APPLIED FOOD DIAGNOSTICS, INC.
18 INDUSTRIAL DRIVE
SUITE 22
BLOOMSBURG, PA 17815
(PHONE) 570-450-7995

Validation of the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay on Frozen/Cooked Shrimp after 24 Hours of Enrichment

An unpaired study was conducted to compare the performance of the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay against the FDA BAM Chapter 10 reference method to detect *Listeria* species and *Listeria monocytogenes* in frozen/cooked shrimp. Samples tested in this study were artificially inoculated at a low level expected to produce fractional results and a high level expected to produce all positive results. The results obtained were analyzed using the probability of detection (POD). The SIMUL-qPCR method demonstrated equivalent performance to the reference method for detecting both *Listeria* species and *monocytogenes*.

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INTRODUCTION

The purpose of this matrix study was to determine if *Listeria* species and *Listeria monocytogenes* could be detected in frozen/cooked shrimp by the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay after 24 hours of incubation. The assay had prevously been validated on this matrix for 30-36 hours of incubation. However, as multiple matrices were evaluated during that validation, the intention was to have the same incubation time for all matrices. It was known that for some of the products, the enrichment time could be shortened, but for uniformity and ease of use by the end user, 30-36 hours was the recommended enrichment time for all products and environmental samples. The design of inoculated challenge study are based on FSIS Guidance for Test Kit Manufacturers, Laboratories (10/15/10). The guidelines outline a comparison of the candidate method (in this case the SIMUL-qPCR Assay) to a known reference method (the FDA BAM Chapter 10 method) using a high inoculation set, a low inoculation set, and a set not inoculated. The results should show no statistically significant difference between the candidate and reference method.

The performances (measurement of specificity (false positive rate), inclusivity, exclusivity, repeatability, reproducibility, and ruggedness) of the SIMUL-qPCR Assays have been subjected to evaluation by an independent organization, and by following guidance provided by the AOAC.

Sample Preparation and Enrichment

Listeria monocytogenes 1/2a (BEI-NR-13229) was the strain used for the inoculation of the frozen/cooked shrimp. A bulk sample of frozen/cooked shrimp was inoculated and a high inoculation level (250 CFU/25g). A second bulk sample of frozen/cooked shrimp was inoculated at a low inoculation level (25CFU/25g). The level of inoculation was low enough to lead to fractional (5 to 15 positives out of 20 samples) results. Each bulk sample was mixed thoroughly so the inoculum was evenly distributed throughout the sample. After that time, the bulk sample that had the high concentration of inoculum was divided into five 25 gram samples per method. The bulk sample containing the lower (fractional) level of inoculum was divided into twenty 25 gram samples per method. Five 25 gram samples that did not contain any inoculum were also weighed out per method. Once inoculated and portioned out, the samples were held at -20°C for two weeks to allow for stabilization of the microorganisms.

For the candidate (SIMUL-qPCR) method, 225mL of *Listeria* Recovery and Enrichment Broth (LREB) was added to each sample and homogenized by hand, before being incubated at 30°C ± 1°C for 24 hours.

For the reference (FDA BAM Chapter 10) method, 225mL of Buffered *Listeria* Enrichment Broth (BLEB) was poured into each of the 25 gram samples and homogenized. All samples were incubated at 30°C ± 1°C for 4 hours. After the initial incubation, three filter sterilized selective agents were aseptically added to the BLEB to achieve the final concentrations of 10 mg/L acriflavin, 40mg/L cyclohexamide, and 50 mg/L sodium nalidixic. After mixing, the samples were put back in the 30°C incubator and incubated for 24-48 hours.

METHOD

For the SIMUL-qPCR method, after 24 hours of incubation, 5µL of each enriched sample was pipetted into 400µL of lysis buffer in labeled microcentrifuge tubes. The tubes were heated at 95°C ± 3°C for 10 minutes and then cooled at room temperature for 5 minutes. 20µL of the lysate was then transferred to individual PCR tubes, capped, and placed into the PCR machine. The PCR tubes were analyzed using the MyGo PCR machine using parameters outlined in the "PCR Set Up Guide".

All samples (regardless of the result obtained via the SIMUL-qPCR method) were confirmed using the FDA BAM Chapter 10 method. For the reference method, all samples were run through the enrichment process and then culturally confirmed following the FDA BAM Chapter 10 method.

RESULTS AND DISCUSSION

The probability of detection (POD) and the difference in POD (dPOD) values were calculated with 95% confidence intervals to evaluate the results between the SIMUL-qPCR method and the FDA BAM method. Table 1 shows the presumptive vs. confirmed results of the SIMUL-qPCR method. Table 2 shows the comparison of the confirmed results of candidate vs. reference method.

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Table 1: SIMUL-qPCR Presumptive vs. Confirmed SIMUL *Listeria* Results

				SIMUL-qPCR *Listeria* species and *monocytogenes* presumptive			SIMUL-qPCR *Listeria* species and *monocytogenes* confirmed				
Matrix	Strain	MPN[a]/test portion	N[b]	x[c]	POD_{CP}[d]	95% CI	x	POD_{CC}[e]	95% CI	$dPOD_{CP}$[f]	95% CI[g]
Frozen/ Cooked Shrimp 24 Hour	*L. monocytogenes* 1/2a (BEI NR-13229)	N/A[h]	5	0	0.00	0.00-0.43	0	0.00	0.00-0.43	0.00	-0.43 – 0.43
		1.27	20	15	0.75	0.53-0.89	17	0.85	0.64-0.95	-0.10	-0.34-0.15
		6.45	5	5	1.00	0.57-1.00	5	1.00	0.57-1.00	0.00	-0.43 – 0.43

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]POD_{CP} = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]Not applicable.

Table 2: SIMUL-qPCR Confirmed vs. FSIS MLG Confirmed *Listeria* Results

				SIMUL-qPCR *E Listeria* species and *monocytogenes* Confirmed			FDA BAM *Listeria* Confirmed				
Matrix	Strain	MPN[a]/test portion	N[b]	x[c]	POD_{CP}[d]	95% CI	x	POD_{CC}[e]	95% CI	$dPOD_{CP}$[f]	95% CI[g]
Frozen/ Cooked Shrimp 24 Hour	*L. monocytogenes* 1/2a (BEI NR-13229)	N/A[h]	5	0	0.00	0.00-0.43	0	0.00	0.00-0.43	0.00	-0.43 – 0.43
		1.27	20	15	0.75	0.53-0.89	13	0.65	0.43, 0.82	0.10	-0.18, 0.36
		6.45	5	5	1.00	0.57-1.00	5	1.00	0.57-1.00	0.00	-0.43 – 0.43

[a]MPN = Most Probable Number is based on the POD of reference method test portions using the LCF MPN calculator, with 95% confidence interval.
[b]N = Number of test potions.
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[e]POD_{CC} = Candidate method confirmed positive outcomes divided by the total number of trials.
[f]$dPOD_{CP}$ = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]Not applicable.

CONCLUSION

For *Listeria* species and *Listeria monocytogenes*, the results of the inoculation challenge show that there is no statistically significant difference between the candidate presumptive and the candidate confirmed results after 24 hours of enrichment. When comparing the candidate to the reference method, the SIMUL-qPCR method performed slightly better than the FDA BAM Chapter 10 method, with no significant difference in results. The inoculated challenge study demonstrated that *Listeria* species and *Listeria monocytogenes* were able to be detected down to fractional levels on frozen/cooked shrimp after 24 hours of incubation using the SIMUL-qPCR *Listeria* species and *monocytogenes* Assay.