Journal of Food Protection, Vol. 79, No. 1, 2016, Pages 138–143
doi:10.4315/0362-028X.JFP-15-122

Research Note

Assessment of *Listeria* sp. Interference Using a Molecular Assay To Detect *Listeria monocytogenes* in Food

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MS 15-122: Received 17 March 2015/Accepted 31 August 2015

ABSTRACT

Detection of *Listeria monocytogenes* in food is currently based on enrichment methods. When *L. monocytogenes* is present with other *Listeria* species in food, the species compete during the enrichment process. Overgrowth competition of the nonpathogenic *Listeria* species might result in false-negative results obtained with the current reference methods. This potential issue was noted when 50 food samples artificially spiked with *L. monocytogenes* were tested with a real-time PCR assay and Canada's current reference method, MFHPB-30. Eleven of the samples studied were from foods naturally contaminated with *Listeria* species other than those used for spiking. The real-time PCR assay detected *L. monocytogenes* in all 11 of these samples; however, only 6 of these samples were positive by the MFHPB-30 method. To determine whether *L. monocytogenes* detection can be affected by other species of the same genus due to competition, an *L. monocytogenes* strain and a *Listeria innocua* strain with a faster rate of growth in the enrichment broth were artificially coinoculated at different ratios into ground pork meat samples and cultured according to the MFHPB-30 method. *L. monocytogenes* was detected only by the MFHPB-30 method when *L. monocytogenes*/*L. innocua* ratios were 6.0 or higher. In contrast, using the same enrichments, the real-time PCR assay detected *L. monocytogenes* at ratios as low as 0.6. Taken together, these findings support the hypothesis that *L. monocytogenes* can be outcompeted by *L. innocua* during the MFHPB-30 enrichment phase. However, more reliable detection of *L. monocytogenes* in this situation can be achieved by a PCR-based method mainly because of its sensitivity.

According to most regulatory agencies, detection of one *Listeria monocytogenes* cell in 25 g of food requires enrichment steps to increase the number of target cells to a detectable level. Modifications of culture methods focus on enrichment processes enhancing first the recovery rate of injured *L. monocytogenes* and then selecting this species over the food background microbiota. A few methods are currently most often used *(12)*. Although the U.S. Food and Drug Administration method *(11)* originally developed to isolate *Listeria* spp. from dairy products, vegetables, and seafood includes incubation in only one type of broth, buffered *Listeria* enrichment broth (BLEB) plated at 24 and 48 h, the U.S. Department of Agriculture method *(1)* employs two enrichment steps and was developed to isolate *Listeria* from meat products. The European and international standard method, ISO 11290-1 *(28),* also employs two different broths, half-strength Fraser broth for a preenrichment culture and full-strength selective Fraser broth for a second enrichment culture. The current Canadian Microbiology Food Health Protection Branch method (MFHPB-30) for detection of *L. monocytogenes* in food is based on the method developed by Lovett *(16)* with further modifications *(20),* which essentially consists of a two-step enrichment as follows. After 24 and 48 h of incubation, an aliquot of the first *Listeria* enrichment broth (LEB) culture is transferred to a second modified Fraser broth (MFB) enrichment culture for up to 48 h, generating three broth cultures to examine (one from the first enrichment and two from the second enrichment) followed by streaking on a selective agar. Additional metabolic and biochemical testing of the cultures is then necessary to confirm the identity of isolated *Listeria* colonies in a labor-intensive and time-consuming protocol. Although a negative result can be confirmed in at least 4 days, the time for a positive result is usually 7 to 9 days from sample collection.

Previous observations in our laboratory using the MFHPB-30 method revealed that the broth cultures generated from food positive for *L. monocytogenes* were not all individually positive in about one-third of spiked samples (105 of 302 samples). A pilot study using the MFHPB-30 and a PCR-based method on 50 food samples spiked with *L. monocytogenes* was designed to determine and investigate the reason for this difference. Of the 50 samples analyzed, 11 were naturally contaminated with another *Listeria* species. When multiple species of *Listeria* are present in food, overgrowth of *L. monocytogenes* by a competitor might result in false-negative results when using the current reference method. In several studies, *L. innocua* has been found more frequently than *L. monocytogenes* in

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TABLE 1. L. monocytogenes *isolates used in this study*

Strain no.	Collection yr	Serotype
421	2009	1/2b
575	2009	4b
576	2009	1/2a
1499	2009	1/2b
2000	2009	1/2a
2272	2009	4b
2753	2009	1/2a
3057	2009	4b
4013	2009	1/2b
4499	2009	1/2b
4534	2009	1/2a
4849	2009	4b
8126	2005	4c
9331	2008	1/2a
9952	2008	4a
10455	2008	1/2a
11609	2008	1/2a

food *(17, 21, 27),* with homogeneous distribution according to the type of food *(23, 29)*. The reasons for this higher recovery rate remain unclear. One hypothesis is that *L. innocua* grows faster. Alternatively, selection during laboratory procedures could favor *L. innocua,* or this species could be naturally more prevalent. *Listeria* spp. may produce several types of inhibitors, such as monocin and bacteriocin, during the enrichment process *(13, 30, 31)*. However, Keys et al. *(14)* recently found that *L. monocytogenes* inhibition by *L. innocua* is not high enough by itself to determine whether this inhibitory activity can affect the recovery of *L. monocytogenes* from food sample enrichment cultures. Inhibitory interactions between these *Listeria* species had less of an impact in food samples when initial contamination levels were low, which is likely the case for most naturally contaminated foods *(6)*.

In recent years, multiple molecular methods for detection of *L. monocytogenes* have been developed *(2, 3, 15, 25, 26),* and real-time PCR has become a promising technique for rapid detection of pathogens in food *(4, 5, 24)*. The aim of the present study was to assess the level of interference by *L. innocua* in assays used to detect *L. monocytogenes* in ground pork meat, i.e., the MFHPB-30 method and a well-characterized real-time PCR assay *(25)*.

MATERIALS AND METHODS

Bacterial strains used for inoculation. Two reference *Listeria* strains were used in this study: *L. monocytogenes* (ATCC 19115) and *L. innocua* (ATCC 33090). Seventeen other *L. monocytogenes* isolates used to spike food samples were collected from clinical specimens (Table 1) isolated at Public Health Ontario (PHO; Toronto, Ontario, Canada). All *Listeria* strains were routinely grown on blood agar media and stored in 12.5% glycerol–Luria-Bertani broth at −80°C.

Artificial contamination of food with *L. monocytogenes* and identification of isolates (MFHPB-30 method). Fifty samples (L902 to L951) from four categories of food (Table 2) were tested. Because of the low prevalence of *L. monocytogenes* in food, the samples analyzed in this study were spiked with strains previously isolated at PHO. To estimate the number of bacteria for spiking, the optical density at 600 nm (OD_{600}) of overnight cultures was measured, and the number of bacteria was estimated by extrapolation to a standard curve generated from known levels of cells. The spiking level was verified by plating dilutions and counting colonies on Trypticase soy agar plates. Target spiking levels of 1 to 5 CFU of the bacteria was added to 50 g of food, and samples were stored at 4 or −20°C (frozen dairy product) for 48 h to simulate domestic storage. After incubation, 450 ml of LEB was added, and the samples were blended for 2 min in a BagMixer (Interscience, Rockland, MA) and then incubated at 30°C for 24 to 48 h according to the MFHPB-30 method *(20)*. Dilutions (1:100, vol/vol) of the LEB in 10 ml of MFB containing ferric ammonium citrate were incubated at 35°C for 24 to 48 h. All *Listeria* species hydrolyze aesculin to aesculetin. Aesculetin reacts with ferric ions, resulting in a color change in the culture from yellow to black (MFB cultures were checked at 24 and 48 h and plated only when aesculin hydrolysis was evident) *(20)*. After 48 h of incubation of the first enrichment culture (LEB 48h) and then two second enrichment cultures in MFB (one inoculated with the 24-h first enrichment culture [L24-M black] and the other inoculated with the 48-h second enrichment culture [L48-M black]), the cultures were plated on selective Oxford and PALCAM agars for *Listeria* isolation. Broth cultures were stored at −20°C. Presumptive *Listeria* colonies were characterized based on results of biochemical and metabolic tests. Motility; hemolysis; fermentation of mannitol, rhamnose, and xylose; Gram staining; and catalase analysis were employed to identify *Listeria* to the species level *(20). L. monocytogenes* was identified following this procedure, and the rest of the species were recorded as *Listeria* spp. for clarity. The food sample was considered positive when at least one colony was found in any of the samples tested *(20)*.

DNA extraction and *L. monocytogenes* real-time PCR detection from enrichment cultures. Broth cultures (750 μl) generated with the MFHPB-30 method were filtered through PrepSEQ Rapid Spin Sample Preparation Kit Tubes (Applied Biosystems, Foster City, CA) to remove food particles. After centrifugation, the pellet was resuspended in 25 μl of nuclease-free water and heated at 95°C for 15 min to obtain crude DNA. DNA samples were stored at −20°C until used. A real-time PCR targeting the *hly* gene from *L. monocytogenes* described previously *(25)* was used with DNA extracted from cultured food samples. DNA (1 μl) was added to PCR Environmental Master Mix 1× (Applied Biosystems) with a final volume of 20 μl, and the PCR was run in a 7900HT system (Applied Biosystems). PCR conditions were slightly modified: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 67°C. As a positive control, 15 ng of *L. monocytogenes* (ATCC 19115) DNA extracted with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) was used. In addition, 5 pg of the subcloned *gfp* gene was routinely used as internal amplification control *(8)*. No inhibition (partial or total) was observed for the samples tested in this study. Negative controls spiked with low levels (1 to 5 CFU) of *Escherichia coli* (ATCC 25922) were included for food samples in each category. *L. monocytogenes* was not detected in the negative controls by either method. The real-time PCR assay detected *L. monocytogenes* in 39 of 50 spiked samples. Because improving the DNA extraction efficiency can increase detection with the real-time PCR assay, DNA from all 11 negative samples was extracted using the QIAamp DNA Mini Kit according to manufacturer's instructions and resuspended in 30 μl of elution buffer. DNA (1 μl) extracted in

TABLE 2. *Characterization of 50 food samples artificially contaminated with* L. monocytogenes *strains*

Category	Inoculation level (CFU)	Strain no(s).	No. of samples		
			Inoculated	Positive by MFHPB-30	Positive by real-time PCR
Meats					
Cured	2–4	2753, 3057, 421	3	3	3
Fermented	2–4	ATCC 19115, 10455, 4534	3	2	2
Miscellaneous	4	2272	2	2	2
Pâté	1	4499	1	1	1
Raw	2–5	421, 575, 1499, 11609	4	3	4
Fish and seafood					
Raw	1–5	421, 576, 2000, ATCC 19115, 10455, 4499, 4849	7	6	7
Salted	2–4	576, 1499, 8126	3	2	2
Smoked	2–5	575, ATCC 19115, 4534	3	0	3
Vegetables					
Coleslaw mixes	3	4849, 8126	2	1	2
Salads	2–5	421, 575, 576, 4534, 9952	5	3	5
Sprouts	2–4	2753, 4499, 9331	3	3	3
Miscellaneous	2–3	ATCC 19115, 4013	2	1	2
Dairy					
Cultured, fermented	2–4	3057, 2000, 11609, 421, 4849, 8126, 9952	7	6	7
Frozen	2	1499	1	1	1
Raw	2–3	2000, 4013, ATCC 19115, 9331	4	4	4
Total	1–5		50	38	48

this way was tested in a 20-μl final volume with the real-time PCR assay as described above.

Screening for *L. monocytogenes* using real-time PCR assay. All samples positive for *L. monocytogenes* by the PCR-based method but negative according to the MFHPB-30 method were further screened by a culture method. L24-M and L48-M broth cultures were plated on Oxford and PALCAM agar, and presumptive *Listeria* colonies were streaked in Trypticase soy agar plates for identification. Equivalent numbers of individual colonies were analyzed by real-time PCR *(25),* pooling four colonies per PCR tube in with the internal amplification control. Individual colonies were isolated by real-time PCR from positives pooled samples. Colonies that were positive by PCR assay were confirmed with MFHPB-30 metabolic and biochemical tests *(20)*.

Growth rate determination. *L. monocytogenes* ATCC 19115 and *L. innocua* ATCC 33090 were cultured overnight, and the OD_{600} was measured. The bacterial level was adjusted to 10^8 cells per ml in 6 ml of LEB ($t = 0$), and cultures were incubated at 30°C with shaking in aerobic culture tubes. Generation time was determined by measuring the OD_{600} of each culture every hour. Least squares linear regression of log-transformed absorbance values were used. Results are reported as means ± standard deviations determined from three independent experiments.

***L. monocytogenes* and *L. innocua* coinoculation experiments.** *L. monocytogenes* ATCC 19115 and *L. innocua* ATCC 33090 were used in the coinoculation experiments. Only ground pork was used for this experiment because the usual gram-negative bacteria in this type of food provides a complex background. Ground pork samples were spiked with *L. innocua* (5, 13, and 22 CFU) and *L. monocytogenes* (3, 15, 30, 60, 150, and 300 CFU) and then analyzed according to the MFHPB-30 method. DNA was extracted from L24-M and L48-M cultures using the QIAamp DNA Mini Kit according to the manufacturer's instructions and analyzed with the real-time PCR method as described above. Control samples spiked with only *L. monocytogenes* were positive by both the real-time PCR and MFHPB-30 methods. Neither method detected *L. monocytogenes* in nonspiked samples (negative control) or in samples spiked with only *L. innocua*.

Statistics. Results of the MFHPB-30 and PCR-based methods used to detect *L. monocytogenes* in the presence of other *Listeria* species were evaluated using Fisher's exact test *(22)*.

RESULTS

Detection of *Listeria* in artificially contaminated food. When 50 food samples spiked with *L. monocytogenes* were analyzed, the MFHPB-30 method detected *L. monocytogenes* in 76% (38 of 50) of the samples, results similar to those previously reported *(9, 18)*. Hayes et al. *(9)* reported a correlation between false-negative results and low levels of *L. monocytogenes* contamination using selective enrichment methods. The PCR-based assay detected *L. monocytogenes* in 48 of 50 spiked samples (96%; $P = 0.004$; Table 2). An overview of these results is shown in Table 3. Only 31 of 50 samples were positive for *L. monocytogenes* in the three broth cultures generated by MFHPB-30 method. The remaining 19 samples (38%) were negative for *L. monocytogenes* in at least one of the three broths, thus confirming previous unpublished results from our laboratory. When the methods were compared, the results for the primary enrichment (LEB 48h) reflected the lower detection rate of the PCR-based method at this stage (25 of 50 samples) compared with the MFHPB-30 method (31 of 50 samples).

TABLE 3. L. monocytogenes *detection in broth cultures of 50 spiked food samples by MFHPB-30 and PCR-based methods*

Culture(s)[a]	No. of samples	
	Positive by MFHPB-30	Positive by real-time PCR
LEB 48h + L24-M + L48-M	31	24
L24-M + L48-M	32	39
LEB 48h	34	25
L24-M	33	42
L48-M	36	44

[a] LEB 48h, 48 h of incubation in the first enrichment step (LEB); L24-M, inoculum from first enrichment culture transferred after 24 h of incubation to the second enrichment culture medium (MFB); L48-M, inoculum from first enrichment culture transferred after 48 h of incubation to the second enrichment culture medium (MFB).

However, when the results of only the second enrichment cultures were analyzed (L24-M and L48-M), the PCR-based method was more sensitive, confirming recent observations *(4)*.

L. monocytogenes was detected using the real-time PCR screening method and subsequently isolated from three of four aesculetin-negative samples (L914, L922, and L949) and confirmed by the MFHPB-30 biochemical and metabolic tests (Table 4). Sample L915 was aesculetin negative in the L24-M culture but aesculetin positive in the L48-M culture, which allowed isolation of *L. monocytogenes* and another *Listeria* sp. by the MFHPB-30 method. However, the PCR assay detected *L. monocytogenes* in both the L24-M and L48-M broth cultures. Further investigation is necessary to establish the reason for the delay in aesculetin production in this group of samples that tested positive by the real-time PCR assay. All of the aesculetin-negative samples (L914, L915, L922, L949, and L951) belonged to the salted or smoked subgroups of the fish and seafood group (Table 4).

According to the results of the 50 food samples spiked with *L. monocytogenes,* the PCR-based method detected *L. monocytogenes* in 10 samples (L902, L904, L905, L914, L917, L922, L926, L930, L949, and L950) that were negative by the MFHPB-30 method (Table 4). A pooled-colony screening test was used to identify presumptive *L. monocytogenes* colonies recovered from these 10 PCR-only positive samples, and isolates were confirmed according to MFHPB-30 biochemical and metabolic tests. Samples L902, L904, L917, L926, and L950 contained another *Listeria* species, according to the MFHPB-30 method. However, the PCR-based method was able to detect *L. monocytogenes* in the 11 samples naturally contaminated with another *Listeria* species ($P = 0.272$), whereas the MFHPB-30 method detected *L. monocytogenes* in only 6 samples ($P = 0.073$), suggesting possible interference of *Listeria* species in the detection of *L. monocytogenes* when using the MFHPB-30 method.

TABLE 4. *Subset of the 50 spiked food samples, including samples naturally contaminated with* Listeria *spp. and samples negative for aesculetin production*

Sample	Inoculation level (CFU)	Type of food	Assay results[a]									
			Real-time PCR[b]					MFHPB-30[c]				
			LEB 48h (C_T)	L24-M (C_T)	L48-M (C_T)	Reported as:	Aesculetin	LEB 48h	L24-M	L48-M	Reported as:	No. of *Listeria* spp. present
L902	2	Mushrooms	–	37	35	Pos	Pos	–	–	–	Neg	1
L904	5	Mixed greens	–	37	28	Pos	Pos	–	–	–	Neg	2
L905	4	Broccoli	–	39	–	Pos	Pos	AT	AT	NG	Neg	
L913	5	Fresh salmon	–	40	37	Pos	Pos	+[d]	–	AT	Pos	1
L914	5	Smoked bloaters	–	27	36	Pos	Neg	AT	NP	NP	Neg	
L915	4	Salted pollock	37	24	21	Pos	Neg/Pos	+	NP	+	Pos	1
L917	3	Soft cheese	–	38	–	Pos	Pos	–	–	AT	Neg	2
L922	2	Smoked herring	–	37	–	Pos	Neg	AT	NP	NP	Neg	
L926	2	Ground pork	34	29	28	Pos	Pos	–	–	–	Neg	1
L928	2	Sprouts	–	–	36	Pos	Pos	+	+[d]	+	Pos	1
L930	3	Coleslaw carrots	–	–	38	Pos	Pos	NG	NG	NG	Neg	
L932	2	Bean sprouts	–	33	34	Pos	Pos	+	+	+	Pos	1
L944	4	Ground lamb	–	–	26	Pos	Pos	–	–	+	Pos	3
L946	4	Octopus	28	28	23	Pos	Pos	+	+	+	Pos	1
L949	4	Smoked herring	–	32	37	Pos	Neg	NG	NP	NP	Neg	
L950	3	Fresh smelts	–	–	35	Pos	Pos	–	–	–	Neg	1
L951	3	Salt cod bits	–	–	–	Neg	Neg	AT	NP	NP	Neg	

[a] Pos, positive for *L. monocytogenes* presence; Neg, negative for *L. monocytogenes* presence; +, *L. monocytogenes* detected; –, *L. monocytogenes* not detected.
[b] C_T, cycle threshold value.
[c] NG, sample plated, colonies did not grow; AT, atypical colonies (not *Listeria* spp.); NP, sample not plated (MFB negative).
[d] Only one colony isolated was identified as *L. monocytogenes*.

TABLE 5. *Sensitivity of* L. monocytogenes *(ATCC 19115) detection in ground pork samples in the presence of* L. innocua *(ATCC 33090)*[a]

Ratio (no. of *L. monocytogenes*/*L. innocua* cells)	Real-time PCR results			MFHPB-30 results			
	Reported as:	L24-M	L48-M	Reported as:	LEB 48h	L24-M	L48-M
0.2 (3/13)	Neg	−	−	Neg	−	−	−
0.6 (3/5)	Pos	+	−	Neg	−	−	−
0.7 (15/22)	Pos	+	+	Neg	−	−	−
1.4 (30/22)	Pos	+	+	Neg	−	−	−
2.3 (30/13)	Pos	+	+	Neg	−	−	−
2.7 (60/22)	Pos	+	+	Neg	−	−	−
6.0 (30/5)	Pos	+	+	Pos	+	−	−
6.8 (150/22)	Pos	+	+	Pos	+	−	−
13.6 (300/22)	Pos	+	+	Pos	+	−	−

[a] Pos, positive for *L. monocytogenes* presence; Neg, negative for *L. monocytogenes* presence; +, *L. monocytogenes* detected; −, *L. monocytogenes* not detected.

Assessment of *L. innocua* interference with *L. monocytogenes* detection in pork meat. To confirm that the presence of other *Listeria* species affected the detection of *L. monocytogenes* in food products, one *L. innocua* strain with a generation time of 45.9 ± 13.0 min and one *L. monocytogenes* strain with a slower generation time (244.6 ± 83 min) for the first enrichment broth were selected and then coinoculated into food to favor the outgrowth of *L. monocytogenes* during the enrichment process. Ground pork samples were then spiked with different ratios of *L. monocytogenes*/*L. innocua* and analyzed by MFHPB-30 and real-time PCR methods (Table 5). When samples were spiked at an *L. monocytogenes*/*L. innocua* ratio as low as 0.7 (15/22 CFU, respectively), the real-time PCR assay consistently detected the presence of *L. monocytogenes* in L24-M and L48-M broth cultures. When the ratio of *L. monocytogenes*/*L. innocua* was lower at 0.6 (3/5 CFU, respectively), *L. monocytogenes* was detected by PCR only in the L24-M culture. The MFHPB-30 method detected *L. monocytogenes* when the ratio of *L. monocytogenes*/*L. innocua* was 6 or higher and only in cultures with the shorter incubation time (LEB 48h).

DISCUSSION

Culture conditions favorable for *L. monocytogenes* growth are also favorable for growth of other *Listeria* species *(10)*. In naturally contaminated food, the incidence of multiple *Listeria* species has been frequently reported *(23, 27, 29)*. Eleven of 50 (22%) food samples analyzed in this study were naturally contaminated with 15 *Listeria* spp. in addition to the spiked species. Four *Listeria* spp. were found in two samples in the meat category. Four and five *Listeria* spp. were found in four samples belonging to the fish-seafood and vegetables categories, respectively. Only two *Listeria* spp. were found in one dairy product. The low number of *Listeria* species found in only one cheese sample in this category possibly reflects the better manufacturing practices for this type of food. The analysis of the 50 spiked samples in which the presence of *Listeria* spp. was indicated suggested that these other *Listeria* species may interfere with the detection of *L. monocytogenes* using the MFHPB-30 reference method.

When *L. innocua* was favored to outcompete *L. monocytogenes* during the enrichment phase, the PCR assay was more sensitive than the reference method (Table 5). The MFHPB-30 method was able to detect *L. monocytogenes* in only the first enrichment culture (LEB 48h) at inoculation ratios of 6.0, but only *L. innocua* strains were isolated from later enrichment cultures (L24-M and L48-M). However, the PCR-based method detected *L. monocytogenes* at the second enrichment stage at inoculation ratios as low as 0.6, indicating under these conditions a higher sensitivity than the reference method. Thus, *L. innocua* strains may compete for nutrients and eventually outgrow *L. monocytogenes* during the enrichment phase, as was previously suggested *(7)*. The superior sensitivity of the PCR assay when *L. monocytogenes* is at low levels as a result of being outcompeted by another *Listeria* species could have a significant impact in food testing. The routine use of *L. monocytogenes* differential agar could be useful for detecting *L. monocytogenes* that has been outcompeted in samples where multiple *Listeria* species are present. However, the use of Oxoid chromogenic *Listeria* agar with BLEB cultures *(14)* and agar *Listeria* according to Ottaviani and Agosti with Fraser broth *(19)* failed to detect *L. monocytogenes* when the *L. monocytogenes*/*L. innocua* ratios were 1 or lower.

The results presented in this article suggest that a combination of early culture times and more sensitive techniques, such as the real-time PCR, are better options for detecting *L. monocytogenes* in the presence of other *Listeria* spp. before the *L. monocytogenes* becomes undetectable, as was previously suggested *(5, 7, 14, 19, 32)*. By combining the enrichment protocol described in the MFHPB-30 method with a real-time PCR assay, it is possible to significantly reduce the time of detection.

ACKNOWLEDGMENTS

We are grateful to Vanessa Allen for helpful discussions and to the Environmental Microbiology section at PHO-L, in particular Mariana Shafer, Erna Follmi-Lieder, and Jennifer Sohar, who provided technical support and assistance. We also thank Gabrielle Gaedecke (PHO-L library) for her diligence in response to each of our requests. This work was supported by internal funding.

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