all test samples, all tubes of PDX-LIB were streaked onto MOX plates at the end of the 48th hour of incubation. Dark gray colonies with black zones were confirmed using the API biochemical panel.

Liv on stainless steel

Serial logarithmic dilutions of overnight culture of Liv in TSB were made into sterile TSB. A one-hundred microliter volume of log-6 and log-7 dilutions was plated onto MOX and TSAIE plates for estimating the number of cells loaded onto 4" × 4" zones on stainless steel. Stainless steel sheets were bought from Metal Supermarket Inc, Roseville, MN. They were wrapped in aluminum foil and autoclaved for 15 min at 120°C. Stainless steel sheets were kept wrapped until the time of inoculation. The inoculation of surfaces were done at 0.250 ml/surface from the log-1 (high) and log-2 (medium) dilutions, corresponding approximately to 7.07 log CFU and 6.07 log CFU consecutively, twenty replicates for each level per method. Inoculations on surfaces were spread within the corresponding 4" × 4" zone with the help of a disposable sterile spreader. The surfaces were left to dry for a minimum of 18 hours at room temperature.

After the samples were dried on the surfaces for a minimum of 18 hours, they were removed by swabbing using Tecra Enviroswabs. Both test method (PDX-LIB-Labor Saver] and reference method (USDA) was done in twenty replicates per level (high and low) of inoculations. In order to confirm the presence-absence of Listeria in all test samples, all tubes of PDX-LIB were streaked onto MOX plates at the end of the 48th hour of incubation. Dark gray colonies with black zones were confirmed using the API biochemical panel.

Lw on plastic

Serial logarithmic dilutions of overnight culture of Lw in TSB were made into sterileTSB. A one-hundred microliter volume of log-6 and log-7 dilutions was plated onto MOX and TSAIE plates for estimating the number of cells loaded onto 4" × 4" zones on plastic. Polypropylene plastic cutting boards were bought from a local department store. 4" × 4" zones were marked on the surface of the cutting boards. They were wrapped in aluminum foil and autoclaved for 15 min at 120°C. Plastic cutting boards were kept wrapped until the time of inoculation. The inoculation of surfaces were done at 0.250 ml/surface from the log-4 (high) and log-6 (low) dilutions, corresponding approximately to 5.18 log CFU and 3.18 log CFU of Lw consecutively, twenty replicates for each level per method. Inoculations on surfaces were spread within the corresponding 4" × 4" zone with the help of a disposable sterile spreader. After that surfaces were left to dry min 18 hours at room temperature.

After the samples were dried on the surfaces for a minimum of 18 hours, they were removed by swabbing using Tecra Enviroswabs. Both test method (PDX-LIB-Labor Saver] and reference method (USDA) was done in twenty replicates per level (high and low) of inoculations. In order to confirm the presence-absence of Listeria in all test samples, all tubes of PDX-LIB were streaked onto MOX plates at the end of the 48th hour of incubation. Dark gray colonies with black zones were confirmed using the API biochemical panel.

Lm in 10x *E.coli* on sealed concrete

Lm in 10x *E. coli* stock culture was made by adding 0.1 ml of overnight grown Lm (10^9 CFU/ml) and 1 ml of overnight grown *E. coli* (10^9 CFU/ml) into 9 ml of TSB. Serial logarithmic dilutions of

Lm in 10x *E. coli* were made into sterile TSB. A one-hundred microliter volume of log-5, log-6 and log-7 (for *E. coli*) dilutions were plated onto MOX and TSAIE plates for estimating the number of cells loaded onto 4" × 4" zones on sealed concrete blocks. Number of cells on MOX was used to estimate number of CFU Lm/ml, and number of beige colonies on TSAIE was used to estimate the number of CFU *E. coli*/ml.

Concrete blocks (15.5" × 7.5" × 3.5"; length × width × depth) were purchased from a local Home Depot. Blocks were sealed with a solvent-based concrete sealer (BW Crete Seal 25 LV, St. Paul, MN). After a minimum of two coats of sealant was placed on the blocks, they were dried in a chemical hood until all the solvent had evaporated (minimum of 24 hours). Zones of 4" × 4" were marked on the sealed side using a permanent marker. Before inoculations, sealed concrete blocks were sprayed with ethanol and allowed to air dry. The ethanol was air dried for at least 20 minutes but no more than an hour. The concrete surfaces were not rinsed after the second application of ethanol was allowed to air dry. The inoculation of sealed concrete surfaces were done at 0.250 ml/surface from the log-4 *E. coli*/log-5 Lm (high) and log-5 *E. coli*/log-6 Lm (low) dilutions, corresponding approximately to 3.85 log CFU and 2.85 log CFU respectively, twenty replicates for each level, per method. Inoculations on surfaces were spread within the corresponding 4" × 4" zone. Surfaces were left to dry for at least 18 hours at RT.

After the samples were dried on the surfaces for a minimum of 18 hours, they were removed by swabbing using Tecra Enviroswabs. Both test method (PDX-LIB-Labor Saver) and reference method (USDA) was done in twenty replicates per level (high and low of inoculations. In order to confirm the presence-absence of Listeria in all test samples, all tubes of PDX-LIB were streaked onto MOX plates at the end of the 48th hour of incubation. Dark gray colonies with black zones were confirmed using the API biochemical panel.

Surface Studies of *L. monocytogenes* with and without Background Microflora

Serial logarithmic dilutions of overnight culture of *Listeria monocytogenes* in BHI were made into sterile peptone water. A one-hundred microliter volume of log-7 and log-8 dilutions was plated onto TSA plates for estimating the number of cells loaded onto 4" × 4" zones on the plastic substrate. Polypropylene plastic cutting boards were bought from a local department store. 4" × 4" zones were marked on the surface of the cutting boards. They were wrapped in aluminum foil and autoclaved for 15 min at 120°C. Plastic cutting boards were kept wrapped until the time of inoculation. The inoculation of surfaces was done at 0.250 ml/surface from the log-5 dilutions, corresponding approximately to 5.18 log CFU of *Listeria monocytogenes* consecutively, twenty replicates for each level per method. Inoculations on surfaces were spread within the corresponding 4" × 4" zone with the help of a disposable sterile spreader. After that surfaces were left to dry min 18 hours at room temperature.

After the samples were dried on the surfaces for a minimum of 18 hours, they were removed by swabbing using Securswabs (Paradigm Diagnostics, Inc). Both the test method PDX-LIB and a modified USDA reference method [4] were done in twenty replicates at an inoculation level which would provide fractional recovery of positive samples. In order to confirm the presence of Listeria in all test samples, all samples were streaked onto MOX after at the end of 48th hour of incubation at 37°C. Dark gray colonies with black zones grown on MOX were picked and confirmed using Microgen Listeria ID kit. The same procedures were followed using three other *Listeria monocytogenes* strains on stainless steel and sealed concrete.

Statistical Data Analysis

The Mantel-Haenszel chi-square formula for unmatched test portions was used for the statistical analysis [17]. A Chi-square value <3.84 indicates that the proportions positive for the alternative and the reference method are not statistically different at the 5% level of significance. This criterion must be satisfied for each level of each surface type. However, a significant difference between the proportions positive for the two methods is acceptable provided that the alternative method demonstrates superior recovery to the reference method.

Chi Square=Mantel-Haenszel: $\chi 2=(n-1)(ad-bc)2/[(a+b)\ (a+c)\ (b+d)\ (c+d)]$, where n =total number of samples tested by the two methods, a=number of samples positive by the test method, b=number of samples negative by the test method, c=number of samples positive by the reference method and d=number of samples negative by the reference method.

Results

Four different *Listeria* sp. were subjected to surface challenge testing per the AOAC test methodology [14]. The test methodology is devised to determine the limit of detection of the analytical method by measuring the fractional recovery of the test samples at the highest possible cell dilution. Table 1, which was excerpted from the original AOAC final report [12], summarizes the results of surface recovery studies of four different *Listeria* sp, *L. welshmerii*, *L. innocua*, *L. ivanovii* and *L. monocytogenes* on four different surface materials. Detailed studies of the method robustness with respect to time and temperature are contained within the AOAC final report. The aforementioned AOAC final report was self-published at the Paradigm Diagnostics' website. The test method yielded higher recovery of *Listeria* sp than the modified reference USDA reference method in three of the four test matrices at the lowest inoculation levels.

Five different *Listeria monocytogenes* isolates were subjected to challenge surface testing studies under drying conditions in the presence of minimal nutrients, simulating real conditions in the food-processing environment. In the first two studies the Listeria were spread on surfaces as monocultures at cell densities sufficient to obtain fractional recovery of the twenty replicate surfaces which in this case were polypropylene cutting boards (plastic). Both studies showed that more Listeria-positive samples were recovered in PDX-LIB than in UVM as the primary selective enrichment media (Table 2).

Three surface studies in which the Listeria were spread as mixed cultures with 10-fold greater competitive micro-flora in the background were conducted. The mixed culture studies revealed profound effects on the recovery of Listeria populations in UVM compared to the recovery of Listeria in PDX-LIB media. In all cases the LIB media yielded superior recovery of the sub-lethally injured Listeria compared to the UVM control cultures; eighteen confirmed LIB positives versus three confirmed UVM positives, ten confirmed LIB positives versus no UVM positives recovered and nine confirmed LIB positives versus no UVM- positives. All three groups of samples containing mixed cultures were judged to be dissimilar using the statistical criteria for comparison of the methods [10].

	Levels	Inoculation (CFU/surface)	Replicates	TEST (PDX-LIB Labor Savor)			REFERENCE (USDA)	Chi Squared Value
				Presumptive Positives	Confirmed	False negatives	Confirmed positives	
Listeria innocua on ceramic tile	-Ctrl	0	5	0	0	0	0	NA
	Medium	4669	20	19	19	0	6	15.36
	High	46688	20	20	20	0	19	0
Listeria ivanovii on stainless steel	-Ctrl	0	5	0	0	0	0	NA
	Medium	1175000	20	20	20	0	5	20.91
	High	11750000	20	19	19	0	7	13.3
Listeria welshmeri on plastic	-Ctrl	0	5	0	0	0	0	NA
	Low	1506	20	15	15	0	17	1.41
	High	150563	20	20	20	0	19	0
Listeria monocytogenes in 10x *E.coli* on sealed concrete	-Ctrl	0	5	0	0	0	0	NA
	Medium	715	20	8	9	1	0	7.66
	High	7156	20	14	15	1	3	10.23

Table 1: Summary of methods comparison at 48 hours incubation [12].

	Inoculation (CFU/surface)	Replicates	PDX-LIB		UVM-USDA	χ^2
			Presumptive Positives	Confirmed positives	Confirmed positives	
1	1.50E+05	20	9	9	2	2.92
2	1.30E+05	20	16	16	1	11.5
3	2.50E+04	20	18	18	3	10.7
4	1.60E+04	20	10	10	0	6.33
5	7.20E+02	20	9	9	0	5.51

Table 2: Summary of methods comparison between PDX-LIB and UVM-USDA.

Discussion

The data presented here are consistent with previously reported findings concerning the potential suppression of *L. monocytogenes* in mixed cultures when cultivated in acriflavin-containing selective enrichment media such as UVM, or Fraser Broth [10,11]. Previous studies suggested that L. innocua in mixed cultures with *L. monocytogenes* permitted the outgrowth of L. innocua while suppressing the growth *L. monocytogenes* in acriflavin-containing media. In fact McClain and Lee stated that using several different acriflavin-containg composition they were unable to isolate *L. monocytogenes* from any of their meat samples inoculated with the pathogen at 20-2500 CFU/g during primary enrichment. They consequently recommended that the USDA method employ a two stage lengthened enrichment [9]. Beumer et al. demonstrated that the proteins and food particles in the food matrix likely bind the acriflavin dye molecules detoxifying the compound permitting the L.monocytogenes present in food samples to survive and grow, albeit at substantially reduced frequency [11]. In environmental samples devoid of proteins and food particles it is more likely that acriflavin being more active in the unbound state could affect greater kill *L. monocytogenes* than in the food product matrix.

PDX-LIB was designed to promote recovery of sub lethally injured Listeria. The media was formulated to contain recovery agents and the acriflavin was supplanted as a selective agent. UVM, BLEB and Fraser Broth do not contain recovery agents such as sodium pyruvate and necessary divalent metal salts to provide nutritional requirements to promote recovery of sub lethally injured Listeria cells. While developing PDX-LIB we found that acriflavin exerted growth retardation effects on several species of Listeria prompting us seek alternative selective agents such as a combination of a third generation cephalosporin and a nitrofuran [13]. These findings were consistent with the observations published by Beumer et al. [11].

The data presented in this study suggest that virtually any competitive micro-flora could suppress the recovery of *L.*

monocytogenes since the surface studies demonstrated this effect in the presence of *E. faecaelis*, *L. lactis* and *E. coli*, common background micro-flora. The results reflected in Table 1 suggest that environmental samples enriched with acriflavin-containing media such as UVM, LEB and Fraser Broth could be under-reporting the presence of *L. monocytogenes*.

In conclusion the essential points of the studies are:

- The enrichment media used in PDX-LIB is a more sensitive method than those methods employing acriflavin- containing formulations such as UVM.
- Use of optimally sensitive enrichment medias, such as LIB, may increase recovery of sub-lethally injured *Listeria* sp. from food processing equipment. This is of potential concern because processing equipment is subjected to sanitizing chemicals and drying.
- Confirmatory methods such as immunoassay and PCR are dependent on the sensitivity of the primary enrichment media employed.

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