Performance Tested Method℠ Study of the ANSR™ for Listeria Assay for Detection of Listeria spp. in a Variety of Foods

Neogen Corporation, Lansing, MI USA

Susan Alles, R. Lucas Gray, Oscar Caballero, Michael Wendorf, Mark Mozola and Jennifer Rice

ABSTRACT

ANSR for *Listeria* is a new isothermal nucleic acid amplification method based on the nicking enzyme amplification reaction (NEAR™) technology. The method received AOAC Performance Tested Method℠ status for detection of *Listeria* spp. in environmental samples in 2012 (PTM #101202). Here we describe results of a validation study covering seven food matrices: pasteurized milk, Mexican-style cheese, ice cream, smoked salmon, cantaloupe, guacamole and lettuce.

Foods were inoculated with individual *Listeria* spp. and stored under conditions typical for the product but also expected to produce inoculum stress. For each food tested, 40 or 50 test portions were prepared at a fractional level, designed to result in 25–75% positive test portions. Half of the test portions were tested by the ANSR method after enrichment in LESS broth for 16 hours and 24 hours at 36±1°C. The remaining portions were tested using the FDA/BAM reference culture procedure. Similarly, five test portions at a higher level and five uninoculated control test portions were prepared for testing by each method.

Results showed that there were no significant differences in method performance between the ANSR and reference methods for any of the foods as determined by probability of detection analysis. It is concluded that the ANSR method is a reliable procedure for rapid detection of *Listeria* spp. in a variety of sample types.

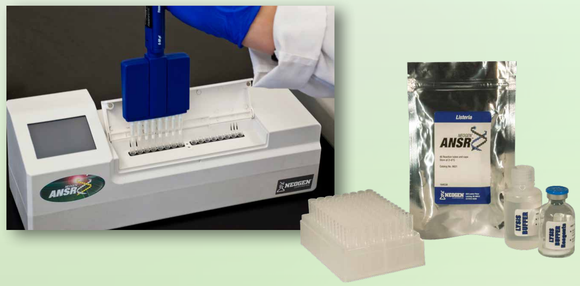
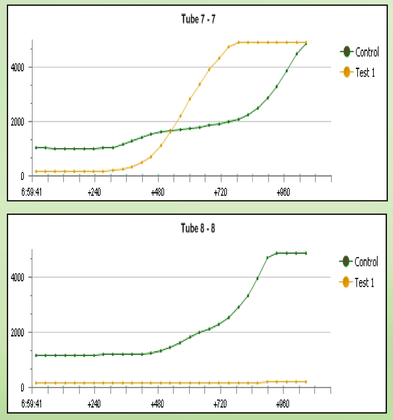


Figure 1. Typical fluorescence curves for the ANSR for *Listeria* assay.



MATERIALS AND METHODS

Inclusivity Testing

Inclusivity testing was performed using a panel of 51 strains of *Listeria* spp., representing the species *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*. Extra weight was given to *L. monocytogenes* as this is the only species associated with human foodborne listeriosis.

Isolates were taken from -80°C frozen storage and cultured on tryptic soy agar (TSA) for 18–24 hours at 36±1°C. Overnight cultures were grown from single-colony isolates and diluted. A tube containing 10 mL of LESS broth was inoculated with the diluted test culture at a level of 10–50 cfu/tube. The cultures were incubated for 16 hours at 36±1°C, diluted in LESS broth to a titer of approximately $1x10^4$ cfu/mL (about 100-fold above the limit of detection of the ANSR assay), and tested in the assay. Inclusivity strains were intermixed with exclusivity strains (see below), randomized, and blind coded prior to testing with the ANSR assay.

Exclusivity Testing

Exclusivity testing was performed using a panel of 30 strains representing 13 genera and 30 species of non-*listeria* Gram-positive bacteria.

Isolates were taken from -80°C frozen storage and cultured on TSA for 18–24 hours at 36±1°C, or otherwise as appropriate for certain strains. A single colony was transferred to a tube containing 10 mL of tryptic soy broth (TSB) and incubated for 16–24 h at 36±1°C, or cultured under alternative conditions when required. Since TSB and certain other enrichment media may produce excessive background fluorescence in the ANSR assay, cells from the overnight cultures were pelleted by centrifugation and resuspended in an equal volume of LESS broth (titers in the range 1 x 10^8–1 x 10^9 cfu/mL). Aliquots of the resuspended cells were tested in the ANSR assay without dilution.

MATERIALS AND METHODS

Food Testing

- **Test Strains** – *Listeria* spp. were obtained from the ATCC, CDC, and other documented sources. A different strain was used with each food product type. Inoculum strains are shown in **Table 3.**
- **Inoculum Preparation** – Test strains were grown as TSB overnight cultures and diluted in Butterfield's phosphate-buffered dilution water to the appropriate titer to use for inoculation.
- **Inoculation** – Solid products such as lettuce, smoked salmon, and cheese were chopped, crumbled or shredded prior to application of the inoculum. Ice cream was thawed before inoculation. Foods were inoculated in bulk and extensively mixed by stirring or hand-mixing.
- **Preparation of Test Portions** – Test portions of 25 g were weighed from bulk inoculated material. Three levels were prepared: uninoculated control (five for each method), fractional level (20 or 25 for each method), and high level (five for each method). All test portions were held at 2–8°C for 48–72 hours prior to analysis, except ice cream which was held at -20°C for a minimum of 14 days. Test portions were randomized and blind-coded prior to analysis by the ANSR and reference methods.
- **ANSR Method** – Test portions were enriched in 225 mL of pre-warmed LESS broth at 36±1°C for 16–24 hours. ANSR assays were performed as described [1] at both the 16-hour and 24-hour time points. All test portions were confirmed by plating to selective/differential agar media, regardless of ANSR assay result.
- **Reference Method** – All foods were tested in parallel following the U.S. FDA Bacteriological Analytical Manual procedure for detection of *Listeria* spp. [2]
- **Statistical Analysis** – A probability of detection (POD) model was used to determine if the difference in the number of positives produced by the ANSR and reference methods was statistically significant [3].

RESULTS

Inclusivity/Exclusivity Testing

Results are shown in **Tables 1** and **2**. All *Listeria* spp. produced positive results in the ANSR assay, and all non-*listeria* produced negative results.

Food Testing

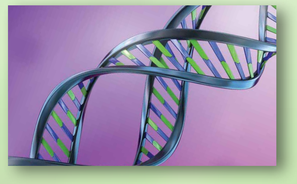
Results, including POD analysis, are shown in **Table 3**. There were no statistically significant differences in the number of positive results by the FDA/BAM reference method and the ANSR method at either 16 hours or 24 hours for any of the foods tested, with the exception of the independent laboratory trial with cantaloupe, where there were significantly more positives by the ANSR method at both 16 hours and 24 hours. In the case of pasteurized milk, there were notably more ANSR positives at 24 hours as compared to 16 hours, indicating that 24 hours of enrichment is required for this product type. For all other foods tested, the number of positive ANSR results was very similar at the two enrichment time points. All uninoculated control test portions produced negative results by the ANSR assay, with the exception of a single lettuce test portion at 24 hours, and a single guacamole test portion at 16 hours.

Table 1. Results of inclusivity testing for the ANSR for *Listeria* assay from LESS broth

Species	No. Strains	No. Positive
L. grayi	3	3
L. innocua	9	9
L. ivanovii	4	4
L. monocytogenes	26	26
L. seeligeri	4	4
L. welshimeri	5	5
TOTAL	**51**	**51**

Table 2. Results of exclusivity testing for the ANSR for *Listeria* assay

Species	No. Strains (Species)	No. Positive
Bacillus	3	0
Brochothrix	1	0
Enterococcus	4	0
Geobacillus	1	0
Gordonia	1	0
Kocuria	2	0
Kurthia	2	0
Lactobacillus	4	0
Lactococcus	1	0
Micrococcus	1	0
Rhodococcus	2	0
Staphylococcus	3	0
Streptococcus	5	0
TOTAL	**30**	**0**



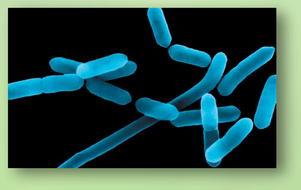


Table 3. Results of food testing by the ANSR for *Listeria* and reference culture methods

Sample	Serovar	Level (cfu/portion)	N[a]	ANSR 16 h[b]	ANSR 24 h[b]	FDA method	dPOD[c] 16 h[c]	dPOD[c] 24 h[c]
Pasteurized 2% milk	*L. welshimeri*	> 11	5	3	5	5	-0.40 (-0.76, 0.11)	0 (-0.43, 0.43)
		< 0.75	25	3	9	5	-0.08 (-0.29, 0.13)	0.16 (-0.09, 0.38)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Pasteurized 2% milk[d]	*L. welshimeri*	4.4	5	4	5	5	-0.20 (-0.62, 0.28)	0 (-0.43, 0.43)
		0.26	20	0	9	4	-0.20 (-0.42, 0.00)	0.25 (-0.04, 0.49)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Mexican style cheese	*L. mono.* 1/2b	23	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		0.89	20	8	9	6	0.10 (-0.18, 0.36)	0.15 (-0.14, 0.41)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Ice cream	*L. mono.* 1/2a	5.7	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		2.3	25	19	18	22	-0.12 (-0.33, 0.10)	-0.16 (-0.37, 0.07)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Smoked salmon	*L. mono.* 4b	30	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		0.89	20	6	8	7	-0.05 (-0.32, 0.23)	0.05 (-0.23, 0.32)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Lettuce	*L. mono.* 1/2a	37	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		1.5	20	13	14	14	-0.05 (-0.32, 0.23)	0 (-0.27, 0.27)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Cantaloupe	*L. mono.* 1/2b	> 275	5	4	5	5	-0.20 (-0.62, 0.28)	0 (-0.43, 0.43)
		5.8	20	11	13	13	-0.10 (-0.37, 0.19)	0 (-0.28, 0.28)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Cantaloupe[d]	*L. mono.* 1/2b	4.38	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		1.3	20	20	20	14	0.30 (0.08, 0.52)	0.30 (0.08, 0.52)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)
Guacamole	*L. innocua*	23	5	5	5	5	0 (-0.43, 0.43)	0 (-0.43, 0.43)
		0.89	20	5	7	4	0.05 (-0.21, 0.30)	0.15 (-0.12, 0.40)
	–	–	5	0	0	0	0 (-0.43, 0.43)	0 (-0.43, 0.43)

a Number of test portions.
b Number of test portions positive by the ANSR assay and confirmed by culture from ANSR-associated enrichments.
c Difference in POD values between ANSR confirmed and reference culture methods, with 95% confidence intervals. If confidence interval does not contain zero, then difference is significant.
d Trial performed by independent laboratory.

DISCUSSION AND CONCLUSIONS

Results of this study show that the ANSR for *Listeria* method can be used as an effective alternative to the FDA/BAM reference culture procedure for detection of *Listeria* spp. in a variety of food products. In testing pure cultures, inclusivity and exclusivity of the ANSR method were both 100%. There were no significant differences in the number of positive results produced by the ANSR and reference methods for any of the foods tested, with the single exception of the independent laboratory trial with cantaloupe, where there were significantly more positives by the ANSR method. Results showed that 24 hours of enrichment is indicated for pasteurized milk, whereas 16 hour and 24 hour ANSR results were similar for all other products tested.

It is recommended that a matrix extension be granted for PTM method 101202 to include pasteurized milk, ice cream, Mexican-style cheese, smoked salmon, lettuce, cantaloupe and guacamole. Validation of the ANSR method for use with processed meat products is in progress.

REFERENCES

[1] Wendorf, M., E. Feldpausch, L. Pinkava, K. Luplow, E. Hosking, P. Norton, P. Biswas, M. Mozola, & J. Rice. (2013) Validation of the ANSR *Listeria* method for detection of *Listeria* spp. in environmental samples. *J. AOAC Intl.*, in press

[2] US FDA (2011) Detection and enumeration of *Listeria monocytogenes*. *Bacteriological Analytical Manual*, chapter 10 http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm071400.htm

[3] AOAC International (2012) AOAC International Method Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, pre-publication draft

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