

Research Note

Increased Detection of *Listeria* Species and *Listeria monocytogenes* in Raw Beef, Using the Assurance GDS Molecular Detection System with Culture Isolation[†]

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ABSTRACT

Testing for *Listeria* is challenging because of its slow growth rate. Recently, we described a rapid *Listeria* culture isolation method. This method can be improved by utilizing a rapid molecular detection test such as the Assurance GDS tests for *Listeria* and *Listeria monocytogenes*. These two methods (culture isolation and Assurance GDS) use different enrichment strategies that may affect the number of *Listeria* and *L. monocytogenes* cells detected. Therefore, after first determining that the Assurance GDS accurately identified common *Listeria* strains isolated from raw beef, the two methods were compared by using paired ground beef samples ($n = 256$) that had been gathered from commercial sources. The agreement of the two methods was >76% for the culture and GDS *Listeria* method and >77% for the culture and GDS *L. monocytogenes* method. The molecular tests then were evaluated as endpoint tests in selected culture isolation enrichments. In this comparison, culture isolation and the molecular *Listeria* test agreed 100 and 84.4% of the time for *Listeria*-positive and -negative enrichments, respectively. An analysis of the discrepant samples in both experiments revealed that ~50% of the samples identified as positive by the molecular method but not by the culture method could be confirmed by subsequent testing, indicating that the immunomagnetic concentration step of the GDS test likely provides a more sensitive level of detection than does culture alone. The culture results were available 2 days earlier when the molecular tests were used instead of plating media. However, because the Assurance GDS *Listeria* test cannot distinguish *L. monocytogenes* from other *Listeria* species such as *Listeria innocua*, samples containing both species could not be distinguished.

Organisms of the *Listeria* genus are present throughout the environment. The species *Listeria monocytogenes* is an intracellular pathogen and the causative agent of epidemic and sporadic listeriosis (9, 18, 24). The consequences from contracting listeriosis can be particularly severe, resulting in spontaneous abortion in pregnant women and meningitis or septicemia in newborns (younger than 1 year), the elderly (>65 years of age), or immunocompromised individuals, with mortality rates approaching 30% (9). *L. monocytogenes* has 11 serovars, but three (1/2a, 1/2b, and 4b) are responsible for more than 95% of reported human listeriosis cases, and serovar 4b has been linked to major outbreaks (8, 17, 18).

L. monocytogenes is routinely isolated from numerous animal sources, including cattle (12, 20), and a clear relationship between food source and disease was established in 1981 when an outbreak of listeriosis was linked to con-

taminated coleslaw (9, 25). The presence of this pathogen in fresh beef that is to be cooked has been considered inconsequential because *Listeria* strains usually are considered a problem associated with ready-to-eat (RTE) foods. However, the producers of RTE products have begun to require *L. monocytogenes*-free materials from their suppliers. A wide range of *L. monocytogenes* contamination in retail ground beef products has been observed. Researchers reported that *L. monocytogenes* was present in 52% of 100 raw ground beef samples from retail markets in Canada (3) and in 3.5% of 512 ground beef samples from retail outlets in the state of Washington (23).

Testing for *Listeria* is challenging because of the slow growth rate of this pathogen compared with that of other pathogens. Although bacteria such as *Escherichia coli* and *Salmonella* have generation times of 20 to 30 min at 37°C, the generation time for *L. monocytogenes* is about 1 h at 37°C (1). Detection tests rely on an enrichment protocol that promotes growth of *Listeria* while suppressing the growth of competitive organisms. We previously reported a more time efficient method for the isolation of *Listeria* and *L. monocytogenes* that shortened the culture detection time from 7 days to 4 days (11). This method used the same sample for the isolation of *E. coli* O157:H7 and *Salmonella*.

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† Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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TABLE 1. Specificity of Assurance GDS test for *Listeria* species and *L. monocytogenes* isolated from beef

Species	Serovar	n	GDS test results ^a	
			<i>Listeria</i> spp.	<i>L. mono-</i> <i>cytogenes</i>
<i>Listeria monocytogenes</i>	1/2a	3	3	3
<i>L. monocytogenes</i>	1/2b	3	3	3
<i>L. monocytogenes</i>	1/2c	3	3	3
<i>L. monocytogenes</i>	4b	3	3	3
<i>L. monocytogenes</i>	4a/4c ^b	1	1	1
<i>L. innocua</i>		13	13	0
<i>L. ivanovii</i>		2	2	0
<i>L. seeligeri</i>		2	2	0
<i>L. welshimeri</i>		2	2	0
Untypeable <i>Listeria</i>		3	3	0
<i>Enterococcus</i> spp. ^c		5	0	0

^a Each isolate was incubated for 16 h in Demi-Fraser broth at 36°C and then used in each Assurance GDS test.

^b The serovar of this *L. monocytogenes* isolate could not be resolved between 4a and 4c.

^c The *Enterococcus* species used were originally identified as suspect *Listeria* based on colony phenotype as grown on *Listeria* CHROMAger.

Additional time may be saved when DNA amplification techniques are used (19) to detect *Listeria*, but the drawback to DNA-based assays is that the isolate is not available for characterization. A few of the rapid molecular tests that have been approved for detection of *Listeria* and *L. monocytogenes* are the TaqMan *L. monocytogenes* detection kit, iQ-Check, Qualicon BAX, Warnex Genevision, GeneQuence *Listeria*, and Assurance GDS (9, 14, 16). The objective of the present study was to determine the efficacy of adding a *Listeria* molecular detection test to our culture isolation method for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* to improve detection sensitivity and shorten the time needed for *Listeria* and *L. monocytogenes* detection to that for the other pathogens in the assay. The GDS Assurance assay was chosen because it incorporates an immunomagnetic separation (IMS) concentration step. We found IMS to be very useful for increasing the detection of *E. coli* O157:H7 and *Salmonella* (2). The resulting IMS products of the Assurance GDS tests also can be used immediately to obtain an isolate, without additional delays and culture steps.

MATERIALS AND METHODS

Experiment 1. The specificity of the Assurance GDS *Listeria* and *Listeria monocytogenes* kits (BioControl Systems Inc., Bellevue, WA) was determined for common *Listeria* species and *L. monocytogenes* isolated from raw beef and production environments. A panel of 40 U.S. Meat Animal Research Center (USMARC) isolates (Table 1) obtained in previous studies was used. These isolates included five *L. monocytogenes* serovars, four additional *Listeria* species, and three *Listeria* isolates that were not identifiable to species. A variety of *Enterococcus* species also were included; these species often have been confused with *Listeria* in the culture method because of similar colony phenotypes on *Listeria* CHROMAger (DRG International, Mountainside, NJ).

Experiment 2. A parallel comparison of the Assurance GDS "30 Hour" method to the USMARC *Listeria* isolation method (11) was performed to determine whether the performances of the two methods were comparable. This comparison utilized 256 samples of ground beef. Two 65-g portions of each sample were processed following either the USMARC protocol or the Assurance GDS protocol. All discrepancies in the results between the two methods were examined two ways. One, each Demi-Fraser or Fraser enrichment was held for 48 h at 4°C until culture results were known, then samples that produced discrepant results with the two methods were restreaked onto *Listeria* CHROMAger to isolate the *Listeria* and/or *L. monocytogenes*. Two, a 5- μ l portion of the Assurance GDS concentration reagent (IMS beads) was removed before the lysis step and held at 4°C in 100 μ l of tryptic soy broth (TSB). The IMS beads from discrepant enrichments were spread plated onto *Listeria* CHROMAger for isolation of any *Listeria* present.

Experiment 3. The Assurance GDS tests (for *Listeria* and *L. monocytogenes*) were evaluated as endpoint detection assays for the USMARC *Listeria* culture isolation method. Selected Fraser enrichments from the USMARC method ($n = 137$) were tested with the Assurance GDS kits to determine the correlation between kit results and culture results. The Fraser enrichments were selected from a large set of ground beef sample enrichments (separate from experiment 2) and were either presumptive negative, as indicated by no color change of the Fraser broth, or presumptive *Listeria* and/or *L. monocytogenes* positive, as indicated by the requisite color change. Discrepancies between the results of USMARC culture and Assurance GDS method were examined as described for experiment 2.

Samples. Samples were portions of ground beef sent to USMARC by 10 different commercial ground beef producers in the United States. After receipt, samples were maintained at -20°C until thawed for testing. To thaw, samples were held at 4°C for 16 to 40 h depending on the size of the sample (chubs required 40 h to thaw, whereas patties needed only 16 h to thaw).

***Listeria* and *L. monocytogenes* isolation and identification by culture method.** *Listeria* and *L. monocytogenes* were isolated as previously described using the USMARC universal TSB enrichment followed by a secondary Fraser broth selective enrichment (11). Suspect *Listeria* and *L. monocytogenes* colonies were visualized on *Listeria* CHROMAger, and these colonies were picked and inoculated into 2 \times yeast extract broth in 96-well deep-well blocks and allowed to grow overnight. The next day, the *Listeria* species and *L. monocytogenes* serovar (if applicable) were determined using a PCR method (5, 6).

***Listeria* and *L. monocytogenes* detection using Assurance GDS test kits.** Detection of *Listeria* and *L. monocytogenes* was performed according to the Assurance GDS test kit package insert. In experiment 3, an additional resuspension wash step was added to remove presumptive inhibitory compounds present in the Fraser broth used in the USMARC *Listeria* isolation protocol. This wash step consisted of retracting the PickPen tool tips and then carefully resuspending the captured IMS beads (concentration reagent) in 1 ml of phosphate-buffered saline containing 0.1% Tween 20. The PickPen tips were replaced with fresh tips, and the washed beads were recaptured as described in the package insert. A dip wash followed, and the beads were collected for use in the subsequent steps of the detection assay.

Statistics. Comparisons of frequencies of *Listeria* and/or *L. monocytogenes* identified by culture isolation versus Assurance

GDS detection tests were made using 2×2 contingency tables to determine *P* values. Two-tailed *P* values were calculated using Fisher's exact test from the GraphPad Prism Software (GraphPad Software, La Jolla, CA).

RESULTS AND DISCUSSION

In experiment 1, the Assurance GDS test kits accurately identified common *Listeria* species and various serovars of *L. monocytogenes* that we previously isolated from raw beef. During studies of beef processing environments and products, we have isolated a number of *Listeria* species (*L. innocua*, *L. seeligeri*, and *L. welshimeri*) and *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c, and 4b and the uncommon serovar 4a/4c. Our enrichment protocol for the isolation of *Listeria* involves the use of Fraser broth, which changes from gold to black in the presence of an esculin hydrolyzing organism such as *Listeria*. A Fraser broth that has turned color is considered a suspect positive result for *Listeria* until confirmation is achieved by isolation on *Listeria* CHROMAgar and subsequent PCR tests. On several occasions, suspect blue colonies were observed on *Listeria* CHROMAgar after streaking a presumptive-positive Fraser broth, but these colonies were not confirmed as *Listeria* by the PCR test. Further analysis has revealed that these blue colonies were various species of *Enterococcus*. Because these *Enterococcus* species can interfere with and delay culture results, they were included in this specificity assay to confirm that they did not also interfere with the molecular detection end point test. The test kits properly identified all *Listeria* species and *L. monocytogenes* (Table 1), including three unusual *Listeria* not previously identified beyond genus.

Experiment 2 was performed to determine whether the performance of the Assurance GDS method of IMS linked to molecular detection was comparable to that of the USMARC culture method for detecting *Listeria* and *L. monocytogenes* in 256 ground beef samples. The GDS tests detected more samples that were positive for *Listeria* and *L. monocytogenes* than did the culture method. The additional number of *Listeria*-positive samples detected was significantly different ($P = 0.0001$) but the additional number of *L. monocytogenes*-positive samples detected was not significantly different ($P = 0.0519$) from the number detected by culture isolation (Table 2). The GDS tests detected an additional 52 *Listeria* spp. and an additional 19 *L. monocytogenes* isolates in samples that were culture negative. However, the GDS tests also did not detect eight *Listeria* and five *L. monocytogenes* that were detected by the culture method. This experiment was anticipated to have a number of discrepancies such as those due to the effects of using IMS in one method and the effects of different enrichment strategies. The IMS step of the Assurance GDS test may have concentrated *Listeria* that were not detected by culture isolation. During development of the USMARC method, Guerini et al. (11) observed differences between *Listeria* isolation methodologies in regard to the media used. They found that Fraser broth with the full complement of supplements was the optimal enrichment medium for isolating *L. monocytogenes* from samples also being screened for *E.*

TABLE 2. Comparison of culture isolation method and molecular tests for detection of *Listeria* and *L. monocytogenes* from 256 ground beef samples^a

GDS result	Culture results, no. (%)	
<i>Listeria</i> spp.		
	Positive (<i>n</i> = 40)	Negative (<i>n</i> = 216)
Positive ^b (<i>n</i> = 84)	32 (80)	52 (24)
Negative (<i>n</i> = 172)	8 (20)	164 (76)
<i>L. monocytogenes</i>		
	Positive (<i>n</i> = 22)	Negative (<i>n</i> = 234)
Positive ^c (<i>n</i> = 36)	17 (77.3)	19 (8.1)
Negative (<i>n</i> = 220)	5 (22.7)	215 (91.9)

^a Each ground beef sample was divided into two portions and processed using the USMARC *Listeria* culture isolation method and the Assurance GDS test kit.

^b The results for detection of *Listeria* by the Assurance GDS test were significantly different ($P = 0.0001$) from those obtained by culture isolation.

^c The results for detection of *L. monocytogenes* by the Assurance GDS test were not significantly different ($P = 0.0519$) from those obtained by culture isolation.

coli O157:H7 and *Salmonella*. Others have reported similar observations about differences in *Listeria* isolation due to different enrichment strategies and media used (4, 7, 9, 22). Some researchers have suggested that to detect all possible positive samples, multiple *Listeria* enrichment protocols would be needed. This approach would be cumbersome and would consume samples, which often are a finite resource; therefore, the use of a single method that detects as many true *Listeria*-positive samples as possible and that can detect other pathogens is most desirable.

Despite the differences in the enrichment strategies, there was close agreement between the molecular test results and culture results in the parallel samples. Culture and GDS agreed 80 and 76% for the *Listeria*-positive and -negative enrichments, respectively, and 77.3 and 91.9% on the *L. monocytogenes*-positive and -negative enrichments, respectively. Others have described the BAX method as a molecular detection technique and compared its use with that of culture isolation in fish and chicken (15, 21). In these studies, variable results were obtained. When the BAX *Listeria* method was compared with culture isolation from chicken nuggets, a large number of false-negative results were obtained, and the efficiency of *Listeria* detection was low (71%) (21). In a different comparison of the BAX method and culture isolation of *Listeria* and *L. monocytogenes* in raw fish (15), the BAX *Listeria* methods had rates of sensitivity and specificity similar to those we observed in the present study.

Culture-positive GDS-negative samples were further analyzed by plating a portion of the IMS beads to *Listeria* CHROMAgar. One of eight *Listeria* spp. culture-positive GDS-negative samples and one of five *L. monocytogenes* culture-positive GDS-negative samples were scored as positive when plated on *Listeria* CHROMAgar. These results

TABLE 3. Efficacy of Assurance GDS as an endpoint tests compared with culture isolation of *Listeria* and *L. monocytogenes* in 137 ground beef samples^a

GDS result	Culture results, no. (%)	
<i>Listeria</i> spp.	Positive (n = 47)	Negative (n = 90)
Positive ^b (n = 61)	47 (100)	14 (15.6)
Negative (n = 76)	0	76 (84.4)
<i>L. monocytogenes</i>	Positive (n = 25)	Negative (n = 112)
Positive ^c (n = 37)	24 (96)	13 (9.5)
Negative (n = 100)	1 (4)	99 (90.1)

^a The same nonselective tryptic soy broth enrichment was used for both culture isolation and the GDS test. Each enrichment was then transferred to a *Listeria*-selective Fraser broth.

^b The results for detection of *Listeria* by the Assurance GDS test were not significantly different ($P = 0.1078$) from those obtained by culture isolation.

^c The results for detection of *L. monocytogenes* by the Assurance GDS test were not significantly different ($P = 0.1118$) from those obtained by culture isolation.

indicate that culturable *Listeria* were present in the IMS beads but were not detected by the molecular test, possibly because of low numbers of the target organism. The Demi-Fraser enrichments of culture-negative GDS-positive discrepant samples also were streaked for suspect growth on *Listeria* CHROMAgar. Half of the 52 culture-negative GDS-positive samples were found to contain *Listeria*, suggesting that those isolates identified by the GDS tests were culturable and viable and likely not detected due to the differences in the enrichment strategies used.

In experiment 3, we examined the efficacy of using the GDS tests as a molecular endpoint assay for the USMARC *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* culture isolation method to improve and shorten the time of detection for *Listeria* and *L. monocytogenes*. USMARC *Listeria* isolation culture enrichments that used the TSB to Fraser broth enrichment method were evaluated using both GDS tests and our culture isolation protocol (Table 3). During the first comparison using the GDS method with the USMARC Fraser broth enrichments, 13 of 64 samples (20%) gave a “no amplification” result, presumably because of the reaction of compounds in the Fraser broth secondary enrichment of the USMARC *Listeria* isolation method. The GDS tests use a manufacturer-supplied Demi-Fraser enrichment broth that does not contain supplements of ammonium iron(III) citrate, acriflavine, and nalidixic acid. These compounds may interfere with amplification and/or detection of DNA and likely were carried over into the reaction tubes during the IMS step. An additional wash step was added to the concentration (IMS) procedure to reduce possible contaminant crossover into the reaction tubes, thereby eliminating the no amplification results. This extra wash step decreased the proportion of samples with a no amplification result to less than 7%. A final set of 137 samples, all of

which were amplified by the molecular test, were used to evaluate the use of the GDS method as an endpoint assay (Table 3).

Results of experiment 3 revealed that detection of *Listeria* and *L. monocytogenes* by the GDS tests was not different from that obtained with the culture method ($P = 0.1078$ for *Listeria* and $P = 0.1118$ for *L. monocytogenes*). Culture isolation and GDS test agreed 100 and 84.4% on the *Listeria*-positive and -negative enrichments, respectively, and 96 and 90.1% on the *L. monocytogenes*-positive and -negative enrichments, respectively. The GDS tests detected 14 *Listeria* spp. and 13 *L. monocytogenes* isolates that were not detected by culture. The finding of an additional 14 positive samples in the group of 90 identified as culture negative was significant ($P = 0.0001$). This finding was likely an effect of the use of IMS to concentrate the *Listeria* and *L. monocytogenes* organisms before PCR detection, thereby giving the molecular tests more target as compared with the standard culturing methodology.

Follow-up plate cultures using portions of the IMS beads confirmed that this step enhanced *Listeria* and *L. monocytogenes* detection. In total, 8 of the 14 *Listeria* spp. and 1 of the 13 *L. monocytogenes* isolates detected by the GDS tests but not by the culture method were confirmed by direct plating of a portion of the IMS beads. Not all of the Assurance GDS positive results were confirmed. These could have been false-positive results or nonconfirmable positive results due to sample size or the selective medium. The direct plating of the IMS beads for confirmation utilized only a small portion (5 μ l) of the beads from the concentration step. If the *Listeria* and *L. monocytogenes* were present at low levels, then detection on *Listeria* CHROMAgar may not have been possible by directly plating only 5 μ l. Alternatively, many *Listeria* grow differently on selective agars (13), and the *Listeria* and *L. monocytogenes* isolates detected by the GDS tests may not have been culturable on *Listeria* CHROMAgar. The addition of other *Listeria*-selective agar such as Oxford, PALCAM (polymyxin, acriflavine, lithium chloride, ceftazidime, aesculin, and mannitol), or LMP (lithium chloride, phenylethanol, and moxalactam) might have brought further resolution to the question of whether the GDS tests detected nonculturable and/or nonviable *Listeria* species. However, because the protocol used limited the amount of remaining IMS beads to 5 μ l, it was not feasible to adequately distribute these across more than one plate of medium.

A single *L. monocytogenes* culture-positive enrichment sample (serovar 1/2a) was repeatedly negative with the molecular *L. monocytogenes* test but positive with the molecular *Listeria* spp. test. The *L. monocytogenes* isolate was phenotypically and biochemically confirmed to be *L. monocytogenes*. Further characterization of this *L. monocytogenes* isolate by testing directly in the GDS assays produced a positive reaction in both tests. It is not likely that this isolate represents a unique form that escaped detection by the *L. monocytogenes* molecular test because it reacted positively when tested alone in the GDS *L. monocytogenes* assay, and because the same IMS beads are used for both tests it probably does not lack the antigen recognized during

IMS. The *L. monocytogenes* present in this sample may have been present at a very low level and was not amplified in the *L. monocytogenes* assay but was detected in the *Listeria* spp. assay.

During these studies, we observed multiple instances of an individual sample that was positive by culture for both a *Listeria* species such as *L. innocua* and for *L. monocytogenes* and of an individual sample that contained more than one serovar of *L. monocytogenes*. This information is very useful when evaluating samples for gross levels of contamination; however, the GDS *Listeria* spp. assay does not distinguish *L. monocytogenes* from *L. innocua* or any other *Listeria* species. Both GDS test kits must be used to determine the prevalence of *Listeria* species that are not *L. monocytogenes*. Differences in the competitive fitness of *L. monocytogenes* serovars 1/2a and 4b in mixed cultures have been identified with the U.S. Food and Drug Administration enrichment protocol (10). A comparison of the *L. monocytogenes* serovars obtained by culture with those detected by the GDS assay for *L. monocytogenes* from the same samples revealed no differences in serovars detected. In experiment 3, *Listeria* enrichments were performed in Fraser broth rather than Demi-Fraser broth, and an additional IMS wash step was required to remove compounds that inhibit the PCR or interfere with PCR product detection. The Assurance GDS should be used with the proper Fraser broth supplied for its protocol because this broth does not interfere with PCR detection.

The established Food Safety and Inspection Service method for detection of *Listeria* and *L. monocytogenes* requires up to 8 days (26). The rapid enrichment strategy described by Guerini et al. (11) reduces detection time to 5 days and allows for the simultaneous isolation of other pathogens such as *E. coli* O157:H7 and *Salmonella* from the same sample. These organisms would not be detected if a Fraser broth (or Demi-Fraser broth) were the only enrichment strategy used. The Assurance GDS method when used alone provides results in 2 days and when used in conjunction with the USMARC *Listeria* culture method reduced detection time to 3 days, providing results in the same time it takes to detect the other pathogens. The GDS or another PCR-based rapid detection method such as the Qualicon BAX or Neogen GeneSequence *Listeria* tests also could be effective as an endpoint test following our culture enrichment if rapid results are required and isolates are not needed for characterization. Antibody-based enzyme-linked immunosorbent assay such as the VIDAS *Listeria*, Gene-Trak, and Assurance-EIA were not investigated here because these tests have been thoroughly evaluated and described elsewhere (14, 16).

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