Research Report

Validation of the use of Composite Sampling for *Listeria monocytogenes* in Ready-to-Eat Meat and Poultry Products

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SUMMARY

Equal numbers of *Listeria monocytogenes* were introduced into ready-to-eat (RTE) meat samples of three sizes--25, 125, and 375 grams--to determine whether the volume of product affected the detection rate. The 25-gram sample represented the standard test unit. The 125- and 375-gram sample sizes simulated compositing schemes of 5 and 15 units of 25 grams, respectively. The USDA (Revisions 1 and 2), ELISA, and PCR methods were used for detection. Overall, 678 inoculated and 108 non-inoculated analytical samples were analyzed per test method. The average inoculation level was 1.01 colony forming units per sample for each of the three samples sizes.

Of 226 inoculated 25-gram samples analyzed by the USDA Rev-1 method, 124 were positive for *L. monocytogenes.* At the 125-gram sample size, 112 samples were positive, a result not significantly different ($p=0.12$) than the result observed at the 25-gram size. However, the number of positives at the 375-gram sample size, 95, was significantly different ($p<0.01$) than the number of positives at the 25-gram size.

For the USDA Rev-2 method, 128 of 226 were positive at the 25-gram test size. At the 125 and 375 gram sample sizes, 115 and 103 were positive, respectively. The result at the 125-gram size was equal to the result at 25-gram ($p=0.09$), but the 375-gram result was not equivalent to the 25-gram result ($p<0.01$).

Tests conducted at the 3 sample sizes using the PCR method showed that the 25-gram results were equivalent to the 125-gram results ($p=0.039$) and just not equivalent to the results for the 375-gram sample size ($p=0.05$). For the ELISA method, neither the result for the 125-gram sample size ($p<0.01$) nor the 375-gram samples size ($p<0.01$) sample size was equivalent to the 25-gram results.

In conclusion, the data support the use of sample compositing up to a total of 125 grams of RTE meat or poultry using the USDA and PCR methods for detection of *L. monocytogenes*. Compositing a total of 375 grams is not recommended for these methods. Compositing is not recommended for the ELISA method.

MATERIALS AND METHODS

Test Organisms

Three *Listeria monocytogenes* isolates were used in the study. The reference number and serotype of the strains were SLR10, 1/2a; SLR31, 1/2b; and SLR1234, 4b (Scott A). Each isolate was maintained at -70°C in 15% glycerol for long term storage. Working stock cultures were kept on Trypticase Soy Agar with Yeast Extract (TSAYE; BBL, Becton Dickinson and Co., Cockeysville, MD). Cultures for inoculation were grown in Trypticase Soy Broth with Yeast Extract (BBL, Becton Dickinson and Co., Cockeysville, MD) at 35°C for 18-24 hours. Numbers of colony forming units were determined on TSAYE incubated at 35°C for 24 hours. Broth cultures were stored at 4°C prior to product inoculation. Dilutions were prepared with Butterfield's phosphate buffer (0.3 mM, pH 7.2).

Preparation of Samples

Three production lots of the test each was prepared in a commercial establishment and shipped to test laboratory for inoculation and testing.

Frankfurters were ground in a Hobart mill and the sliced deli products were hand trimmed into small strips. Portions of ground or trimmed product were used for inoculation. Each inoculated portion consisted of 1150 gram. After inoculation, the sample was mixed thoroughly and stored overnight at 4°C to adapt the inoculum to the product. From the inoculated material, 27 samples of 25-gram were prepared. These were combined with 0, 100, or 350 grams of non-inoculated meat just prior to analysis to form 9 test samples at each of three sample sizes: 25, 125, and 375 grams. The remaining inoculated product was used for determination of the *Listeria* contamination level. Inoculations were performed for each of 4 inoculation levels for each of 3 strains for each of 3 product lots for each of 3 product types.

Non-inoculated portions of each product were prepared as negative controls. There were nine replicate samples for each sample size for each product lot.

Microbial Analyses

Samples were analyzed for *Listeria* species by four enrichment methods, USDA-FSIS (revision 1 and revision 2), VIDAS LIS ELISA (St. Louis, MO) method, and BAX PCR (Wilmington, DE) method. The pre-enrichments for the test methods were modified to accommodate the 125 and 375-gram sample sizes. The added broth volumes were 1125 mL

and 3375 mL, respectively. These modifications maintained the 1:9 sample to broth ratio specified by each test method.

Standard Culture Procedures

USDA Method, Revision 1 (February 8, 1994): Each sample was combined with 9 volumes of prewarmed UVM (University of Vermont Medium) broth, stomached (Stomacher 400 or 3500, Tekman Co., Cincinnati, Ohio) for 2 min, and incubated for 20-24 h at 30 ± 1 °C. Following incubation, 0.1 mL pre-enrichment was transferred to 10 mL Fraser broth and incubated at 35 ± 1 °C for 48 h. At 24 and 48 h, enrichments were streaked to modified Oxford medium (MOX) for isolation. Suspect colonies were streaked for purification and identification on TSAYE plates.

USDA Method Revisions 2 (August 1999): The 20-24 h at 30 ± 1 °C pre-enrichment prepared for the USDA Revision 1 method was employed for the Revision 2 method. Following incubation, 10 microliters of enriched broth was streaked to modified Oxford medium (MOX) and incubated for 24 and 48 hr at 35°C. At 24 and 48 h, plates were read and suspect colonies were confirmed. An additional 0.1 mL of pre-enrichment was transferred to 10mL Fraser broth and incubated at 35 ± 1 °C for 48 h. At 24 and 48 h, blackened Fraser enrichments were streaked to MOX for isolation. Suspect colonies were streaked for purification on TSAYE plates and then confirmed.

PCR Procedure

Each test samples was combined with 9 volumes of pre-warmed Demi-Fraser broth, stomached for 2 min, and incubated for 22-24 h at 30 ± 1 °C. Following incubation, 0.1 mL pre-enrichment was transferred to 9.9 mL MOPS-BLEB and incubated at 30 ± 1 °C for 20 - 24 h.

The PCR method was followed according to manufacturer's instructions. Enrichments from suspect samples from the assay were streaked for purification on TSAYE plates and then confirmed.

ELISA Procedure

Each samples was enriched in pre-warmed bLEB with sodium pyruvate solution per BAM 8th Edition (1995). Sample mixture was stomached for 2 min and incubated for 48-50 h at 30°C. After incubation, 1-2 mL of each enrichment broth was transferred to a test tube and heated in boiling water bath at 95-100°C for 15 min. Tubes were cooled to ambient temperature and tested with the VIDAS LIS assay per manufacturer's instructions. Remaining unheated enrichments were stored at 2-8°C for confirmation of any *Listeria*-positive assay results.

Most Probable Number Calculation and Statistical Analysis

A 4 dilution, 3 tube Most Probable Number (MPN) table (Garthright, 1995) was used to determine MPN index values. Microsoft Excel (Redmond, Washington) functions were used

to calculate X^2 test statistics. The Yates correction factor for small numbers of samples was used in the calculation. Differences were considered significant at the p=0.05 level.

Results and Discussion

For the purpose of evaluating the efficacy of compositing for each method, the results for the 25-gram sample size were treated as the expected results. The compositing results at the 125-gram and 375-gram sample sizes were compared to the 25-gram results using the X^2 test. The compositing scheme was interpreted as equal to non-compositing result if the probability (p value) of the X^2 was greater than or equal to 0.05.

Frankfurters

All 324 test results for non-inoculated frankfurters samples were negative for *L. monocytogenes*. Negative results were independent of frankfurter lot, sample size or test method.

Test results were grouped into according to the *L. monocytogenes* counts for the low, medium, and high inoculum levels. Grouping was based upon the MPN results for each inoculated set of samples. The mean MPN results for the low, medium, and high inoculum samples were 0.18, 0.95 and 10.13 colony forming units per enrichment (Table 1). Using the USDA Revision 1 method for *L. monocytogenes,* compositing results were not equivalent to the 25-gram results for the 125-gram size at the low inoculum level or the 375-gram size at the high inoculum level. At the low inoculum level, the Poisson predicts 4 positive samples.

The observed number of positives at the 375-gram level was closer to the predicted value than the observed value. As such the calculated significant difference may not be real. When the results for the 3 levels were totaled, both compositing methods proved equivalent to the 25-gram method.

The results for the USDA Revision 2 method indicated equivalence between the 25-gram results and either the 125-gram or 375-gram results for the medium and high inoculum levels (Table 1). At the low inoculum level, equivalence was observed with the 375-gram size, but not the 125-gram size. The reason for this difference may reflect an higher than expected number of positives for the 25-gram sample size compared to the number of positives predicted for the mean inoculum level. Results totaled across all 3 inoculum levels supported compositing at both of the larger samples sizes.

With PCR (Table 1), results for both compositing sizes provided equivalent to the 25-gram results. For the ELISA method (Table 1), equivalence was observed at low inoculum level and for the 125-gram size at the medium inoculum level. However, when results were totaled, compositing was not significant ($p<0.01$) at either inoculum levels.

The results for the frankfurter samples support compositing 25-gram sample portions into single 125-gram and 375-gram analytical units for testing with the USDA or PCR methods. The ELISA method results for these samples did not support compositing.

Deli Turkey

All non-inoculated deli turkey samples tested negative for *L. monocytogenes*. Negative results were independent of sample log, sample size, and test method.

The mean inoculation levels for the low, medium and high samples were 0.12, 1.39, and 11.07 Listeria per enrichment (Table 2). Using the USDA Revision 1 method for *L. monocytogenes*, compositing results were not equivalent to the 25-gram results for the 375-gram size at the medium inoculum level. Approximately 24 negative results were expected at the this inoculum level. The results at the 25 and 125-sample sizes were close to the expected result, whereas, many fewer were observed at the 375-gram size. Thus, the predicted result agrees with the statistic that the compositing was not equivalent at the 375-gram size. When results for the 3 levels were combined, equivalence was only observed with the 125-gram size and not the 375-gram size.

The results for the USDA Revision 2 method indicated equivalence between the 25-gram results and either the 125-gram or 375-gram results for the low and high inoculum levels. The medium inoculum level, equivalence was only observed with the 125-gram size. Results totaled across all 3 inoculum levels supported compositing at both of the larger sample sizes.

With the PCR test method, results for both compositing sizes provided equivalent to the 25-gram results at all 3 inoculum levels and again when results were totaled across inoculum levels. For the ELISA method, equivalence was observed at only the low inoculum level at the 125-gram and 375-gram sample sizes.

The results for deli turkey samples support compositing 25-gram portions into single 125-gram analytical units for testing using the USDA (Revisions 1 and 2) or PCR methods, and 375-gram analytical units using USDA Revision 2 and PCR methods. The ELISA method results for these samples did not support compositing.

Deli Ham

Non-inoculated deli ham tested at all three samples sizes tested negative for *L. monocytogenes* by all four test methods.

The mean inoculation levels for the low, medium and high samples were 0.13, 1.41, and 8.57 Listeria per enrichment (Table 3). Using the USDA Revision 1 method, compositing results were not equivalent to the 25-gram results for the 125-gram size at the medium inoculum level and the 375-gram size at the both the medium and high inoculum levels. The expected number of positives at the medium and high inoculum levels based upon the MPN results were 16 and 24, respectively. When the results for the 3 levels were combined, compositing results were not equivalent at either the 125-gram or 375-gram size.

The results for the USDA Revision 2 method indicated compositing results were not equivalent to the 25-gram results for the 125-gram size at the medium inoculum level and the 375-gram size at the both the medium and high inoculum levels. When the results for the 3 levels were combined, compositing results were not equivalent at either the 125-gram or 375-gram size.

With PCR, compositing results were not equivalent to 25-gram results for the 375-gram size at the medium inoculum level. When the results for the 3 levels were combined, compositing results proved equivalent to the 25-gram results for the 125-gram size only.

For the ELISA method, compositing results indicated equivalence between the 25-gram results and either the 125-gram or 375-gram results for both the low and high inoculum levels.

Combining results for the 3 levels indicated compositing results were not equivalent at the 125-gram or 375-gram size.

The results for the deli ham samples support compositing 25-gram portions into single 125-gram analytical unit for testing using only the PCR method. None of the methods supported compositing at the 375-gram analytical unit.

Conclusion

Results tabulated for all 3 products are presented in Table 4. Compositing up to five 25-gram portions proved acceptable for analysis by the USDA culture methods and the PCR method. At the 375-gram size, compositing was marginally acceptable with the PCR method. Compositing is not recommended for the ELISA method.

References

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Table 1. Validation of compositing frankfurter samples for *L. monocytogenes* analysis.

Cells per enrichment				No.	USDA Rev-1		USDA Rev-2		PCR		ELISA	
Level	Log Mean	Range	AU	Samples	No. pos.	p	No. pos.	p	No. pos.	p	No. pos.	p
Low	0.18	0.09-0.375	25	25	10	-	10	-	6	-	6	-
			125	25	2	<0.01 **	2	<0.01 **	10	0.10	3	0.24
			375	25	5	0.07	6	0.15	8	0.48	2	0.10
Medium	0.95	0.525-2.325	25	21	10	-	12	-	7	-	9	-
			125	21	12	0.51	14	0.51	9	0.49	6	0.27
			375	21	11	0.83	12	0.83	6	0.82	4	0.05 *
High	10.13	5.25-37.5	25	27	26	-	26	-	25	-	27	-
			125	27	27	0.61	27	0.61	26	0.71	22	1.00
			375	27	22	<0.01 **	24	0.13	26	0.71	14	1.00
All	1.23	0.09-37.5	25	73	46	-	48	-	38	-	42	-
			125	73	41	0.28	43	0.27	45	0.13	31	0.01 *
			375	73	38	0.07	42	0.17	40	0.73	20	<0.01 **

Table 2. Validation of compositing deli turkey samples for *L. monocytogenes* analysis.

Cells per enrichment				No.	USDA Rev-1		USDA Rev-2		PCR		ELISA	
Level	Log Mean	Range	AU	Samples	No. pos.	p	No. pos.	p	No. pos.	p	No. pos.	p
Low	0.12	0.09-0.375	25	33	2	-	3	-	6	-	2	-
			125	33	4	0.27	4	0.76	4	0.50	1	0.72
			375	33	2	0.72	3	0.76	2	0.11	1	0.72
Medium	1.39	0.5-3.75	25	33	19	-	19	-	18	-	14	-
			125	33	20	0.86	20	0.86	17	0.86	5	<0.01 **
			375	33	9	<0.01 **	11	<0.01 **	15	0.38	6	<0.01 **
High	11.07	5.75-23.25	25	15	15	-	15	-	15	-	14	-
			125	15	15	1.00	15	1.00	14	1.00	10	<0.01 **
			375	15	14	1.00	14	1.00	15	1.00	7	<0.01 **
All	0.74	0.09-23.25	25	81	36	-	36	-	39	-	30	-
			125	81	39	0.58	39	0.74	35	0.44	16	<0.01 **
			375	81	25	0.02 *	28	0.06	32	0.15	14	<0.01 **

Table 3. Validation of compositing deli ham samples for *L. monocytogenes* analysis.

Cells per enrichment				No.	USDA Rev-1		USDA Rev-2		PCR		ELISA	
Level	Log Mean	Range	AU	Samples	No. pos.	p	No. pos.	p	No. pos.	p	No. pos.	p
Low	0.13	0.075-0.375	25	33	4	-	4	-	2	-	2	-
			125	33	6	0.42	6	0.42	3	0.72	2	0.72
			375	33	4	0.79	4	0.79	1	0.72	1	0.72
Medium	1.41	1.075-2.325	25	21	15	-	16	-	10	-	11	-
			125	21	4	<0.01 **	5	<0.01 **	12	0.51	4	<0.01 **
			375	21	10	0.03 *	10	<0.01 **	3	<0.01 **	4	<0.01 **
High	8.57	5.75-60	25	24	23	-	23	-	22	-	21	-
			125	24	22	0.61	22	0.61	23	0.71	18	0.12
			375	24	18	<0.01 **	19	<0.01 **	20	0.27	20	0.76
All	0.97	0.075-60	25	78	42	-	43	-	34	-	34	-
			125	78	32	0.03 *	33	0.03 *	38	0.42	24	0.03 *
			375	78	32	0.03 *	33	0.03 *	24	0.03 *	25	0.05

Table 4. Validation of compositing RTE meat and poultry samples for *L. monocytogenes* analysis.

Cells per enrichment				No.	USDA Rev-1		USDA Rev-2		PCR		ELISA	
Level	Log Mean	Range	AU	Samples	No. pos.	p	No. pos.	p	No. pos.	p	No. pos.	p
Low	0.14	0.075-0.375	25	91	16	-	17	-	14	-	10	-
			125	91	12	0.34	12	0.23	17	0.47	6	0.24
			375	91	11	0.22	13	0.35	11	0.47	4	0.07
Medium	1.25	0.5-3.75	25	75	44	-	47	-	35	-	34	-
			125	75	36	0.08	39	0.07	38	0.56	15	<0.01 **
			375	75	30	<0.01 **	33	<0.01 **	24	0.02 *	14	<0.01 **
High	9.68	5.25-60	25	66	64	-	64	-	62	-	62	-
			125	66	64	0.72	64	0.72	63	0.80	50	<0.01 **
			375	66	54	<0.01 **	57	<0.01 **	61	0.80	41	<0.01 **
All	0.95	0.075-60	25	232	124	-	127	-	111	-	106	-
			125	232	112	0.13	115	0.10	118	0.39	71	<0.01 **
			375	232	95	<0.01 **	103	<0.01 **	96	0.06	59	<0.01 **