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Use of Whole Genome Sequencing and Patient Interviews To Link a Case of Sporadic Listeriosis to Consumption of Prepackaged Lettuce

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Abstract

We report on a case of listeriosis in a patient who probably consumed a prepackaged romaine lettuce-containing product recalled for *Listeria monocytogenes* contamination. Although definitive epidemiological information demonstrating exposure to the specific recalled product was lacking, the patient reported consumption of a prepackaged romaine lettuce-containing product of either the recalled brand or a different brand. A multinational investigation found that patient and food isolates from the recalled product were indistinguishable by pulsed-field gel electrophoresis and were highly related by whole genome sequencing, differing by four alleles by whole genome multilocus sequence typing and by five high-quality single nucleotide polymorphisms, suggesting a common source. To our knowledge, this is the first time prepackaged lettuce has been identified as a likely source for listeriosis. This investigation highlights the power of whole genome sequencing, as well as the continued need for timely and thorough epidemiological exposure data to identify sources of foodborne infections.

Keywords

Lettuce; Listeriosis; Whole genome sequencing

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Listeria monocytogenes (Lm) infection (listeriosis) is typically a foodborne disease that can cause meningitis or sepsis in elderly and immunocompromised persons. Infection during pregnancy often leads to fetal loss or invasive listeriosis in the newborn. Although listeriosis is rare, >90% of patients require hospitalization, and it is estimated as the third leading cause of death from foodborne pathogens in the United States (21).

Most listeriosis illnesses are not linked to an outbreak. However, identification of food sources has largely been limited to outbreak settings because several patients with epidemiological linkages are usually needed to identify a common source.

Lm can be found in many foods. Most outbreaks have been associated with contaminated delicatessen meat, frankfurters, and cheese (2). However, the first recognized foodborne outbreak of listeriosis was associated with coleslaw (22). Since 2008, produce items implicated in outbreaks have included sprouts (3), celery (9), cantaloupe (17), and apples (5). Despite the emergence of produce-associated listeriosis outbreaks, we are not aware of any cases or outbreaks linked to contaminated lettuce, even though surveys of retail foods have shown lettuce to have higher rates of Lm contamination than several other produce items (8, 10, 16).

In March 2014, the Canadian Food Inspection Agency (CFIA) identified, through routine retail product testing, Lm in a prepackaged lettuce mix imported from the United States. We describe a multinational investigation that used whole genome sequencing (WGS) and patient interviewing to identify human illnesses likely associated with consumption of this recalled product.

MATERIALS AND METHODS

United States

As part of the *Listeria* Initiative, state and local health department personnel use a standard case report form to collect and report data on patient demographics, clinical information, exposure history for >40 higher-risk foods (e.g., foods previously associated with Lm outbreaks), and laboratory information (6). When investigators suspect that a food item not listed on the *Listeria* Initiative form is linked to illness, they administer a supplemental exposure questionnaire to the patient. Investigators also request shopper card data, when available, to verify brand and lot information of suspect foods. When Lm is isolated from food, the U.S. Food and Drug Administration (FDA) inspects the firm.

Laboratories speciate *Listeria* isolates using Accuprobe per manufacturer's instructions (Hologic, Inc., Marlborough, MA). State and federal laboratories certified by PulseNet, the national molecular subtyping network for foodborne disease surveillance, perform pulsed-field gel electrophoresis (PFGE) using the standardized PulseNet *Listeria* protocol on clinical, food, and environmental Lm isolates (4). PulseNet is an international collaboration. In addition to PFGE, in September 2013, near real-time characterization of all available patient, food, and environmental Lm isolates by WGS began in the United States. This project is a joint effort between the Centers for Disease Control and Prevention (CDC), FDA, the U.S. Department of Agriculture's Food Safety and Inspection Service, the

National Center for Biotechnology Information (NCBI) of the National Institutes of Health, and state and international partners. Sequencing is performed by several states, CDC, and FDA (20). Sequence data are uploaded to NCBI's publicly available Sequence Read Archive (18). When PulseNet identifies highly related isolates, investigators use both sequence and exposure data to investigate cases.

CDC assesses relatedness of isolates by high-quality single nucleotide polymorphisms (hqSNPs) (Lyve-SET (13)) and by whole genome multilocus sequence typing (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium). The purpose of the Lyve-SET hqSNP pipeline is to create a whole genome phylogeny from WGS reads. Single nucleotide polymorphisms obtained from Lyve-SET are considered high quality because they have a higher confidence level due to heightened thresholds, mainly 10× coverage and 75% consensus. Lyve-SET uses SMALT (<http://www.sanger.ac.uk/resources/software/smalt/>) for read mapping and VarScan (15) for single nucleotide polymorphisms identification. It then uses RAxML v8 (23) with rapid bootstrap analysis and best-scoring maximum likelihood, using the ASC_GTRGAMMA model to infer a phylogeny. Before running Lyve-SET, we used a read-cleaning tool from the CG-Pipeline package (14) called `run_assembly_trimClean.pl`. We used reference genome PNUSAL000564 (available in NCBI Sequence Read Archive).

Canada

Provinces perform PFGE using the standardized PulseNet *Listeria* protocol on all listeriosis cases and submit data in real-time to the PulseNet Canada Network operated by the Public Health Agency of Canada's (PHAC) National Microbiology Laboratory. PulseNet Canada identifies and reports clusters of indistinguishable isolates to its laboratory and epidemiology partners. CFIA, as does FDA, routinely tests various food products for foodborne bacterial pathogens, including Lm, and further characterizes any isolates by PFGE. CFIA performs WGS of Lm isolated from food on a case-by-case basis. When Lm is isolated from food, CFIA samples additional lots of the foods that yielded Lm to determine the extent of product contamination. WGS of the lettuce isolate was performed at the PHAC National Microbiology Laboratory Genomics Core facility. Sample libraries were prepared using Nextera XT library preparation kit (Illumina, Inc., San Diego, CA). Sequencing was performed on the Illumina MiSeq platform with the MiSeq Reagent Kit V2 to achieve average genome coverage of greater than 50× for all isolates. Core genome analysis was performed using the PHAC National Microbiology Laboratory bioinformatics custom Single Nucleotide Variant Phylogenomic pipeline consisting of open-source software (19).

RESULTS

On 12 March 2014, CFIA detected *Listeria* in a composite sample of five unopened bags of brand A Italian blend prepackaged lettuce (romaine lettuce and radicchio) imported from the United States. On 13 March, company A, in consultation with FDA, recalled the product (24), which had been distributed to 15 states and three Canadian provinces.

During 28 March to 5 April, a 74-year-old man from Ohio (a state where recalled product was sold) was hospitalized for fever. A blood culture collected on 31 March yielded Lm. On

7 April, a local investigator interviewed the patient's wife using the *Listeria* Initiative standardized case report form.

After the initial interview, Ohio investigators determined that the patient's isolate was indistinguishable from the lettuce isolate by PFGE. The pattern combination (GX6A16.1244/GX6A12.0112) was among the most common (sixth of >2,500) in the PulseNet USA database, and the number of patient isolates with that pattern combination in the preceding 120 days was not above baseline. An investigator recontacted the patient and his wife on 25 April and, using a supplemental questionnaire, asked them to recall whether the patient had eaten various types of lettuce (e.g., mesclun, iceberg, romaine, and red leaf) before illness began, brand names of lettuce products consumed, and places of purchase. They reported that the patient ate prepackaged brand A or brand B romaine lettuce purchased every other week from one of two stores. Shopper card data for one store showed no record of lettuce purchases during the 7 weeks before the patient's hospitalization. The other store, a retail location of a large regional grocery, did not have a shopper card program, and the couple did not have receipts of purchases.

Because patient and food isolates were indistinguishable by PFGE, and because the patient might have consumed the recalled product, PHAC sequenced the food isolate and compared WGS results with those from the patient isolate uploaded to NCBI. Analysis by PHAC found no hqSNP differences between the two isolates among 2,734,200 nucleotide sites compared. Similarly, analysis at CDC revealed a highly similar sequence homology between these two isolates, which differed by five hqSNPs among 9,655 high-quality positions with variant sites (Fig. 1). Twenty-six additional patient isolates with specimen collection dates from February 2013 to June 2014 that were indistinguishable by PFGE from the lettuce isolate were compared by hqSNP and by whole genome multilocus sequence typing analyses. These isolates differed from the lettuce isolate by 30 hqSNPs and 30 alleles, with similar resolution by each method.

After the recall, CFIA sampled 60 bags of brand A Italian blend prepackaged lettuce with "use-by" dates 6, 8, and 10 days later than the date on the implicated bags; none yielded *Listeria*. Additionally, neither of the two environmental samples (which consisted of 177 and 138 subsamples, respectively) and none of the three product samples collected at the processing facility by FDA yielded *Listeria*. No environmental samples were collected at the field where the lettuce was grown.

DISCUSSION

Whole genome sequencing data and epidemiological exposure information provided strong circumstantial evidence linking a sporadic case of listeriosis to a prepackaged lettuce product that had been recalled for Lm contamination. To our knowledge, this case is the first known instance of Lm infection likely attributable to the consumption of contaminated prepackaged lettuce mix. Lm has been shown to multiply on lettuce under storage conditions (1). Thus, consumption of prepackaged lettuce contaminated with Lm is a plausible cause of listeriosis. This product recall, in accordance with FDA's "zero tolerance" policy for Lm in ready-to-eat foods, likely prevented additional cases.

This investigation highlights the power of molecular subtyping of food and patient isolates, particularly by WGS, to identify linkages between human illness and food sources. Although 27 patient isolates with specimen collection dates from February 2013 through June 2014 had PFGE patterns indistinguishable from the lettuce isolate, only one of these isolates was closely related to the lettuce isolate by WGS, demonstrating the increased discriminatory power of this technology. However, even with the increased resolution of WGS, detailed epidemiologic data were essential to assessing exposure and causation.

Although purchase of the recalled product could not be confirmed through shopper card data or receipts, the findings of 0 to 5 hqSNP differences between the clinical and food isolates indicate that the two isolates were essentially identical and, thus, were highly likely to share a common source. To put this in context, the degree of genetic difference is commensurate with the differences observed among isolates attributed to common sources in solved foodborne outbreaks of listeriosis (7, 12). To maximize the public health value of WGS, listeriosis cases must be reported expeditiously to public health officials so that interviews can occur as soon as possible to ensure that exposure recall is as complete as possible and to prevent additional cases.

The identification of only one illness likely associated with this recall suggests that contamination was limited. Regulatory agencies were unable to identify *Listeria* in subsequent product lots or in the processing facility. Additionally, the short shelf life of this item might have limited the amount of growth in the product before consumption. Although Lm was not identified in more than one product lot, contamination could have occurred at any point in the food production chain, whether on the farm, during transportation, or in the processing facility (11).

With WGS-based surveillance that integrates clinical, food, and environmental data, continued collaborative multiagency responses will likely result in greater recognition of links between sporadic cases and contaminated foods, if high-quality epidemiologic exposure data are also obtained. In addition to preventing more cases in the short-term, these efforts have the potential to identify new sources of illness, such as lettuce, that can lead to industry-wide prevention measures that will decrease illnesses.

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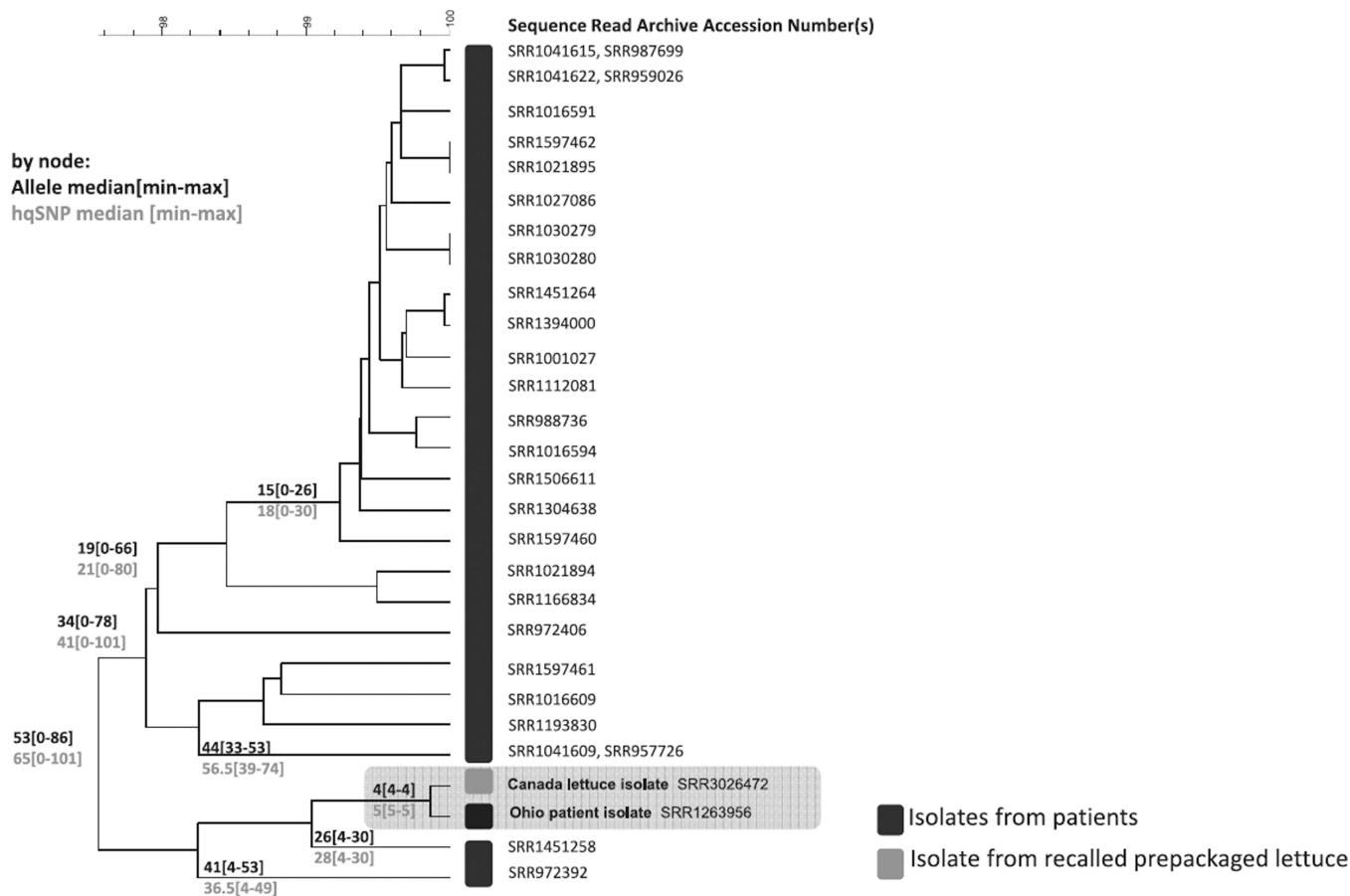


FIGURE 1. Phylogenetic tree showing high-quality single nucleotide polymorphism (hqSNP) and whole genome multilocus sequence typing differences among *Listeria monocytogenes* isolates with pulsed-field gel electrophoresis (PFGE) pattern GX6A16.1244/GX6A12.0112 and specimen collection dates from February 2013 to June 2014 ($n = 28$). The phylogeny was inferred using the unweighted pair group method with arithmetic mean algorithm; allelic differences and hqSNP differences are overlaid on the phylogeny. The top scale bar displays the percentage of common alleles between two genomes. The within-clade allelic differences are shown at several ancestor nodes in the format of median (minimum to maximum). For example, in the two-member clade containing the Canadian lettuce isolate and the Ohio clinical isolate, there are only four allelic differences.