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Prevalence and Level of *Listeria monocytogenes* in Ice Cream Linked to a Listeriosis Outbreak in the United States

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ABSTRACT

A most-probable-number (MPN) method was used to enumerate *Listeria monocytogenes* in 2,320 commercial ice cream scoops manufactured on a production line that was implicated in a 2015 listeriosis outbreak in the United States. The analyzed samples were collected from seven lots produced in November 2014, December 2014, January 2015, and March 2015. *L. monocytogenes* was detected in 99% (2,307 of 2,320) of the tested samples (lower limit of detection, 0.03 MPN/g), 92% of which were contaminated at <20 MPN/g. The levels of *L. monocytogenes* in these samples had a geometric mean per lot of 0.15 to 7.1 MPN/g. The prevalence and enumeration data from an unprecedented large number of naturally contaminated ice cream products linked to a listeriosis outbreak provided a unique data set for further understanding the risk associated with *L. monocytogenes* contamination for highly susceptible populations.

Key words: Enumeration; Ice cream; Listeriosis outbreak

Listeria monocytogenes is a facultative intracellular foodborne pathogen that can survive and persist in a variety of foods and food processing environments *(10)*. Various ready-to-eat foods contaminated with *L. monocytogenes* have been linked to outbreaks and sporadic human illnesses *(1, 6, 10)*. In February 2015, routine surveillance revealed *L. monocytogenes* in two ice cream novelty products, a single serving ice cream bar and an ice cream sandwich, and later in a third product, individually packaged scoops of vanilla flavored ice cream, all from the same brand *(2)*. All three products came from the same production line (production line A). In March 2015, listeriosis infections in five elderly patients were reported from a hospital (hospital X) *(2)*, which served milkshakes prepared from contaminated ice cream scoops, and these infections triggered an outbreak investigation *(2)*. DNA fingerprinting by pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS) of *L. monocytogenes* isolates from these patients and from various ice cream products linked four of the five patients (illness onset was January 2014 to January 2015) to contaminated ice cream scoops made on production line A *(2)*. A fifth patient from hospital X was included in the initial epidemiologic investigation by the Centers for Disease Control and Prevention based on the food history. However, the isolates recovered from a blood culture from this patient did not match the isolates from ice cream recovered by PFGE or WGS; thus, this patient might not have been part of this outbreak *(2)*. The investigation and food analysis led to multiple recalls of these ice cream products since mid-March 2015 *(2)*. The four listeriosis case patients from hospital X had a median age of 78 years and were infected with *L. monocytogenes* while hospitalized for other medical conditions (K.K., personal communication).

The prevalence and level of *L. monocytogenes* in food samples associated with a listeriosis outbreak provide a unique data set for further understanding the risk associated with *L. monocytogenes* contamination. An understanding of this risk is particularly relevant for products that generally do not support *L. monocytogenes* growth, e.g., ice cream. *L. monocytogenes* populations in ice cream can remain stable for an extended period of time at freezing temperatures *(5, 7, 13)*; therefore, the *L. monocytogenes* levels in ice cream at the time of laboratory analysis should be very similar to those at the time of consumer purchase. The prevalence and level of *L. monocytogenes* from contaminated samples could help researchers estimate consumer exposure to *L. monocytogenes* from the incriminated ice cream products. Here, we

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report the results of the simultaneous detection and enumeration of *L. monocytogenes* in 2,320 individually packaged scoop samples of vanilla-flavored ice cream produced on production line A, which was implicated in the 2015 listeriosis outbreak.

MATERIALS AND METHODS

Ice cream products manufactured on production line A. Scoops of ice cream from seven lots were made available by the manufacturer. These seven lots, each of which represented 1 day of production by the implicated production line A, were manufactured from November 2014 to March 2015 (Table 1). The production dates partially overlapped with the illness onset dates of the four case patients (January 2014 to January 2015), although information on the exact lots of ice cream consumed by the case patients was not available. All samples were produced before the recalls were issued *(2),* and the samples with production dates between November 2014 and January 2015 were produced before the initial finding of *L. monocytogenes* from ice cream produced on production line A *(2)*. Three to 53 boxes were made available from each lot; each box contained 60 individually wrapped scoops in 3-oz (80- to 85-g) portions. All ice cream samples had been stored at −20 to −30°C. Ten individually wrapped scoops from each box were randomly selected for *L. monocytogenes* analysis. For one lot from which only three boxes were available, 20 samples were randomly selected from each box (Table 1). Within the same lot, the boxes of ice cream reflected different packing units and were not associated with any changes in production. The water activity and pH were measured for three randomly selected scoops per lot using a dew point water activity meter (AquaLab 4TE, Decagon Devices, Pullman, WA) and a pH meter (Orion Star A211, ThermoScientific, Waltham, MA).

Simultaneous *L. monocytogenes* detection and enumeration. Most-probable-number (MPN) analysis was used to detect and enumerate *L. monocytogenes*. Each individually packaged sample was aseptically opened, and the contents were transferred to sterile Whirl-Pak stomacher bags (Nasco, Fort Atkinson, WI). The ice cream was then left at room temperature for 20 to 40 min until fully melted. Each sample was then homogenized at high speed for 1 min in a Stomacher 400 circulator (Seward, Worthington, UK), and 40 g was collected and serially diluted (1:10) in buffered *Listeria* enrichment broth (BLEB; CM0897, ThermoScientific). MPN tubes were then prepared and incubated at 30°C. Selective supplements (10 mg/liter acriflavin hydrochloride, 40 mg/liter nalidixic acid, and 50 mg/liter cycloheximide; SR0149, Oxoid, Basingstoke, UK) were added after 4 h, and incubation continued for an additional 44 h. Enriched cultures were subsequently streaked onto RAPID'*L. mono* agar (3563694, Bio-Rad Laboratories, Hercules, CA) or Agar *Listeria* Ottavani & Agosti (ALOA; AEB150072, bioMérieux, Hazelwood, MO) plates, which were incubated at 37°C for up to 48 h. The presence of typical *L. monocytogenes* colonies on an agar plate was considered a positive result for the corresponding MPN tube. A subset of typical colonies were picked and all confirmed as *L. monocytogenes* by the real-time PCR assay and/or the API *Listeria* test (10300, bioMérieux) as described in the *Bacteriological Analytical Manual (8).*

The choice of appropriate MPN dilution schemes required knowledge of *L. monocytogenes* levels in the samples. During initial testing, the levels of *L. monocytogenes* in the samples were unknown, and thus, several different MPN schemes were evaluated for 260 samples randomly selected from the seven lots: scheme I was 3 tubes of 10 g of the sample diluted 1:10 in BLEB, 3 tubes of 1 g, and 3 tubes of 0.1 g; scheme II was 3 tubes of 10 g, 16 tubes of 1 g, and 3 tubes of 0.1 g; scheme III was 3 tubes of 10 g, 5 tubes of 1 g, and 5 tubes of 0.1 g; and scheme IV was 3 tubes of 10 g, 5 tubes of 1 g, 5 tubes of 0.1 g, and 5 tubes of 0.01 g. This led to the development of scheme V (3 tubes of 10 g, 5 tubes of 1 g, 8 tubes of 0.1 g, and 8 tubes of 0.01 g), which was appropriate for the levels of *L. monocytogenes* in these samples and provided a balance between the labor required and a narrow confidence interval. We therefore used scheme V for the remaining 2,060 samples. For scheme V, the lower limit of detection (LOD; a single tube positive at the lowest dilution) was 0.03 MPN/g and the maximum enumerable value (all tubes but one positive at the highest dilution) was 208 MPN/g. Deepwell 96-well plates (278743, ThermoScientific), which can hold 2.0 ml of liquid, were used for the analysis, which allowed convenient preparation of the eight 0.1-g and eight 0.01-g dilutions by using multichannel micropipettes *(11)*. Direct plating was used for enumeration of *L. monocytogenes* levels for the majority of samples in anticipation that the levels in some of the samples might exceed the maximum enumerable value (all MPN dilution tubes positive). A total of 0.72 to 1.52 g of melted ice cream was spread onto 2 to 16 RAPID'*L. mono* agar or ALOA plates with an easySpiral automatic spiral plater (Interscience, Saint-Nom-la-Bretèche, France) set to constant volume plating, and the plates were subsequently incubated at 37°C for up to 48 h. Colonies typical of *L. monocytogenes* on these selective agar plates were counted manually, and the identities of a subset of presumptive colonies were confirmed as described above. All colonies picked were confirmed as *L. monocytogenes.*

TABLE 1. *Lot information and number of vanilla ice cream samples analyzed in the present study*

Lot no.	Production date	No. of boxes[a]	No. of samples analyzed per box
110616A	6 Nov. 2014	3	20
122316A	23 Dec. 2014	52	10
122416A	24 Dec. 2014	38	10
012117A	21 Jan. 2015	53	10
012217A	22 Jan. 2015	53	10
030417A	4 Mar. 2015	15	10
030517A	5 Mar. 2015	15	10
Total		229	2,320

[a] 60 samples per box.

Statistical analysis. When needed for descriptive statistics, some values had to be assumed for samples for which the MPN could not be determined (either no tubes positive or all tubes positive). Thirteen samples produced no positive tubes, and direct plating did not yield *L. monocytogenes* for these 13 samples; thus, for analysis we recorded the levels in these samples as half of the LOD (i.e., 0.016 MPN/g for scheme V). For 10 samples, all tubes were positive (1 by scheme V and 9 by schemes I through IV), and we recorded the level as the larger of the direct plating result or the maximum enumerable MPN for each sample. Comparisons of *L. monocytogenes* levels were made using the nonparametric Mann-Whitney test *(16)*. The statistical analyses were performed with R 2015 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Simultaneous *L. monocytogenes* detection and enumeration by the MPN method. In the present study, the prevalence and levels of *L. monocytogenes* in 2,320

TABLE 2. *Prevalence and levels of* L. monocytogenes *in seven lots of ice cream from production line A*

Lot no.	No. of samples	No. of negative samples	*L. monocytogenes* level						
			Minimum (MPN/g)	Q25%	Median (MPN/g)	Arithmetic mean (MPN/g)	Geometric mean (MPN/g)	Q75%	Maximum (MPN/g)[a]
110616A	60	0	0.45	2.33	3.36	5.62	3.86	6.16	38.49
122316A	520	0	0.45	3.36	5.76	8.68	5.80	10.30	91.78
122416A	380	0	0.72	3.36	6.16	9.48	6.24	11.14	207.90
012117A	530	0	0.07	1.54	3.24	6.53	3.35	6.16	98.17
012217A	530	0	0.37	3.36	6.16	11.99	7.12	12.08	356.94
030417A	150	8	<0.03[b]	0.08	0.14	0.27	0.15	0.30	3.36
030517A	150	5	<0.03[b]	0.13	0.41	1.21	0.39	1.49	11.45
Total	2,320	13	<0.03[b]	1.90	4.52	7.97	3.57	8.41	356.94

[a] For 10 samples for which all tubes were positive for *L. monocytogenes,* the level was recorded as the larger of the direct plating result for that sample or the maximum enumerable MPN value, which is the value when all tubes except one at the highest dilution are positive.
[b] For 13 samples for which no tubes were positive for *L. monocytogenes,* the level was recorded as one-half of the LOD, which was the MPN value when one tube was positive at the lowest dilution.

individually wrapped scoops of ice cream were determined by the MPN method. The scoops, when melted, had a pH (mean ± standard deviation) of 6.36 ± 0.08 and a water activity of 0.970 ± 0.003 at room temperature. Direct plating results were obtained for the majority of the samples; however, most of the plates had fewer than 25 colonies, which made the results questionable for statistical analysis *(12)*. Therefore, we used MPN data for statistical analysis. During initial testing, 260 samples were tested for the level of *L. monocytogenes* using MPN schemes I, II, III, and IV, which involved fewer dilution levels and/or tubes than did MPN scheme V. The MPN values for these 260 samples did not have confidence intervals as narrow as those for the other 2,060 samples, although the MPN values were still valid and thus were included in the statistical analysis. For 10 samples, all MPN tubes were positive for *L. monocytogenes*. Among these samples, one was analyzed by scheme V (maximum enumerable MPN value of 208 MPN/g; direct plating value of 357 CFU/g), four samples were analyzed by schemes I and II (maximum enumerable MPN value of 11 MPN/g; direct plating values of 3, 8, 10, and 26 CFU/g), and five samples were analyzed by scheme III (maximum enumerable MPN value of 16 MPN/g; direct plating values of 11, 20, 26, 31, and 89 CFU/g). The result was assumed to be the larger of the direct plating value and the maximum enumerable MPN value for each of these 10 samples. Thus, direct plating results for six of the samples were used for statistical analysis. For 13 samples, all tubes were negative for *L. monocytogenes*; therefore, half of the LOD was assumed as the level of *L. monocytogenes* in these samples and was used for statistical analysis (Table 2).

High prevalence and low levels of *L. monocytogenes* on different production dates. Descriptive statistics from all seven lots of samples are listed in Table 2. *L. monocytogenes* was detected in 2,307 (99.4%) of the total 2,320 samples (LOD, 0.03 MPN/g). Of the 300 samples tested from the two lots manufactured in March 2015, after recognition of the outbreak *(2),* 13 samples (4.3%) did not yield *L. monocytogenes* (<0.03 MPN/g). The geometric and arithmetic mean levels of *L. monocytogenes* in all samples were 3.57 and 7.97 MPN/g, respectively. Based on the cumulative percentages from evaluation of all 2,320 samples, 92.3% (2,142 samples), 98.4% (2,282 samples), and 99.8% (2,316 samples) contained *L. monocytogenes* at <20, 50, and 100 MPN/g, respectively (Table 3). Of all the samples analyzed, four contained *L. monocytogenes* at >100 MPN/g (139, 208, 208, and >208 MPN/g). For the sample that contained *L. monocytogenes* at >208 MPN/g, all MPN tubes were positive; thus, we used the direct plate count estimate (357 CFU/g) for that sample.

The box plot in Figure 1 illustrates the enumeration data for each lot according to the production date. Each color represents a different lot, and the production dates for each lot are listed in the *x* axis. Each box represents 10 or 20 samples from each box of ice cream. The levels of *L. monocytogenes* among the five lots produced in November 2014, December 2014, and January 2015, i.e., before the initial finding of *L. monocytogenes* and the cleaning and overhaul of production line A *(2),* had a geometric mean per lot of 3.9 to 7.1 MPN/g. The two lots produced in March 2015 had substantially lower *L. monocytogenes* levels

TABLE 3. *Percentage of ice cream samples yielding various levels of* L. monocytogenes

L. monocytogenes level[a]	No. of samples	% of samples	Cumulative % of samples
<0.03	13	0.56	0.56
(0.03, 0.1]	73	3.15	3.71
(0.1, 1]	242	10.43	14.14
(1, 5]	986	42.50	56.64
(5, 10]	479	20.64	77.28
(10, 20]	349	15.04	92.32
(20, 50]	140	6.03	98.35
(50, 100]	34	1.47	99.82
(100, 200]	1	0.04	99.86
(200, 400]	3	0.13	100

[a] Levels are given in MPN per gram except for six samples for which direct plating results (CFU per gram) were used.

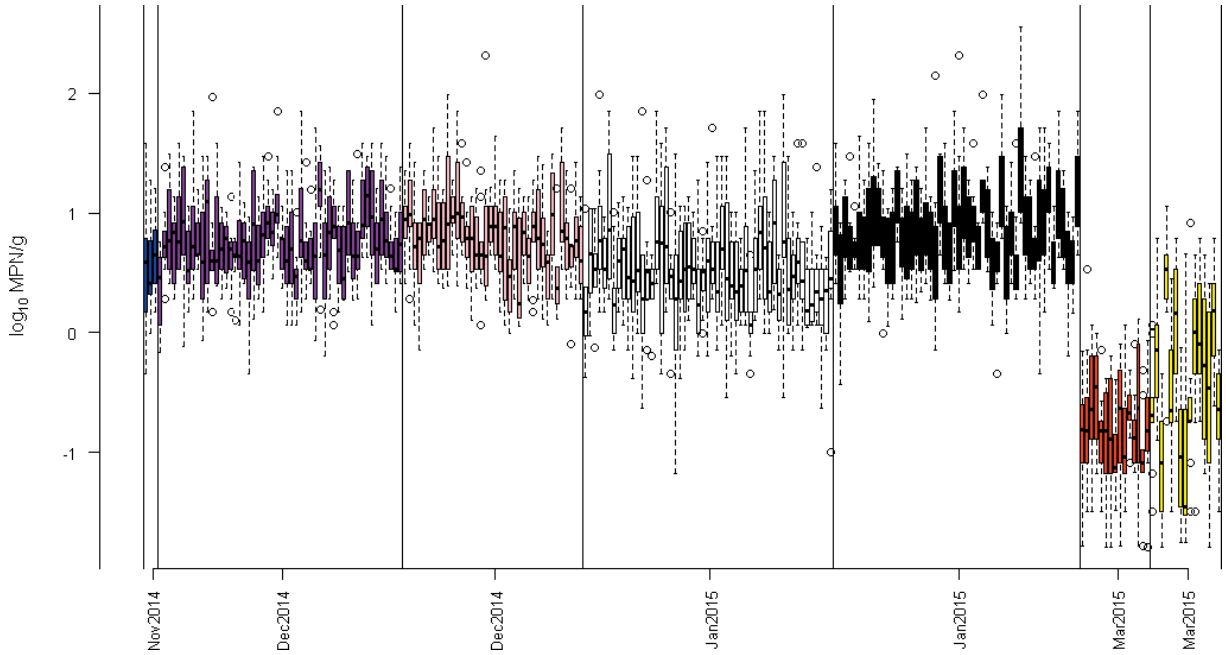

FIGURE 1. *Box plot of* L. monocytogenes *enumeration data from each ice cream lot. Each color represents a different lot, and the production date for each lot is listed in the* x *axis. Each box represents 10 or 20 samples of scoops from each box of ice cream. The lots (from left to right) are 110616A (geometric mean, 3.86 MPN/g), 122316A (5.80 MPN/g), 122416A (6.24 MPN/g), 012117A (3.35 MPN/g), 012217A (7.12 MPN/g), 030417A (0.15 MPN/g), and 030517A (0.39 MPN/g).*

(Mann-Whitney test, $P < 0.0001$) than did the previous lots, with geometric means of 0.15 and 0.39 MPN/g.

DISCUSSION

We obtained a large number of products manufactured from the production line implicated in the manufacture of products associated with the listeriosis outbreak reported in 2015 *(2)*. During listeriosis outbreak investigations, products consumed by the case patients or from the same lot or same production line are rarely available because of the long incubation time of the illness and/or short shelf life of the implicated products. In the present study, *L. monocytogenes* levels in individually wrapped scoops of ice cream produced between November 2014 and March 2015 (Table 1) by production line A were determined. The production dates partially overlapped with the listeriosis onset dates in the case patients *(2),* although information on the exact lots of ice cream consumed by the case patients was not available. WGS analysis using the Center for Food Safety and Applied Nutrition single nucleotide polymorphism pipeline *(4, 14)* revealed that isolates recovered from both MPN enrichment cultures and directly plated cultures of ice cream samples from all seven lots (Table 1) differed by ≤16 single nucleotide polymorphisms from any of the four clinical isolates obtained from the four confirmed case patients in hospital X (Y.C., unpublished data). All ice cream isolates and clinical isolates were grouped in a single WGS cluster that was distinct from epidemiologically unrelated isolates (Y.C., unpublished data). The levels of *L. monocytogenes* in five lots of tested samples produced between November 2014 and January 2015 had a geometric mean per lot of 3.9 to 7.1 MPN/g, and 92.3% of all seven lots of tested samples were contaminated at <20 MPN/g. These data provide an estimate of the range of *L. monocytogenes* contamination in scoops produced before the onset of listeriosis in the case patients because these samples were all produced on the same production line. A retrospective analysis identified listeriosis cases linked to the ice cream products made in a separate facility operated by the same company *(2)*; however, the PFGE and WGS results indicated that the *L. monocytogenes* isolates from ice cream products produced in that second facility did not match those from the ice cream produced on production line A or those from the case patients in hospital X *(2)*. Therefore, ice cream products made in the second facility were not included in this study.

This study is to our knowledge the first time such a large number of samples from a naturally contaminated nongrowth food linked to a listeriosis outbreak was gathered and analyzed. Results of previous studies have indicated that *L. monocytogenes* populations can remain stable in ice cream products stored at freezing temperatures *(5, 7, 13)*. The ice cream products analyzed in the present study had been stored at freezing temperatures, and *L. monocytogenes* was enumerated between April and May 2015. Our subsequent studies for other purposes involving *L. monocytogenes* enumeration in July through September 2015 and May 2016 revealed that the levels of *L. monocytogenes* in the scoops of the seven lots (Table 1) were in the same range as those found in this study (Y.C., unpublished data; *3*), confirming previous findings *(5, 7, 13)*. Thus, we had a unique opportunity to assess the levels of *L. monocytogenes* in the contaminated ice cream at the time the product was purchased.

The prevalence and enumeration data generated in the present study offer a unique data set for future risk-based characterization of *L. monocytogenes* contamination. The

case patients from hospital X consumed milkshakes made from the contaminated ice cream, but environmental samples collected from the kitchen of hospital X during the epidemiologic investigation, including those from the milkshake maker, did not yield *L. monocytogenes (2, 9),* and no evidence of temperature abuse of milkshakes in hospital X was reported *(2)*. Chen et al. *(3)* evaluated the recovery and growth of *L. monocytogenes* in 30 milkshake samples prepared from ice cream scoops randomly picked from the five contaminated lots produced between November 2014 and January 2015 (Table 1), assuming an extreme scenario that milkshakes were unintentionally left at room temperature. The researchers used the same milkshake recipe as that used in hospital X, used a milkshake maker with the same agitator design as that used in hospital X, and monitored the growth of *L. monocytogenes* every hour. They determined that when milkshakes were left at room temperature for 14 h the *L. monocytogenes* lag phase (determined as the time for the initial population to increase twofold) was 9.05 h and the growth rate was 0.186 log CFU/g/h *(3)*. These recovery and growth kinetics results could be incorporated into any risk assessment in which this outbreak is used as a case study. However, any conclusions obtained from such an assessment might apply only to subpopulations of *L. monocytogenes* that are similar to the isolates involved in this outbreak and may not necessarily apply to all populations of *L. monocytogenes*.

In summary, high prevalence and low levels of *L. monocytogenes* contamination were found in the 2,320 tested ice cream samples that were produced from November 2014 to March 2015 on production line A, which was implicated in a listeriosis outbreak. The data suggest the possibility of a high number of individual consumers exposed to low levels of *L. monocytogenes* from these products. Overall, distribution of ice cream produced on production line A resulted in four reported ice cream–associated cases of listeriosis, including two deaths, among members of a highly susceptible population of elderly persons hospitalized for other medical conditions.

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