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Original article

Two listeria outbreaks caused by smoked fish consumption—using whole-genome sequencing for outbreak investigations

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ABSTRACT

Listeria monocytogenes may contaminate and persist in food production facilities and cause repeated, seemingly sporadic, illnesses over extended periods of time. We report on the investigation of two such concurrent outbreaks. We compared patient isolates and available isolates from foods and food production facilities by use of whole-genome sequencing and subsequent multilocus sequence type and single nucleotide polymorphism analysis. Outbreak cases shared outbreak strains, defined as *Listeria monocytogenes* isolates belonging to the same sequence type with fewer than five single nucleotide polymorphism differences. We performed routine food consumption interviews of *L. monocytogenes* patients and compared outbreak cases with sporadic cases. Two outbreaks were defined, each consisting of ten outbreak cases in the period 2013–15. Seven outbreak cases and a fetus in gestational week 38 died. *Listeria monocytogenes* isolates from cold smoked or gravad fish products or their two respective production environments were repeatedly found to belong to the outbreak strains. Outbreak cases more often than sporadic cases stated that they consumed the relevant fish products, odds ratio 10.7. Routine collection and typing of food isolates was key to solving the outbreaks. Furthermore, these outbreaks illustrate the value of whole-genome sequencing for outbreak definition and investigation. Whole-genome sequencing combined with epidemiological investigations provided the discriminatory power to recognize low-intensity, extended time-period outbreaks and link them to food products from two different contaminated production facilities with sufficient strength for food authorities to intervene on. Cold smoked and gravad fish constitute risk products and may be responsible for more listeriosis cases than previously recognized. **S. Gillesberg Lassen, CMI 2016;22:620**

Introduction

Listeriosis is a serious food-borne infection. Invasive infections with *Listeria monocytogenes* mainly present as sepsis or meningitis and are most commonly seen among the elderly or patients with an underlying illness that impairs immunity. In pregnant women, listeriosis can cause abortions, stillbirths or neonatal infections. The case-fatality rate is between 20% and 30%. *Listeria monocytogenes* is a ubiquitous Gram-positive bacterium that can grow at temperatures below 5°C. Potential vehicles are ready-to-eat foods that provide a favourable growth matrix and have a shelf life of several weeks. Food sources frequently reported in the literature include deli-meats, soft cheeses and fish products, besides fruits and vegetables [1–3]. Though most cases of listeriosis are sporadic, outbreaks also occur. In the USA, 24 outbreaks were reported over the 11-year period from 1998 to 2008 [4]. In 2011, a US multi-state outbreak with contaminated cantaloupe melons caused 140 illnesses and 39 deaths [5]. In Denmark in 2014, a cold cut deli-meat product caused 41 cases and 17 deaths [6]. The potential to cause outbreaks is of concern because of the high case fatality and the inherent difficulty in finding outbreak sources. Reasons for this include the long incubation period of typically 1–4 weeks [7] and

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the fragile state of patients, making it difficult to establish pre-onset food histories.

Next-generation whole-genome sequencing (WGS) is a technique that, due to technical development and decreasing cost, is increasingly becoming part of infectious diseases surveillance. In Denmark, WGS was introduced for the surveillance of *L. monocytogenes* in 2013. As the first organism undergoing routine real-time use of WGS, *L. monocytogenes* was chosen because of the severity of the infections and the relatively modest incidence compared with other food-borne infections. Using WGS, we identified two genetic clusters of *L. monocytogenes* in 2013–15 with different serogroups and sequence types but traced to the same type of source. Here we describe the identification and investigation of these outbreaks and discuss the potential benefit of using WGS for surveillance and outbreak investigations.

Methods

Listeriosis is laboratory notifiable in Denmark. All patient *L. monocytogenes* isolates are sent to Statens Serum Institut (SSI) for typing. Samples of food or from production environments taken by official control are analysed for listeria at the Danish Veterinary and Food Administration (DVFA) laboratory in Ringsted. Isolates obtained by private laboratories through companies own control programmes are not available for typing. Starting in 2013, typing of patient isolates went from pulsed-field gel-electrophoresis [8] to WGS, followed in 2014 also by WGS typing of isolates obtained from the national mandatory surveillance of food and by routine epidemiological follow up on patients as described below.

Isolates received at SSI from January 2013 onwards were genome sequenced to at least 25× depth using the MiSeq sequencer (Illumina, San Diego, CA, USA). Analysis was performed as previously described [6]; in short, data were assigned multilocus sequence type (MLST) and analysed for single nucleotide polymorphism (SNP) differences using an in-house pipeline built on mapping reads via the Burrows–Wheeler Aligner BWA-MEM [9] and using the Genome Analysis Toolkit [10] for variant calling. Quality control was performed in two steps to evaluate the run (Sequence Analysis Viewer; Illumina) as well as the mappings. The reference for the analysis of each cluster was an isolate with the same MLST sequence type (ST). Each isolate was expressed as the ST with further subdivision by the described SNP analysis. For each cluster, we created maximum parsimony trees using all Danish sequenced isolates within that same ST. *Listeria monocytogenes* isolates from food or environment at food-producing companies were sequenced at SSI in 2014 but from January 2015 this was done at the National Food Institute at the Technical University of Denmark using the same equipment as at SSI. The joint WGS analyses of human and food sequences were performed both at SSI using the aforementioned pipeline and at Technical University of Denmark using the Centre for Genomic Epidemiology web tools MLST and CSI Phylogeny version 1.1 [11,12]. For the analysis with CSI Phylogeny, default settings were used and a food isolate of the same ST was used as reference for each cluster. The two analysis pipelines lead to the same clustering of isolates.

For listeriosis patients from 2014 onwards, we obtained clinical information through hospital physicians or nurses. We then interviewed patients using a structured questionnaire including questions on travel, food purchase habits and venues, food intake and food handling during the 30-day period before disease onset. An outbreak case was defined as a Danish listeriosis patient with isolation of an outbreak strain; an *L. monocytogenes* strain clustering with fewer than five SNPs and either belonging to ST391 in the period 1 June 2013 to 1 September 2015 or to ST6 in the period from 1 May 2013 to 1 September 2015. The five SNP cut-off was not chosen *a priori*, but was based on a situation-dependent evaluation of the dendrograms. We defined a fatal case as a patient no longer alive 30 days after the date of diagnosis as assessed through registrations of death in the Danish Civil Registration System [13].

Trace-back investigations were conducted using the information gathered through the structured interviews. The local food authorities inspected restaurants, food businesses and the food-producing companies linked to the consumed products. Product and environmental samples were taken before and after cleaning at the food-producing companies.

Because of the low number of cases, formal case–control studies were not conducted. Instead, we performed a case–case comparison of food consumption between the cases in the two outbreaks combined on one side and two other groups of interviewed listeria patients. These were: (a) cases from an ST224 outbreak in 2014 [6] where the source was a cold cut deli-meat, and (b) sporadic listeriosis cases that did not belong to any cluster by WGS analysis. We compared relevant food categories available from the questionnaires. The cold smoked fish category included consumption of cold smoked halibut, trout, salmon or gravad salmon. The soft cheese category included blue cheese, camembert, brie, feta, buffalo mozzarella or raw milk cheese. We calculated the crude OR and its 95% CI using Stata12 (Stata Corp, College Station, TX, USA).

Results

We identified two separate *L. monocytogenes* clusters of ST391 (serotype IIa) and ST6 (serotype IVb) as shown in Fig. 1. Below, their identification, investigating and subsequent management are described separately for each.

Outbreak 1: L. monocytogenes serotype IIa, ST391

The ST391 outbreak cluster was identified in April 2014 and an outbreak investigation was begun. The initial cluster contained four patients with the earliest laboratory sample date being 22 June 2013. Over the following several months, a new case was identified approximately every other month (Fig. 2). In June 2015, two *L. monocytogenes* isolates from environmental sampling at Company X were found by WGS to be of the outbreak strain (Fig. 1). The company had been inspected in the follow up of a recall of cold smoked salmon found positive for *L. monocytogenes* and sold in Supermarket A. Review of case interviews supported a hypothesis of the outbreak source being cold smoked salmon. This led the DVFA to implement production stop at Company X and a sales ban on cold smoked and gravad fish products. Smoked and gravad fish products were sampled as was the production environment, including 100 swab samples from equipment. The outbreak strain was isolated from an environmental sample taken from non-product touching areas and additionally, *L. monocytogenes* of ST121 was found. Following extensive cleaning and disinfection of the production area, the marketing and production bans were lifted. Nevertheless, within weeks a new case was identified. This patient reported consumption of hot smoked salmon produced by Company X. The DVFA re-inspected Company X to assess the probability of cross-contamination between cold and hot smoked fish products. Product samples were taken from hot smoked salmon and mackerel; all were negative for *L. monocytogenes*. The company was allowed to continue to operate; however, production of cold smoked fish products was discontinued.

As of 1 September 2015, ten cases had been identified (Table 1). Cases lived across Denmark and all belonged to known risk-groups of *L. monocytogenes* infection, one case was pregnancy-associated. The pregnant woman delivered a stillborn baby in gestation week 38. Four adult cases died.

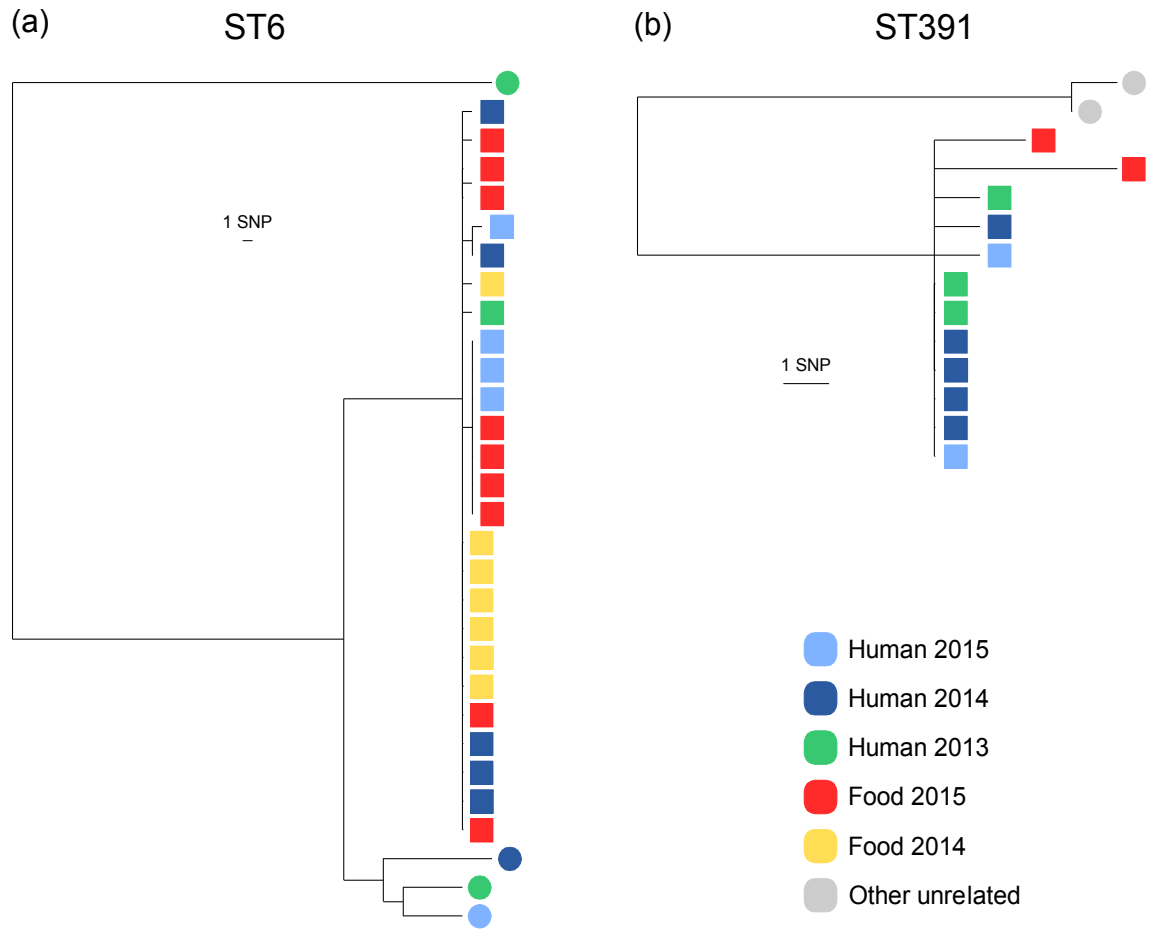

Fig. 1. Maximum parsimony trees defining the two outbreaks. (a) ST6 outbreak cluster, all ST6 isolates from the years 2013–15 are included. (b) ST391 outbreak cluster. In addition to all ST391 isolates from 2013–15 it includes two strains, 'Other unrelated', which are two ST391 human isolates from 2006. These were used as an outgroup for rooting the tree. Colours denote the year of isolation and if the strains were of patient (Human) or food or food production environment (Food) origin. Square symbols denote isolates belonging to the outbreak strains (ST6 or ST391), circles denote isolates that are not part of the clusters and hence not of the outbreak strains.

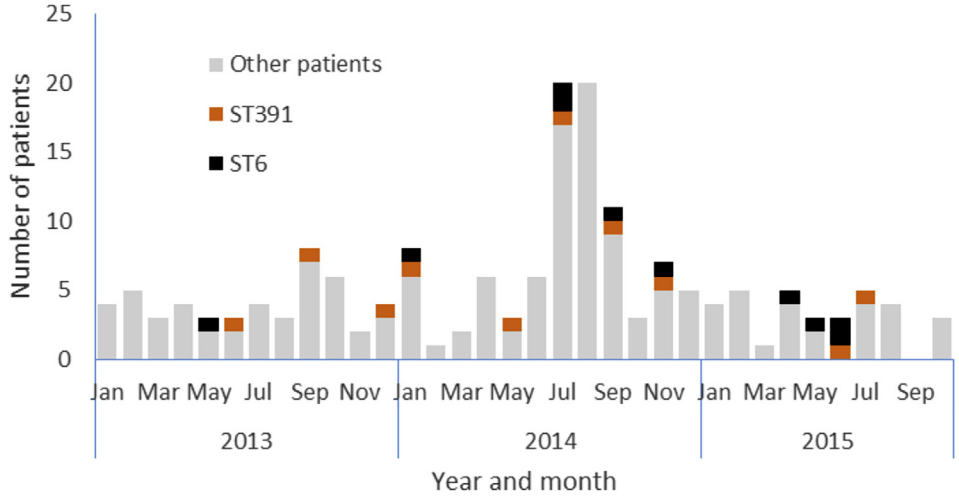

Fig. 2. Notified patients with *Listeria monocytogenes* infection in Denmark by month of diagnosis, January 2013 to September 2015. Outbreak cases belonging to the ST391 and ST6 outbreaks are marked separately.

Table 1
Characteristics of cases in the ST391 and ST6 *Listeria monocytogenes* outbreaks, Denmark, January 2013 to August 2015

Characteristic	ST391 outbreak	ST6 outbreak
No. of cases	10	10
Age (years), median (range)	69 (12–89)	73 (43–90)
Women (%)	50	60
Sepsis	8	6
Meningitis	0	3
Sepsis + meningitis	1	1
Pregnancy-associated	1	0
Fatal cases	4[a]	3

[a] In addition to the four adult fatal cases, the unborn baby (not counted among cases) of the pregnant case died in gestation week 38.

Outbreak 2: L. monocytogenes serotype IVb, ST6

On 10 September 2014, an SNP-cluster of five ST6 *L. monocytogenes* patient isolates, where the earliest dated back to 15 May 2013 (Fig. 2), was identified and an outbreak investigation was initiated. On 22 September 2014, WGS analysis found isolates from cold smoked halibut and trout produced by Company Y to be of the outbreak strain. These isolates originated from a batch that had been recalled because it tested positive for *L. monocytogenes* following a DVFA inspection. Upon identification of a new case in April 2015, the DVFA re-inspected Company Y and took product and environmental samples. Environmental samples were negative, whereas samples taken from gravad salmon and from frozen halibut were positive for *L. monocytogenes* of the outbreak strain as determined by WGS (Fig. 1). As more cases were identified and interviewed it became apparent that all cases had eaten cold smoked fish bought in supermarkets supplied by Company Y. The DVFA re-inspected Company Y and again product and environmental samples were collected. Product samples were negative, but six of 22 environmental samples yielded *L. monocytogenes* of the outbreak strain. As of 1 September 2015, the DVFA continues to follow-up on initiatives put in place at Company Y and an extensive sampling scheme is maintained. Ten outbreak cases were identified (Table 1). Cases lived across Denmark, all belonged to known risk-groups of *L. monocytogenes* infection. Three cases died.

Case–case comparison

Epidemiological follow up was available for 120 listeriosis patients from 1 January 2014 to 1 September 2015, of whom 76 (63%) had answered questions related to consumption of cold smoked fish. Of these, five were part of the ST391 outbreak, eight of the ST6 outbreak, 25 of the ST224 outbreak [6], 34 were sporadic cases while four cases belonged to two other small outbreaks. Twelve (92%) of the 13 cases belonging to the ST391/ST6 outbreaks had eaten cold smoked fish, compared with 13 (52%) of the ST224 outbreak cases (OR 11.1; 95% CI 1.2–510), and 18 (53%) of the sporadic patients (OR 10.7; 95% CI 1.3–480). The ST391/ST6 cases were therefore associated with consumption of cold smoked fish both when compared with the ST224 cases and the sporadic listeria patients, whereas this was not the case when looking at consumption of two other well-known *L. monocytogenes* infection sources: cheeses or a deli-meat product (Table 2).

Discussion

Beginning in 2014, the surveillance of *L. monocytogenes* infections in Denmark was adapted to comprise a systematic follow up with interviews of patients, and WGS of available isolates from patients, foods and food production environments. This initiative has proven valuable for outbreak investigations, as exemplified by this report. Patient food histories obtained from interviews helped to provide analytical epidemiological evidence by pooling case data and performing case–case comparisons. The use of WGS provided the possibility of linking cases that occurred over a period of years, bring about the understanding that they were in fact continuous-source outbreaks and establish links with two different food production facilities. In fact, the investigations provided evidence of sufficient strength for the Danish food authorities to intervene accordingly, possibly preventing future cases. WGS in particular was valuable in casting light on a complex outbreak structure with foods that were: (a) of different types, (b) from more than one production line, (c) only intermittently positive and (d) most likely only low-grade contaminated. Even if one or both outbreak clusters could have been defined using pulsed-field gel-electrophoresis, this typing method would probably not have provided sufficient discriminatory power to warrant a detailed outbreak investigation, nor to establish the link with the food production environments.

The annual incidence of invasive listeriosis in the EU in 2008 to 2012 ranged from 0.3 to 0.4 per 100 000 [14]. In comparison, Denmark had a high incidence, ranging from 0.8 to 1.8 in the last decade [15,16]. The high incidence in Denmark may in part be explained by a well-functioning system for diagnostics and surveillance, but other factors are probably also important. Open sandwiches (*smørrebrød*) served for lunch are a frequent part of the Danish diet. These sandwiches are typically prepared with a variety of cold cuts including cold smoked fish products, gravad salmon and sliced deli-meats, all recognized as risk products for listeriosis [6,17,18]. Many of these products are packaged with modified atmosphere and hence have a longer shelf life and a greater risk of *L. monocytogenes* growth to levels above 100 CFU/g at the end of shelf life [17]. Smoked fish products have been associated with outbreaks in other countries [19,20]. However, it has previously been difficult to document the relative significance of the various suspected products, because epidemiological data have often been limited and the characterization of *L. monocytogenes* isolates based on pulsed-field gel-electrophoresis has been ambiguous, in part due to the widespread occurrence of *L. monocytogenes* in food and the environment. The investigations described here corroborate our suspicion that ready-to-eat fish products are an important source of *L. monocytogenes* infections in Denmark and possibly neighbouring countries. This has obvious public health implications. In Denmark, current guidelines do not advise pregnant women and the elderly against consumption of cold smoked fish products. Following the occurrence of these outbreaks and the large Danish 2014 outbreak, an expert panel reviewing measures to reduce listeriosis in Denmark [6] has, however, recommended that

Table 2
Univariable *Listeria monocytogenes* case–case comparison of food consumption of ST391/ST6 outbreak cases with previous ST224 outbreak cases and sporadic cases respectively, Denmark, January 2013 to August 2015

Exposure	ST391 & ST6 cases No./all (%)	ST224 cases No./all (%)	OR (95% CI)	Sporadic casesNo./all (%)	OR (95% CI)
Cold smoked or gravad fish	12/13 (92)	13/25 (52)	11.1 (1.2–510)	18/34 (53)	10.7 (1.3–480)
Cold-cut deli-meat (*rullepølse*)	5/13 (38)	19/26 (73)	0.2 (0.4–1.2)	16/24 (44)	0.8 (0.2–3.4)
Soft cheese	9/13 (69)	14/24 (58)	1.6 (0.3–9.1)	22/33 (67)	1.1 (0.2–6.1)

this be changed. An additional problem that these outbreak investigations underline is the well-known ability of *L. monocytogenes* to persist in fish production environments [21,22]. They suggest that once significant numbers of *L. monocytogenes* are found in products, the whole production site should be subject to a thorough inspection and sampling with special attention to all the possible contamination/cross-contamination issues before implementing corrective measures.

The outbreaks described here were severe, comprising eight deaths including a stillborn baby. They were however also 'low-intensity' outbreaks, running over periods of years. In all likelihood, they would therefore not have been solved with purely epidemiological methods unless substantially more cases had occurred. Instead, typing of routinely collected food isolates was key to solving the outbreaks. It is important that laboratory methods leading to isolation of *L. monocytogenes* from contaminated foods are used alongside PCR or quick-test technology. It is also important to cooperate across different disciplines and sectors, to be able to have access to strains obtained through surveillance, including strains from food products and production environments. A systematic and cross-disciplinary approach to surveillance of *L. monocytogenes* infections improves the ability to find and solve outbreaks but also improves the evidence base for preventing such infections and for providing safer food for groups at risk, such as the elderly, people with chronic diseases and pregnant women.

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Contribution to Authorship

SGL and SE drafted the paper. SGL contributed to the clinical and patient follow up and the epidemiological investigations and conducted the case–case analysis. JTB conducted the typing and comparison of human isolates and of environmental and product isolates from 2014 as well as comparison between human, environmental and product isolates. SE and KM contributed to the epidemiological investigations. LM and AKJ contributed to the clinical follow up and the epidemiological investigations. TJ was in charge of the trace-back investigations. GS was in charge of typing of environmental and product isolates from 2015 and their comparison to human isolates. EMN contributed to the laboratory and typing investigations.

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Transparency Declaration

The authors declare no conflicts of interest.

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