



Eurosurveillance

Europe's journal on infectious disease epidemiology, prevention and control

Special edition:
**Food- and waterborne
outbreaks caused by
Salmonella and
*Listeria***

May 2018

Featuring

- Ongoing nationwide outbreak of *Salmonella* Agona associated with internationally distributed infant milk products, France, December 2017
- Cross-border outbreak of listeriosis caused by cold-smoked salmon, revealed by integrated surveillance and whole genome sequencing (WGS), Denmark and France, 2015 to 2017
- and more...

Editorial team

Based at the European Centre for Disease Prevention and Control (ECDC),
169 73 Stockholm, Sweden

Telephone number

+46 (0)8 58 60 11 38

E-mail

eurosurveillance@ecdc.europa.eu

Editor-in-chief

Dr Ines Steffens

Senior editor

Kathrin Hagmaier

Scientific editors

Janelle Sandberg

Karen Wilson

Assistant editors

Alina Buzdugan

Ingela Rumenius

Associate editors

Tommi Asikainen, Brussels, Belgium

Magnus Boman, Stockholm, Sweden

Mike Catchpole, Stockholm, Sweden

Natasha Crowcroft, Toronto, Canada

Christian Drostén, Berlin, Germany

Karl Ekdahl, Stockholm, Sweden

Johan Giesecke, Stockholm, Sweden

David Heymann, London, United Kingdom

Irena Klavs, Ljubljana, Slovenia

Karl Kristinsson, Reykjavik, Iceland

Daniel Lévy-Bruhl, Paris, France

Jacob Moran-Gilad, Beer-Sheva, Israel

Panayotis T. Tassios, Athens, Greece

Hélène Therre, Paris, France

Henriette de Valk, Paris, France

Sylvie van der Werf, Paris, France

Design / Layout

Fabrice Donguy / Dragos Platon

Online submission system

<http://www.editorialmanager.com/eurosurveillance/>

www.eurosurveillance.org

© Eurosurveillance, 2018

Editorial advisors

Albania: Alban Ylli, Tirana

Austria: Maria Paulke-Korinek, Vienna

Belgium: Koen de Schrijver, Antwerp; Tinne Lernout, Brussels

Bosnia and Herzegovina: Sanjin Musa, Sarajevo

Bulgaria: Iva Christova, Sofia

Croatia: Sanja Music Milanovic, Zagreb

Cyprus: Maria Koliou, Nicosia

Czech Republic: Jan Kynčl, Prague

Denmark: Peter Henrik Andersen, Copenhagen

Estonia: Kuulo Kutsar, Tallinn

Finland: Outi Lyytikäinen, Helsinki

France: Judith Benrekassa, Paris

Germany: Jamela Seedat, Berlin

Greece: Rengina Vorou, Athens

Hungary: Ágnes Hajdu, Budapest

Iceland: Gudrun Sigmundsdóttir, Reykjavík

Ireland: Lelia Thornton, Dublin

Italy: Paola De Castro, Rome

Latvia: Dzintars Mozgis, Riga

Lithuania: Saulius Čaplinskas, Vilnius

Luxembourg: Thérèse Staub, Luxembourg

The former Yugoslav Republic of Macedonia: Aziz Pollozhani, Skopje

Malta: Tanya Melillo Fenech, Msida

Montenegro: Senad Begić, Podgorica

Netherlands: Barbara Schimmer, Bilthoven

Norway: Emily MacDonald, Oslo

Poland: Malgorzata Sadkowska-Todys, Warsaw

Portugal: Paulo Jorge Nogueira, Lisbon

Romania: Daniela Pitigoi, Bucharest

Serbia: Mijomir Pelemis, Belgrade

Slovakia: Lukáš Murajda, Bratislava

Slovenia: Maja Sočan, Ljubljana

Spain: Josefa Masa Calle, Madrid

Sweden: Anders Wallensten, Stockholm

Turkey: Fehminaz Temel, Ankara

United Kingdom: Nick Phin, London

World Health Organization Regional Office for Europe: Robb Butler, Copenhagen

Contents

SPECIAL EDITION: FOOD- AND WATERBORNE OUTBREAKS CAUSED BY *SALMONELLA* AND *LISTERIA*

RAPID COMMUNICATION

Ongoing nationwide outbreak of *Salmonella* Agona associated with internationally distributed infant milk products, France, December 2017 2

Jourdan-da Silva N et al.

Cross-border outbreak of listeriosis caused by cold-smoked salmon, revealed by integrated surveillance and whole genome sequencing (WGS), Denmark and France, 2015 to 2017 7

Schjørring S et al.

SURVEILLANCE AND OUTBREAK REPORT

Outbreak of *Salmonella* Bovismorbificans associated with the consumption of uncooked ham products, the Netherlands, 2016 to 2017 12

Brandwagt D et al.

Re-evaluation of a 2014 multi-country European outbreak of *Salmonella* Enteritidis phage type 14b using recent epidemiological and molecular data 18

Hörmansdorfer S et al.

Investigation using whole genome sequencing of a prolonged restaurant outbreak of *Salmonella* Typhimurium linked to the building drainage system, England, February 2015 to March 2016 25

Mair-Jenkins J et al.

Outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* 1/2a in sliced cold beef ham, Italy, May 2016 34

Maurella C et al.

RESEARCH ARTICLES

Rise and fall of outbreak-specific clone inside endemic pulsotype of *Salmonella* 4,[5],12:i:-; insights from high-resolution molecular surveillance in Emilia-Romagna, Italy, 2012 to 2015 43

Morganti M et al.



© Eurosurveillance

Ongoing nationwide outbreak of *Salmonella* Agona associated with internationally distributed infant milk products, France, December 2017

Nathalie Jourdan-da Silva¹, Laetitia Fabre², Eve Robinson^{1,3}, Nelly Fournet¹, Athinna Nisavanh¹, Mathias Bruyand¹, Alexandra Mailles¹, Estelle Serre², Magali Ravel², Véronique Guibert², Sylvie Issenhuth-Jeanjean², Charlotte Renaudat², Mathieu Tourdjman¹, Alexandra Septfons¹, Henriette de Valk¹, Simon Le Hello²

1. Santé publique France, Saint-Maurice, France

2. Institut Pasteur, Centre National de Référence des Salmonella, Paris, France

3. European Programme for Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden

Correspondence: Nathalie Jourdan-da Silva (Nathalie.JOURDAN-DASILVA@santepubliquefrance.fr)

Citation style for this article:

Jourdan-da Silva Nathalie, Fabre Laetitia, Robinson Eve, Fournet Nelly, Nisavanh Athinna, Bruyand Mathias, Mailles Alexandra, Serre Estelle, Ravel Magali, Guibert Véronique, Issenhuth-Jeanjean Sylvie, Renaudat Charlotte, Tourdjman Mathieu, Septfons Alexandra, de Valk Henriette, Le Hello Simon. Ongoing nationwide outbreak of *Salmonella* Agona associated with internationally distributed infant milk products, France, December 2017. *Euro Surveill.* 2018;23(2):pii=17-00852. <https://doi.org/10.2807/1560-7917.ES.2018.23.2.17-00852>

Article submitted on 21 Dec 2017 / accepted on 11 Jan 2018 / published on 11 Jan 2018

On 1 December 2017, an outbreak of *Salmonella* Agona infections among infants was identified in France. To date, 37 cases (median age: 4 months) and two further international cases have been confirmed. Five different infant milk products manufactured at one facility were implicated. On 2 and 10 December, the company recalled the implicated products; on 22 December, all products processed at the facility since February 2017. Trace-forward investigations indicated product distribution to 66 countries.

Identification of the outbreak

At the end of November 2017 the French National Reference Centre (NRC) for *Salmonella* noted an unusual increase in *Salmonella* Agona among infants, with 22 cases identified between August and November 2017 in children younger than six months. On 1 December, interviews with the caregivers of eight of these infants by the French National Institute for Public Health (Santé Publique France (SPF)) identified infant milk products from a single company as a potential source. All but one of the first eight investigated cases had consumed products manufactured at a single facility operated by this company. This facility had previously been associated with an outbreak of *Salmonella* Agona in 2005 [1]. An outbreak investigation is currently ongoing in conjunction with the NRC, SPF, the Ministry of Health and the Ministry of Economy in charge of consumers' affairs.

Epidemiological investigations

The operational definition for probable cases as at 11 January 2018 includes children younger than two years with a laboratory-confirmed *Salmonella* Agona infection and with a date of onset of symptoms since 1

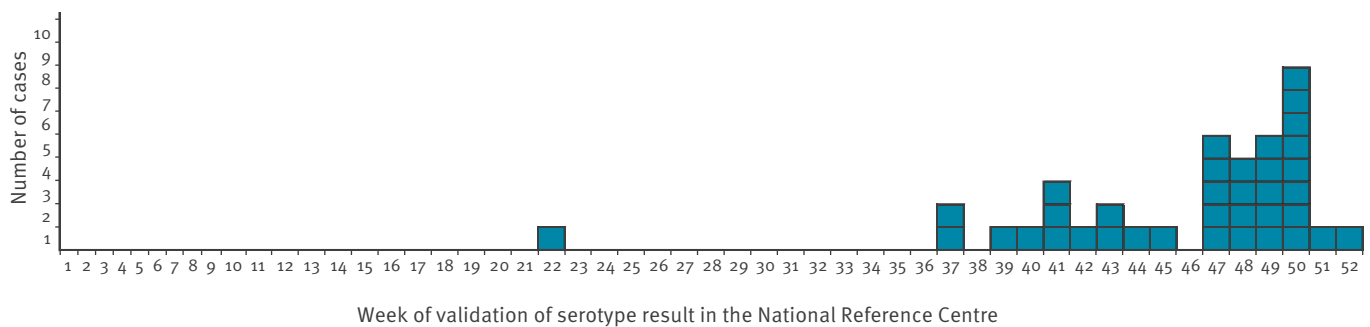
January 2017. A confirmed case is a probable case with a *Salmonella* Agona isolate belonging to the outbreak cluster by whole genome sequencing (WGS) or, if WGS has not been performed, an isolate which does not produce H₂S and gas after 18 h incubation on Kligler iron agar (see *Microbiological investigations* for further details). Cases were excluded if they had a history of travel abroad in the 7 days before symptom onset and probable cases were excluded if after WGS, they were not within the outbreak cluster.

As at 11 January 2018, 37 cases have been identified in France, all of whom were confirmed (Figure 1). All cases had gastrointestinal symptoms, and there was no case of bloodstream infection or meningitis. Almost all cases had diarrhoea (34 cases), 23 had bloody diarrhoea, 26 fever and 13 vomiting. One case had symptom onset in April while the remaining cases had onset of symptoms between mid-August and 2 December. The cases included 21 girls and 16 boys. The median age was 4 months (range: 2 weeks–9 months). The cases were scattered across 10 different regions of France.

Among 36 cases whose caregivers have been interviewed to date, 18 were hospitalised for their illness, all of whom have recovered at the time of writing this report. Eleven cases had underlying medical conditions. These conditions were not associated with an increased risk of salmonellosis. However, they may have increased the likelihood of presentation to a healthcare provider and of diagnostic investigations. In the 3 days before symptom onset, 35 of the 36 cases had consumed infant milk products intended for babies aged 0–6 months manufactured by the same company. Five different products have been implicated to date:

FIGURE 1

Epicurve of *Salmonella* Agona confirmed cases in infants by week of diagnosis at the National Reference Centre, France, 2017 (n = 37)



one product was associated with 26 cases (MILK-A), one product was associated with five cases (MILK-B), one product was associated with two cases (MILK-E) and two products were associated with one case each (MILK-C and MILK-D). All products were manufactured at the same facility. In one confirmed case, exclusive breast-feeding was reported.

No other common foods or drinks were identified among cases. Given the young age of the cases, the majority were still exclusively fed with infant milk products. A variety of types of water (tap and different brands of bottled water) were used to prepare these products. Caregivers were questioned on their practices in relation to the preparation of bottles of milk (e.g. advance preparation, storage, cleaning of equipment) and no issues were identified which would increase the risk of bacterial contamination or bacterial propagation.

Microbiological investigations

WGS of *Salmonella* isolates has been performed routinely at the French NRC since April 2017. DNA extraction, libraries and high-throughput genome sequencing for our cases were carried out at PIBnet, Institut Pasteur, Paris on the Illumina NextSeq500 platform. Molecular serotyping was done by in-house scripts based on MLST-7, *fliC* and *fliB* gene databases. For all 88 *S. Agona* isolates received in the NRC between 1 January and 31 December 2017, the filtered paired-end reads were aligned with the Agona SL483 reference genome (GenBank accession number NC_011149) [2]. Phylogenetic analysis was performed on the single nucleotide polymorphism (SNP) filtered alignment using the maximum-likelihood method, and the final tree was visualised as previously described [3]. Raw reads of a representative strain are available under the European Nucleotide Archive number ERR2219379 and under Enterobase name SAL_NA11229AA.

The phylogenetic analysis of all 88 genomes of *S. Agona* human isolates indicated a unique ST13 type with a high number of 9,519 SNPs in total and revealed that outbreak isolates clustered within a maximum distance of 26 SNPs (Figure 2).

Interestingly, distinctive microbiological traits were also found in the outbreak strain: it did not produce H_2S and gas in 18 h incubation on Kligler iron agar. *Salmonella* isolates do typically produce H_2S and gas in 18 h incubation on Kligler iron agar.

To date, 36 of the 37 confirmed cases belonged to this SNP cluster on WGS. For the remaining case, WGS results are pending but the isolate does not produce H_2S and gas after 18 h incubation on Kligler iron agar.

Environmental and product investigations

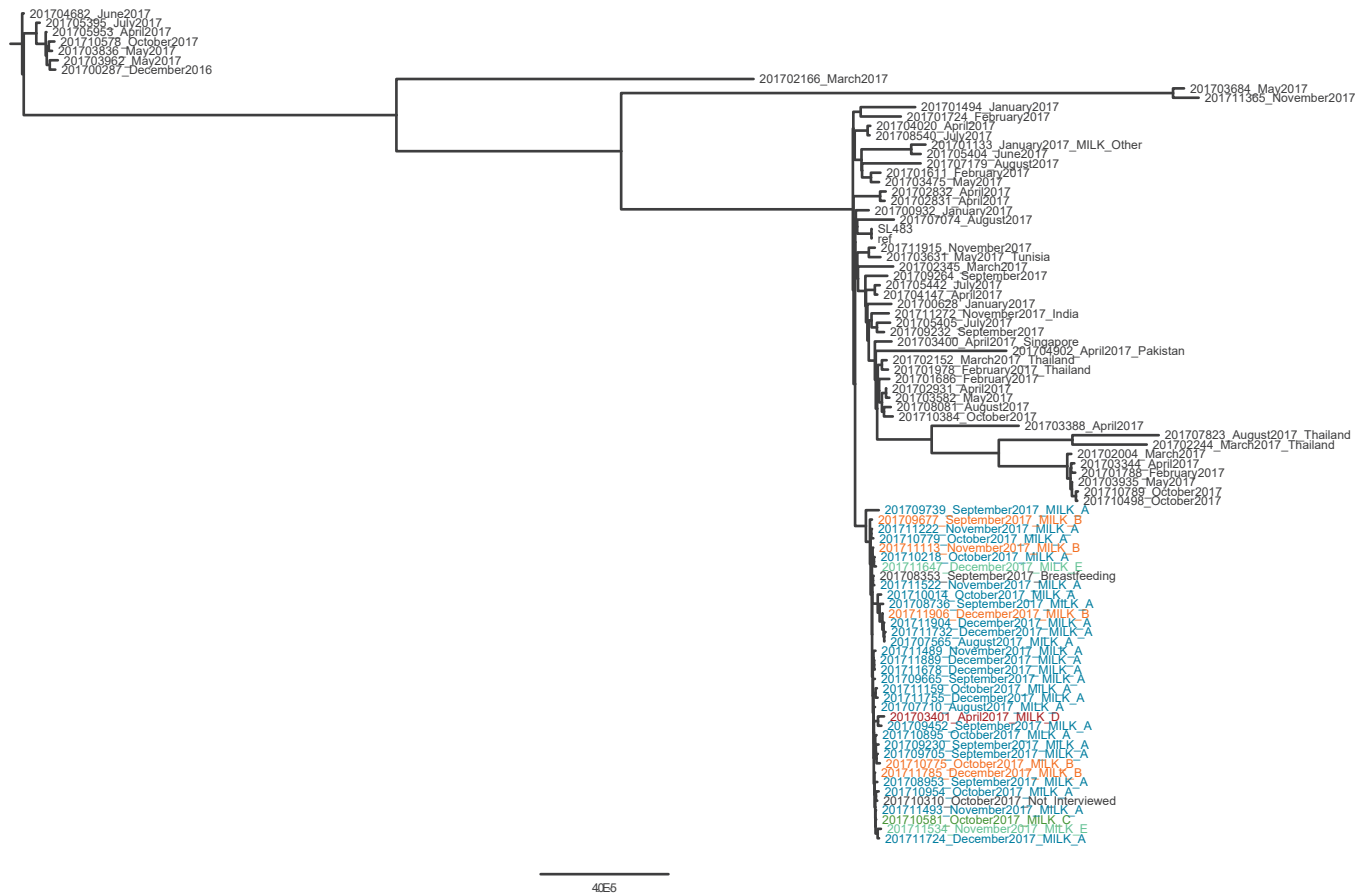
Environmental and product investigations by the company and the Ministry responsible for consumers' affairs are still ongoing. However, epidemiological information strongly suggests products manufactured at one facility as the source of infections. The facility manufactures a wide range of infant and toddler foods. Routine microbiological tests of products undertaken by the facility as part of normal production practices were negative. On 2 December 2017, the company voluntarily recalled batches of the three initially implicated products (MILKS-A, B, C) [4]. On 10 December, after parents of one case reported exclusive use of a fourth product (MILK-D), and in light of preliminary results of environmental investigations at the facility, the company recalled all products manufactured at the facility since mid-February 2017 and for which production was associated with one drying tower at the facility [5,6]. On 13 December, five further batches omitted from the recall list issued on 10 December were recalled [7]. On 22 December, as a precautionary measure, the company voluntarily recalled all products manufactured or processed in the facility since February 2017 [6].

Potential for international spread and international alerts

As at 8 January 2018, trace-forward investigations indicated that recalled products were distributed to 66 countries, including 12 countries in the European Union (EU). An alert was issued on the European Rapid Alert System for Food and Feed (RASFF) on 4 December, and updated as further information on the international distribution network of the products became available [8].

FIGURE 2

Phylogenetic tree of *Salmonella* Agona strains received at the National Reference Centre, France, 2017 (n = 88)



S. Agona genomes were mapped against the ST13 SL483 reference strain [2] using Bowtie2 software and filtering low-quality single nucleotide polymorphisms with in-house scripts. Maximum likelihood phylogeny reconstruction was done using IQ-TREE v.1.5.1 with GTR+I+G model with the fast bootstrapping option with 1,000 values. Names include the National Reference Centre identification number, month and year of isolation, source of contamination if provided in patient interview, and travel indication. The tree was rooted on the 201704682 genome.

The alert was also issued through the Infosan network operated by the World Health Organization. European public health authorities and microbiologists were alerted about the outbreak on 6 December 2017 by SPF via an *urgent inquiry* for information issued through the Epidemic Intelligence Information System for Food and Waterborne Diseases and Zoonoses hosted by the European Centre for Disease Prevention and Control.

To date, two cases in infants who consumed implicated exported products have been identified in EU countries other than France. The isolate from a case in Spain belonged to the outbreak SNP cluster in WGS performed at the NRC (data not shown in Figure 2). The isolate from a case in Greece did not produce H₂S or gas after 18 h incubation on Kligler iron agar, and the results of WGS are pending [9].

Control measures in France

In France, multiple media platforms have been used since 2 December 2017, to inform parents and caregivers about the outbreak, to advise them not to use the

implicated products and to recommend appropriate hygiene practices when preparing infant milk products [5]. All recalled products have been published on the website of the French ministry responsible for consumers' affairs [4-7]. As the initial public alert was issued on a weekend, advice was also given on how to mitigate the risk if alternative products were unavailable over the weekend. In cooperation with the French Society of Paediatrics, advice was issued on alternative infant milk product options [10]. The French Ministry of Health put in place a telephone help service for concerned parents and caregivers. Paediatric and maternity facilities as well as healthcare professionals were also informed.

Discussion

In France, surveillance of *Salmonella* infections is undertaken by the NRC and SPF. Between 2011 and 2016, *Salmonella* Agona accounted for 2.1% of *Salmonella* isolates identified in infants and received in the NRC, with between eight and 13 isolates received by the NRC a year. The detection of eight isolates

of *Salmonella* Agona in infants within a period of 8 days alerted the NRC to the outbreak. No increase in the number of *Salmonella* Agona isolates was observed in other age groups. Prompt investigation provided strong epidemiological evidence pointing to infant milk products manufactured by the same company as the source of the outbreak.

This is the third outbreak of *Salmonella* associated with infant milk products reported in France [1,11]. Similar outbreaks have also been reported elsewhere [12-16]. A *Salmonella* Agona outbreak affecting 141 confirmed cases occurred in France in 2005 and was associated with two different products manufactured within the same facility implicated in the current outbreak [1]. During the 2005 outbreak, samples of the implicated products and environmental samples from the facility yielded isolates with the same PFGE pattern as the clinical isolates. However, only one of 176 and four of 27 samples from the two implicated food products and six of 420 environmental samples tested positive for *Salmonella* Agona, suggesting a low level contamination. The production dates of the positive food samples suggested a persistent environmental contamination. The source of the contamination in the facility was not identified. In the current outbreak, the environmental investigations are still ongoing, and at this stage no obvious source of contamination has been identified within the facility.

The number of cases associated with this outbreak is probably underestimated as cases with mild symptoms may not have consulted a healthcare professional or had comprehensive diagnostic investigations (stool sample, culture, identification of serotype, confirmation at the NRC). Despite this possibility, given the wide distribution of products and the small number of reported cases, the level of contamination of products is probably low. The fact that the current outbreak involves the same serotype as the previous outbreak raises the question whether the organism has persisted in the facility for 12 years. The persistence of *Salmonella* Agona in a dry food production environment in the United States, resulting in two outbreaks 10 years apart, 1998 and 2008, has been reported before [17]. Comparison of human and environmental isolates from 2005 with isolates from the current outbreak is ongoing to investigate this possibility.

Acknowledgements

We thank all French medical laboratories participating to the clinical network of *Salmonella*. We acknowledge all the team of the NRC for their excellent work in *Salmonella* processing.

We warmly thank Edith Laurent, Isabelle Billecoq and epidemiologists located in Agence Régionale de Santé and in Regional offices of SPF in interviewing parents of cases.

We thank Silvia Herrera Leon for sending the Spanish strains, and Kassiani Mellou for sharing information about the Greek strain.

Conflict of interest

None declared.

Authors' contributions

N Jourdan Da Silva is the coordinator of the epidemiological investigation and revised the manuscript. E Robinson drafted the manuscript. A Nisavanh, N Fournet, M Bruyand, M Tourdjman, A Septfons, A. Mailles and H De Valk gathered the data from patients, participated in the investigation and revised the manuscript.

L Fabre, M Rave I, E Serre, V Guibert, S Issenhuth-Jeanjean S, C Renaudat analysed the bacterial strains to confirm the diagnosis of *S. Agona* infection, carried out the genome sequencing and analysis of the sequences, and revised the manuscript. S le Hello coordinated the microbiological investigations and revised the manuscript.

References

1. Brouard C, Espié E, Weill FX, Kérouanton A, Brisabois A, Forgue AM, et al. Two consecutive large outbreaks of *Salmonella enterica* serotype Agona infections in infants linked to the consumption of powdered infant formula. *Pediatr Infect Dis J*. 2007;26(2):148-52. <https://doi.org/10.1097/01.inf.0000253219.06258.23> PMID: 17259878
2. Zhou Z, McCann A, Litrup E, Murphy R, Cormican M, Fanning S, et al. Neutral genomic microevolution of a recently emerged pathogen, *Salmonella enterica* serovar Agona. *PLoS Genet*. 2013;9(4):e1003471. <https://doi.org/10.1371/journal.pgen.1003471> PMID: 23637636
3. Fonteneau L, Jourdan Da Silva N, Fabre L, Ashton P, Torpdahl M, Müller L, et al. Multinational outbreak of travel-related *Salmonella* Chester infections in Europe, summers 2014 and 2015. *Euro Surveill*. 2017;22(7):30463. <https://doi.org/10.2807/1560-7917.ES.2017.22.7.30463> PMID: 28230522
4. Retrait et rappel de laits infantiles 1er âge en raison d'une possible contamination par *Salmonella* Agona. [Withdrawal and recall of infant formula because of possible *Salmonella* Agona contamination]. Paris: Direction générale de la Santé, Direction générale de la concurrence, de la consommation et de la répression des fraudes; 2 Dec 2017. French. Available from: https://www.economie.gouv.fr/files/files/directions_services/dgccrf/presse/communique/2017/239-CP-Salmonelle.pdf
5. Contamination à *Salmonella* Agona de jeunes enfants: extension des mesures de retrait - rappel de produits de nutrition infantile. [Salmonella Agona contamination of young children: extension of withdrawal measures - recall of nutritional products for infants]. Paris: Direction générale de la Santé, Direction générale de la concurrence, de la consommation et de la répression des fraudes; 10 Dec 2017. French. Available from: https://www.economie.gouv.fr/files/files/directions_services/dgccrf/presse/communique/2017/251-CP-produits-de-nutrition-extension.pdf
6. Rappel par le groupe Lactalis de nouveaux produits fabriqués ou conditionnés sur son site de Craon. [Recall by the Lactalis group of new products manufactured or packaged at its Craon site]. Direction générale de la concurrence, de la consommation et de la répression des fraudes; 21 Dec 2017. French. Available from: https://www.economie.gouv.fr/files/files/directions_services/dgccrf/presse/communique/2017/cp_extension_retrait_par_lactalis.pdf
7. Contamination à *Salmonella* Agona de jeunes enfants [Salmonella Agona contamination of young children]. Paris: Direction générale de la concurrence, de la consommation et de la répression des fraudes; 18 Dec 2017. French. Available from: <https://www.economie.gouv.fr/dgccrf/contamination-a-salmonella-agona-jeunes-enfants>
8. *Salmonella enterica* ser. Agona (presence /250ml) in infant formula from France. Brussels: European Commission RASFF Portal; 2017. Available from: https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationDetail&NOTIF_REFERENCE=2017.2095
9. European Centre for Disease Prevention and Control (ECDC). Communicable diseases threats report: Week 1, 31 December 2017-6 January 2018. Stockholm: ECDC; 2017. Available from: <https://ecdc.europa.eu/sites/portal/files/documents/communicable-disease-threats-report-06-jan-2018.pdf>.

10. Française de Pédiatrie S. Recommandations de la Société Française de Pédiatrie (SFP) concernant le remplacement des préparations infantiles du groupe Lactalis Nutrition Santé (LNS). [Recommendations of the French Society of Pediatrics concerning the replacement of infant formulas of the Lactalis Nutrition Santé group]. Paris: Ministère des Solidarités et de la Santé; 2017. Available from: http://solidarites-sante.gouv.fr/IMG/pdf/recommandations_de_la_societe_francaise_de_pediatrie_vdef_corrige.pdf
11. Jourdan N, Le Hello S, Delmas G, Clouzeau J, Manteau C, Désaubliaux B, et al. Nationwide outbreak of *Salmonella enterica* serotype GIVE infections in infants in France, linked to infant milk formula, September 2008. *Euro Surveill.* 2008;13(39):18994. PMID: 18822245
12. Rowe B, Gilbert RJ, Begg NT, Hutchinson DN, Dawkins HC, Jacob M, et al. *Salmonella* ealing infections associated with consumption of infant dried milk. *Lancet.* 1987;2(8564):900-3. [https://doi.org/10.1016/S0140-6736\(87\)91384-5](https://doi.org/10.1016/S0140-6736(87)91384-5) PMID: 2889093
13. Park JK, Seok WS, Choi BJ, Kim HM, Lim BK, Yoon SS, et al. *Salmonella enterica* serovar London infections associated with consumption of infant formula. *Yonsei Med J.* 2004;45(1):43-8. <https://doi.org/10.3349/ymj.2004.45.1.43> PMID: 15004867
14. Centers for Disease Control and Prevention (CDC). *Salmonella* serotype Tennessee in powdered milk products and infant formula--Canada and United States, 1993. *MMWR Morb Mortal Wkly Rep.* 1993;42(26):516-7. PMID: 8515742
15. Usera MA, Echeita A, Aladueña A, Blanco MC, Reymundo R, Prieto MI, et al. Interregional foodborne salmonellosis outbreak due to powdered infant formula contaminated with lactose-fermenting *Salmonella virchow*. *Eur J Epidemiol.* 1996;12(4):377-81. <https://doi.org/10.1007/BF00145301> PMID: 8891542
16. Threlfall EJ, Ward LR, Hampton MD, Ridley AM, Rowe B, Roberts D, et al. Molecular fingerprinting defines a strain of *Salmonella enterica* serotype Anatum responsible for an international outbreak associated with formula-dried milk. *Epidemiol Infect.* 1998;121(2):289-93. <https://doi.org/10.1017/S0950268898001149> PMID: 9825779
17. Russo ET, Biggerstaff G, Hoekstra RM, Meyer S, Patel N, Miller B, et al. A recurrent, multistate outbreak of salmonella serotype agona infections associated with dry, unsweetened cereal consumption, United States, 2008. *J Food Prot.* 2013;76(2):227-30. <https://doi.org/10.4315/0362-028X.JFP-12-209> PMID: 23433369

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2018.

Cross-border outbreak of listeriosis caused by cold-smoked salmon, revealed by integrated surveillance and whole genome sequencing (WGS), Denmark and France, 2015 to 2017

Susanne Schjørring¹, Sofie Gillesberg Lassen², Tenna Jensen³, Alexandra Moura⁴, Jette S Kjeldgaard⁵, Luise Müller², Stine Thielke³, Alexandre Leclercq⁴, Mylene M Maury⁴, Mathieu Tourdjman⁶, Marie-Pierre Donguy⁷, Marc Lecuit^{4,8}, Steen Ethelberg², Eva M Nielsen¹

1. Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark
2. Department of Infectious Disease Epidemiology and Prevention, Statens Serum Institut, Copenhagen, Denmark
3. The Danish Veterinary and Food Administration, Copenhagen, Denmark
4. Institut Pasteur, National Reference Centre and WHO collaborating Center for Listeria, Biology of Infection Unit, Paris, France
5. National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark
6. Santé Publique France, the French Public Health Agency, Saint-Maurice, France
7. Ministry of Agriculture, and Forestry, Paris, France
8. Paris Descartes University, Sorbonne Paris Cité, Division of Infectious Diseases, Necker-Enfants Malades University Hospital, Institut Imagine, F-75743 Paris, France

Correspondence: Susanne Schjørring (SSC@ssi.dk)

Citation style for this article:

Schjørring Susanne, Gillesberg Lassen Sofie, Jensen Tenna, Moura Alexandra, Kjeldgaard Jette S, Müller Luise, Thielke Stine, Leclercq Alexandre, Maury Mylene M, Tourdjman Mathieu, Donguy Marie-Pierre, Lecuit Marc, Ethelberg Steen, Nielsen Eva M. Cross-border outbreak of listeriosis caused by cold-smoked salmon, revealed by integrated surveillance and whole genome sequencing (WGS), Denmark and France, 2015 to 2017. *Euro Surveill.* 2017;22(50):pii=17-00762. <https://doi.org/10.2807/1560-7917.ES.2017.22.50.17-00762>

Article submitted on 15 Nov 2017 / accepted on 14 Dec 2017 / published on 14 Dec 2017

In August 2017, an outbreak of six listeriosis cases in Denmark was traced to cold-smoked salmon, using epidemiological investigations and whole-genome sequencing (WGS) analyses. Exchange of genome sequences allowed identification in France of a food isolate from a salmon-derived product and a human isolate from 2016 within the same cgMLST cluster as the Danish isolates (L2-SL8-ST8-CT771). The salmon product came from a third European Union country. WGS can rapidly link human cases and food isolates across Europe.

Identification of the outbreak

In Denmark, on 23 August 2017, Statens Serum Institut (SSI) identified a genetic cluster of four human *Listeria monocytogenes* sequence type (ST) 8 isolates by core genome multilocus sequence typing (cgMLST) [1]. The allele calling was performed in BioNumerics (v7.6.2, Applied Maths, Belgium). We initiated an epidemiological investigation and notified the Danish Central Outbreak Management Group (collaboration between the Danish Veterinary and Food Administration (DVFA), the National Food Institute at the Technical University of Denmark (DTU) and SSI). On 25 August, two additional human isolates were found to belong to the same genetic cluster.

Raw sequence data of four outbreak isolates, SSI-AC382–5, are deposited at the European Nt Archive (ERS2039635-8).

Case definition

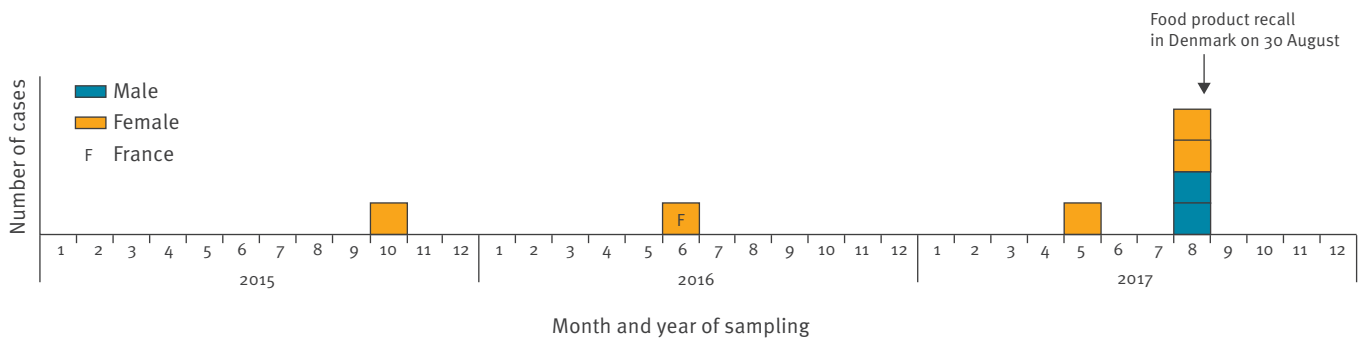
A confirmed case was defined as a person clinically diagnosed with listeriosis after 1 January 2017 with laboratory-confirmed *L. monocytogenes* ST8 clustering using cgMLST (≤ 5 allelic distance, single linkage). Cases diagnosed before 1 January 2017 with an isolate belonging to this cluster were defined as probable cases.

Case description and food exposure of Danish patients

As of 25 August 2017, the genetic cluster comprised six cases; five confirmed and one probable. Laboratory sample dates ranged from 25 October 2015 to 21 August 2017 (Figure 1). The age of the cases ranged from 59 to 96 years (median 80 years) and four were women. All patients had underlying illness and no travel history. One patient died within 30 days of diagnosis. Epidemiological investigations including a standard questionnaire on exposures showed that all five confirmed cases had consumed cold-smoked and/or cured salmon in the 30 days before disease onset. Four cases had bought the salmon in retail chain X. No other food-item was reported as consumed in high frequencies among cases. Epidemiological follow-up for

FIGURE 1

Number of probable and confirmed cases of listeriosis, by month and year of laboratory sample date, sex and country, Denmark and France, 2015–2017 (n=7 cases)



Unless otherwise specified with F, cases are from Denmark.

the probable case did not include information on fish consumption.

Food investigation and control measures

On 29 August 2017, a comparison between the human outbreak isolates and 16 *L. monocytogenes* ST8 food- and environmental isolates identified in Denmark from 2014 to August 2017 showed that the human isolates clustered with a food isolate from cold-smoked salmon, cut and packaged at company Y in Poland (zero to two allelic differences using cgMLST). *L. monocytogenes* had been detected on 31 July 2017 at levels of 110 CFU/g (threshold: 100 CFU/g) at the end of shelf life. The product was widely sold in Denmark and had been sampled by the DVFA in retail chain X, as part of a consumer exposure survey (i.e. analyses project on retail packages). Because the *L. monocytogenes* concentration had been just above the accepted limit and found at the end of the product shelf life a recall of this batch was not conducted. However, due to the positive finding, follow-up sampling had been performed on the 9 and 10 August 2017 from the central storage unit of retail chain X. *L. monocytogenes* had been isolated from two batches analysed before end of shelf life. In one sample from the same batches, which was also analysed at the end of the shelf life, on 28 August 2017 a *L. monocytogenes* level of 240 CFU/g was found. Isolates from the follow-up samples had zero to four allelic differences to the human outbreak isolates using cgMLST.

The human outbreak sequences were also compared to all *L. monocytogenes* ST8 genomes derived from clinical samples in Denmark from 2012 onwards. Although ST8 genomes from Danish patients in the period 2012–2017 showed high diversity, the outbreak isolates clearly formed a distinct cgMLST cluster with 16 allelic differences to the nearest isolates outside the genetic outbreak cluster and a maximum of nine allelic differences within the cluster (Figure 2a). We investigated the relatedness of outbreak isolates further by

single-nucleotide polymorphisms (SNP) analysis performed by both SSI and DTU using two analysis pipelines: Northern Arizona SNP Pipeline (NASP) [2] and CSI Phylogeny version 1.4 from Center for Genomics Epidemiology (CGE), DTU [3] leading to the same conclusion. The SNP analysis showed a maximum of nine SNPs between any two isolates in the cluster (Figure 2b). The food isolate sampled in Denmark in July was identical by SNP analysis to one of the Danish patient outbreak isolates from August 2017.

On 30 August 2017, DVFA advised retail chain X to recall all cold-smoked salmon produced at company Y. This advice was based on the elevated number of *L. monocytogenes* (240 CFU/g) found in the product at the end of shelf-life and the link to the outbreak. Retail chain X voluntarily recalled both cold-smoked and cured salmon produced at company Y. As part of the recall procedure, retail chain X informed company Y on the situation. Information from company Y, provided by the Polish food authorities via the European Union Rapid Alert System for Food and Feed (RASFF), showed that the implicated batches were exclusively sold via retail chain X and only in Denmark.

International enquiry and investigation in France

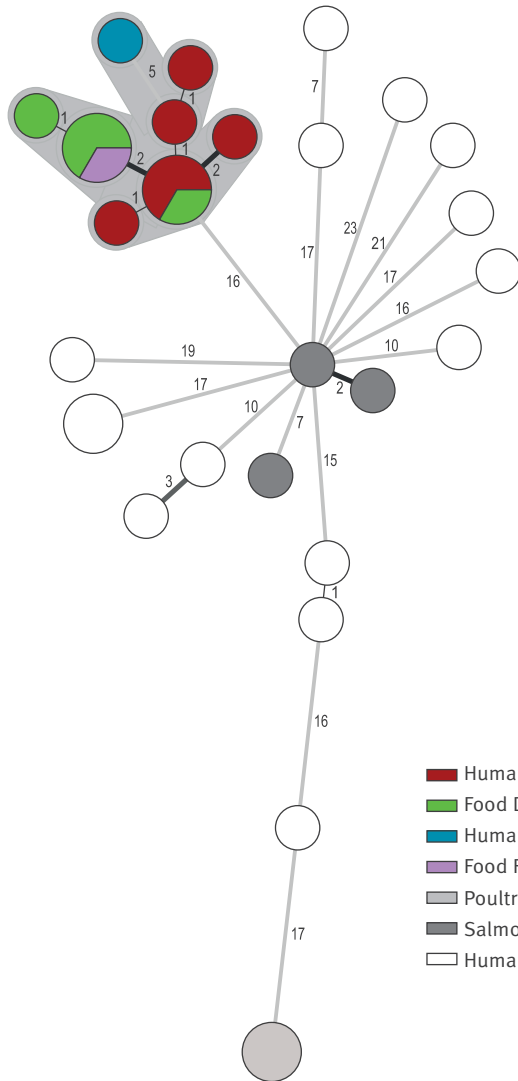
On 31 August 2017, the outbreak was notified internationally on different communication platforms: (i) the European Epidemic Intelligence Information System for food- and waterborne diseases (EPIS-FWD) reference UI-426, (ii) Early Warning and Response System (EWRS) notification reference EWRS20170831DK0001 and (iii) RASFF notification reference RASFF-2017.1319. The EPIS-FWD platform allowed sharing of files with assembled genomic sequence data. SSI distributed raw sequence data of four outbreak isolates (SSI-AC382-AC385) on a local ftp server at SSI.

The French National Reference Centre (NRC) for *Listeria* (Institut Pasteur, Paris), compared the

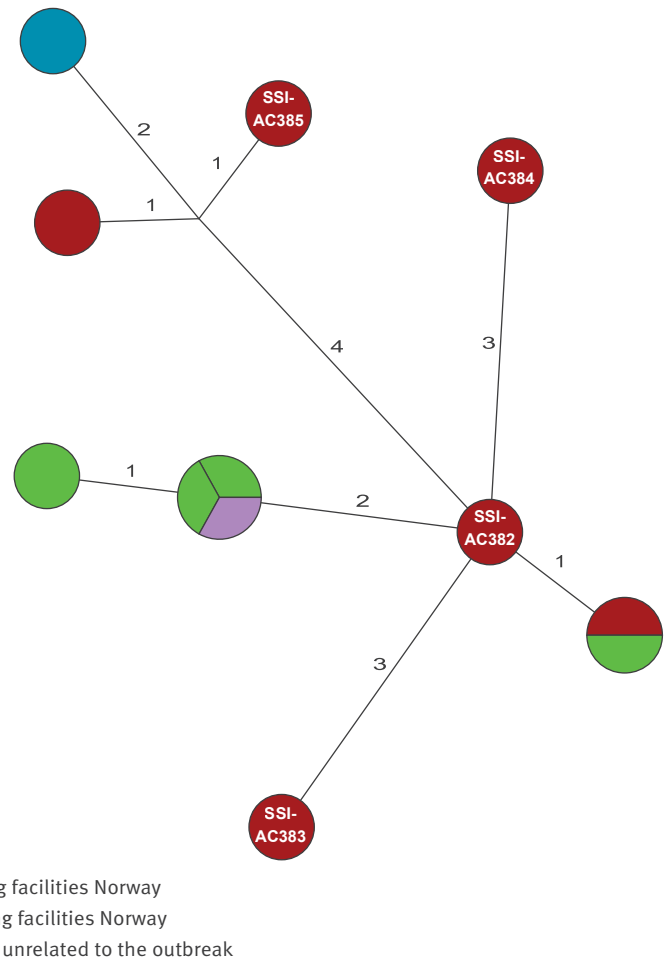
FIGURE 2

Whole genome sequencing based typing of *Listeria monocytogenes* ST8 isolates as part of a cross-border listeriosis outbreak investigation, Denmark and France, 2015–2017

A. Minimum spanning tree of cgMLST allelic profiles^a



B. Maximum parsimony tree based on whole-genome sequencing (WGS) data^b



cgMLST: core genome multilocus sequence typing; SSI: Statens Serum Institut.

The size of nodes corresponds to the number of isolates. Branch lengths represent the SNP difference.

^a Minimum spanning tree of cgMLST allelic profiles of 21 *L. monocytogenes* ST8 isolates from Danish patients in the period 2012–2017, 1 isolate from a probable French case 2016 and food isolates sampled in Denmark (4 isolates; July–August 2017) and France (1 isolate; September 2017) from products produced by company Y. The outbreak cluster is shaded in grey. Published sequences of ST8 isolates (2001–2013) from a study [17] on persistence in a poultry processing facility (SRR3099221, SRR3099222) and a salmon processing facility (SRR3099223, SRR3099224, SRR3099225) were included.

^b Maximum parsimony tree based on whole-genome sequencing (WGS) data showing single-nucleotide polymorphisms (SNPs) within the *L. monocytogenes* ST8 isolates from patients and food sources in the genetic outbreak cluster. One isolate (SSI-AC382, ERS2039635) from the cluster was used as the reference when using NASP.

sequences of the Danish human isolates against its database, using cgMLST as previously described [1,4]. A human isolate from a French resident belonged to the same cluster (L2-SL8-ST8-CT771) as the Danish isolates. This French probable case, a female patient in her mid-80s, was diagnosed in June 2016. Epidemiological investigations carried out by Santé Publique France were inconclusive, since food consumption history was not available at the time of diagnosis nor could information on travel to Denmark be retrieved, as the person had since died.

On 6 September 2017, an official control by the Ministry of Economy was carried out at a French retailer where a kosher chilled cured salmon was sampled for analysis. The sample was contaminated with *L. monocytogenes* at the level of 460 CFU/g and the salmon producer was company Y. An isolate was sent to the French NRC for typing and showed to belong to the same cgMLST type as the Danish outbreak (Figure 2). Further investigations on the food product confirmed that it had not been further processed after production in Poland. The product was recalled and no human cases were linked to its consumption as of beginning of December 2017.

The other nine countries that replied to the EPIS-FWD UI-426 notification (Austria, Finland, Germany, Luxembourg, the Netherlands, Norway, Slovenia, Sweden, United Kingdom) did not report any human or food isolates linked to the Danish outbreak. However, after submission of this report, at the end of November, we were informed through EPIS about three genetically linked human isolates in Germany.

Discussion

Here we report on a listeriosis outbreak and highlight the value of rapidly comparing the genomes of human and food/environmental isolates at the national and international levels.

The fact that the contaminated salmon products identified in Denmark and France were from different batches suggests environmental contamination possibly at the production facility at company Y. It is too early to assess whether any measures taken at company Y have been effective in controlling the outbreak. However, experiences from previous investigations suggest that once *L. monocytogenes* is detected in one product, 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 [5,6]. Moreover, the risk for *L. monocytogenes* persistent strains in the production environment requires the close monitoring for several years to ensure the elimination of these [7,8].

Since WGS was introduced for routine surveillance in Denmark, a number of listeriosis outbreaks have been detected and solved, including outbreaks involving

cold-smoked ready-to-eat sliced fish products [5]. The present investigation further reinforces the suspicion that ready-to-eat fish products are important sources of *L. monocytogenes* infections in Denmark, as well as in other countries.

Though only involving a low number of isolates, WGS *L. monocytogenes* surveillance and communication between countries allowed us to detect and rapidly solve this salmon-associated outbreak, leading to food product recall in two European countries. Compared with previous typing methods, WGS has a higher discriminatory power and the ability to determine genetic distance between isolates. The introduction of WGS for surveillance of food-borne infections has shown that it improves outbreak detection and facilitates outbreak investigations and likely helps reduce the number of infections [4,9-16]. The EPIS-FWD communication platforms allowed for the communication to link cases across borders. However, currently cross-border outbreaks are only detected when case numbers in at least one country exceed normal levels and are notified internationally. Therefore, a possible future system for easy exchange of and comparison of WGS data, e.g. by the use of an agreed cgMLST nomenclature, across borders will enable the identification of more dispersed outbreaks as well as cross-border links between food samples and human infections. This report highlights that by the application of cross-disciplinary and real-time cross-border comparison of WGS data, *L. monocytogenes* infections can be prevented and thereby providing safer food for at-risk groups such as the elderly, immunodeficient individuals and pregnant women.

Acknowledgements

We thank the Danish Clinical Microbiological Laboratories for rapid referral of isolates and support of the surveillance system. In addition, we thank the physicians and other health personnel for supplying clinical information; Marie Walther, Charlotte Munkstrup, Louise La Barbera Kastberg, for their assistance in interviewing patients. Furthermore, we thank Christian Vråby Pedersen and Anette Holm (SSI), Anne Mette Seyfarth (DFVA) the French National reference center technicians, and the PIBnet platform (Institut Pasteur) for excellent technical expertise in performing whole-genome sequencing. We also thank the staff at the DFVA Laboratory in Ringsted, Denmark and the regional food inspection units for collecting the environment and product samples. Part of this work was supported by the European Union's Horizon 2020 research and innovation program through COMPARE under grant agreement No. 643476.

Conflict of interest

None declared.

Authors' contributions

SS and SGL: participated in the outbreak investigation and drafted the manuscript. SS: conducted the typing of human isolates and comparison of human isolates as well as

comparison between human, environmental and product isolates. SGL: contributed to the clinical follow-up, patient interviews and the epidemiological investigation. TJ: was in charge of the trace-back investigations. AM, MMM, AL, ML: conducted the typing of human and food isolates in France and contributed to the writing of the manuscript. JSK: compared human, environmental and product isolates. LM: contributed to the clinical follow-up and the epidemiological investigations. ST: was involved in the trace-back investigations. AL: conducted the typing of human isolates and comparison of human isolates as well as comparison between human, environmental and product isolates in France and participated in the writing of the manuscript. MT, MD: conducted the human and food epidemiological investigations in France. SE: contributed to the epidemiological investigations and contributed to the writing of the manuscript. EMN: contributed to the laboratory and typing investigations as well as contributed to the writing of the manuscript.

References

- Moura A, Criscuolo A, Pouseele H, Maury MM, Leclercq A, Tarr C, et al. Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nat Microbiol*. 2016;2:16185. <https://doi.org/10.1038/nmicrobiol.2016.185> PMID: 27723724
- Sahl JW, Lemmer D, Travis J, Schupp JM, Gillette JD, Aziz M, et al. NASP: an accurate, rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. *Microb Genom*. 2016;2(8):e000074. <https://doi.org/10.1099/mgen.0.000074> PMID: 28348869
- Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One*. 2014;9(8):e104984. <https://doi.org/10.1371/journal.pone.0104984> PMID: 25110940
- Moura A, Tourdjman M, Leclercq A, Hamelin E, Laurent E, Fredriksen N, et al. Real-Time Whole-Genome Sequencing for Surveillance of *Listeria monocytogenes*, France. *Emerg Infect Dis*. 2017;23(9):1462-70. <https://doi.org/10.3201/eid2309.170336> PMID: 28643628
- Kvistholm Jensen A, Nielsen EM, Björkman JT, Jensen T, Müller L, Persson S, et al. Whole-genome Sequencing Used to Investigate a Nationwide Outbreak of Listeriosis Caused by Ready-to-eat Delicatessen Meat, Denmark, 2014. *Clin Infect Dis*. 2016;63(1):64-70. <https://doi.org/10.1093/cid/ciw192> PMID: 27025820
- Gillesberg Lassen S, Ethelberg S, Björkman JT, Jensen T, Sørensen G, Kvistholm Jensen A, et al. Two listeria outbreaks caused by smoked fish consumption-using whole-genome sequencing for outbreak investigations. *Clin Microbiol Infect*. 2016;22(7):620-4. <https://doi.org/10.1016/j.cmi.2016.04.017> PMID: 27145209
- Holch A, Webb K, Lukjancenko O, Ussery D, Rosenthal BM, Gram L. Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Appl Environ Microbiol*. 2013;79(9):2944-51. <https://doi.org/10.1128/AEM.03715-12> PMID: 23435887
- Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J Food Prot*. 2014;77(1):150-70. <https://doi.org/10.4315/0362-028X.JFP-13-150> PMID: 24406014
- Yde M, Naranjo M, Mattheus W, Stragier P, Pochet B, Beulens K, et al. Usefulness of the European Epidemic Intelligence Information System in the management of an outbreak of listeriosis, Belgium, 2011. *Euro Surveill*. 2012;17(38):20279. PMID: 23040965
- Ronholm J, Naseri N, Petronella N, Pagotto F. Navigating Microbiological food safety in the Era of Whole-Genome sequencing. *Clin Microbiol Rev*. 2016;29(4):837-57. <https://doi.org/10.1128/CMR.00056-16> PMID: 27559074
- Kwong JC, Mercouliou K, Tomita T, Easton M, Li HY, Bulach DM, et al. Prospective whole-genome sequencing enhances national surveillance of *Listeria monocytogenes*. *J Clin Microbiol*. 2016;54(2):333-42. <https://doi.org/10.1128/JCM.02344-15> PMID: 26607978
- Jackson BR, Tarr C, Strain E, Jackson KA, Conrad A, Carleton H, et al. Implementation of Nationwide Real-time Whole-genome Sequencing to Enhance Listeriosis Outbreak Detection and Investigation. *Clin Infect Dis*. 2016;63(3):380-6. <https://doi.org/10.1093/cid/ciw242> PMID: 27090985
- Kleta S, Hammerl JA, Dieckmann R, Malorny B, Borowiak M, Halbedel S, et al. Molecular Tracing to Find Source of Protracted Invasive Listeriosis Outbreak, Southern Germany, 2012-2016. *Emerg Infect Dis*. 2017;23(10):1680-3. <https://doi.org/10.3201/eid2310.161623> PMID: 28930013
- Comandatore F, Corbella M, Andreoli G, Scaltriti E, Aguzzi M, Gaiarsa S, et al. Genomic Characterization Helps Dissecting an Outbreak of Listeriosis in Northern Italy. *PLoS Curr*. 2017;9:ecurrents.outbreaks.633fd8994e9f06f31b3494567c7e504c680c. PMID: 28856063
- Dahl V, Sundqvist L, Hedenström I, Löfdahl M, Alm E, Ringberg H, et al. A nationwide outbreak of listeriosis associated with cold-cuts, Sweden 2013-2014. *Infect Ecol Epidemiol*. 2017;7(1):1324232. <https://doi.org/10.1080/20008686.2017.124232> PMID: 28649304
- Schmid D, Allerberger F, Huhulescu S, Pietzka A, Amar C, Kleta S, et al. Whole genome sequencing as a tool to investigate a cluster of seven cases of listeriosis in Austria and Germany, 2011-2013. *Clin Microbiol Infect*. 2014;20(5):431-6. <https://doi.org/10.1111/1469-0691.12638> PMID: 24698214
- Fagerlund A, Langsrud S, Schirmer BC, Møretrø T, Heir E. Genome Analysis of *Listeria monocytogenes* Sequence Type 8 Strains Persisting in Salmon and Poultry Processing Environments and Comparison with Related Strains. *PLoS One*. 2016;11(3):e0151117. <https://doi.org/10.1371/journal.pone.0151117> PMID: 26953695

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2017.

Outbreak of *Salmonella* Bovismorbificans associated with the consumption of uncooked ham products, the Netherlands, 2016 to 2017

Diederik Brandwagt^{1,2,3}, Cees van den Wijngaard¹, Anna Dolores Tulen¹, Annemieke Christine Mulder¹, Agnetha Hofhuis¹, Rianne Jacobs⁴, Max Heck¹, Anjo Verbruggen¹, Hans van den Kerkhof¹, Ife Slegers-Fitz-James⁵, Lapo Mughini-Gras¹, Eelco Franz¹

1. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
2. European Programme for Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
3. Municipal Health Service (GGD) Utrecht Region, Zeist, the Netherlands
4. Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
5. Netherlands Food and Consumer Product Safety Authority (NVWA), Utrecht, the Netherlands

Correspondence: Diederik Brandwagt (diederik.brandwagt@rivm.nl)

Citation style for this article:

Brandwagt Diederik, van den Wijngaard Cees, Tulen Anna Dolores, Mulder Annemieke Christine, Hofhuis Agnetha, Jacobs Rianne, Heck Max, Verbruggen Anjo, van den Kerkhof Hans, Slegers-Fitz-James Ife, Mughini-Gras Lapo, Franz Eelco. Outbreak of *Salmonella* Bovismorbificans associated with the consumption of uncooked ham products, the Netherlands, 2016 to 2017. *Euro Surveill.* 2018;23(1):pii=17-00335. <https://doi.org/10.2807/1560-7917.ES.2018.23.1.17-00335>

Article submitted on 19 May 2017 / accepted on 04 Oct 2017 / published on 04 Jan 2018

In January 2017, an increase in reported *Salmonella* *serica* serotype Bovismorbificans cases in the Netherlands was observed since October 2016. We implemented a case–control study to identify the source, including all cases after December 2016. Adjusted odds ratios were calculated using logistic regression analysis. We traced back the distribution chain of suspected food items and sampled them for microbiological analysis. Human and food isolates were sequenced using whole genome sequencing (WGS). From October 2016 to March 2017, 54 *S. Bovismorbificans* cases were identified. Sequencing indicated that all were infected with identical strains. Twenty-four cases and 37 controls participated in the study. Cases were more likely to have consumed ham products than controls (aOR = 13; 95% CI: 2.0–77) and to have shopped at a supermarket chain (aOR = 7; 95% CI: 1.3–38). Trace-back investigations led to a Belgian meat processor: one retail ham sample originating from this processor tested positive for *S. Bovismorbificans* and matched the outbreak strain by WGS. All ham products related to the same batch were removed from the market to prevent further cases. This investigation illustrates the importance of laboratory surveillance for all *Salmonella* serotypes and the usefulness of WGS in an outbreak investigation.

Introduction

With ca 27,000 infections per year, salmonellosis is among the most frequent zoonotic infections in the Netherlands [1]. Individual cases of salmonellosis are not notifiable in the Netherlands, except for (para)

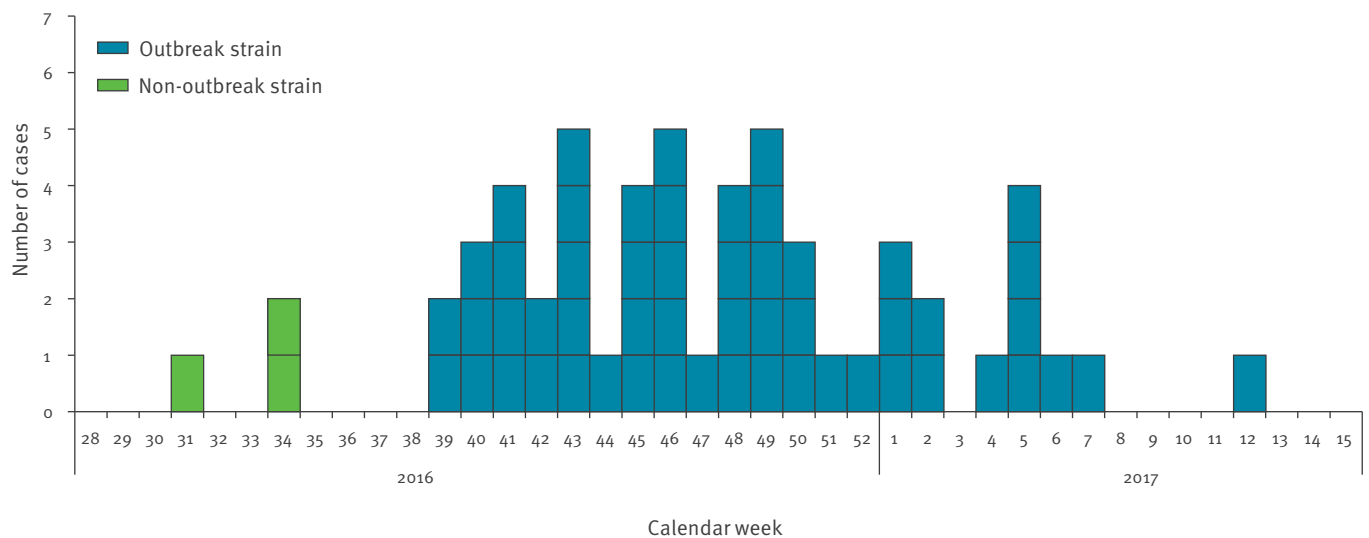
typhoid fever. The detection of trends in *Salmonella* serotype distribution therefore depends on the nationwide laboratory surveillance network for gastroenteric pathogens, established in 1987 and covering ca 64% of the population of the Netherlands [2].

The event

In January 2017, an increase in the number of cases of *Salmonella* Bovismorbificans infections in the Netherlands was reported. From week 41 of 2016 (9 October) until January 2017, 32 *S. Bovismorbificans* cases (one to five per week) were reported to the Center for Disease Control (Cib) of the Dutch National Institute for Public Health and the Environment (RIVM). This number exceeded the expected three to 14 cases of this serotype per year as observed from 2005 to 2015 and was the first possible outbreak of this serotype reported in the Netherlands since the implementation of the laboratory surveillance network [1]. Through an urgent inquiry (UI-393) in the European Centre for Disease Prevention and Control's Epidemic Intelligence Information System (EPIS), a concurrent increase was reported in Belgium to 32 cases in 2016. An increase in the number of *S. Bovismorbificans* cases was also observed in France where 47 cases were noted in 2016. An outbreak investigation was initiated to find the source of the outbreak and thereby prevent further cases.

FIGURE 1

Weekly number of reported cases of *Salmonella Bovismorbificans* by date of symptom onset, the Netherlands, 2016–2017 (n = 54 outbreak cases, n = 3 non-outbreak cases)



Methods

Epidemiological investigation

Case definition

A case was defined as a person with laboratory-confirmed *S. Bovismorbificans* infection, reported since October 2016, in the Netherlands. This date was selected because the increase in cases was seen since week 41 (starting on 9 October 2016). To generate hypotheses about the source of infection, cases were interviewed using a standardised trawling questionnaire. This questionnaire covered the consumption of different meat products, fish, dairy products, vegetables and fruits, snacks; establishments where food was purchased; if there was contact with a person with diarrhoea and if there was contact with animals during the 7 days before onset of gastro-intestinal symptoms. To address possible recall bias, we only interviewed cases reported since December 2016.

Case-control study

As these interviews did not lead to a clear hypothesis, a case-control study was initiated to further explore likely sources. Controls, matched to cases by age, sex and residence municipality, were randomly selected from population registers. Univariable and multivariable odds ratios (OR) and 95% confidence intervals (95% CI) for putative risk factors for *S. Bovismorbificans* infection were calculated using logistic regression analysis. All factors associated in the univariable analysis at $p < 0.05$ were included in the multivariable analysis based on backward variable selection to yield a model with the most relevant independent risk factors.

Cases who returned the questionnaire were requested to provide additional information on suspected

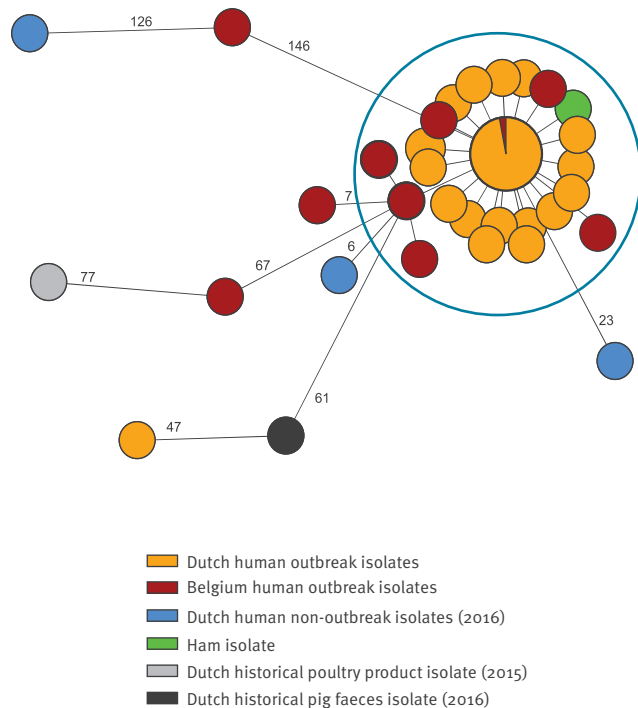
products consumed. This included information on the supermarket chain, producer, product type and European product identification marks of products they bought in the week before symptom onset or that they were buying regularly. This detailed information was shared with the Netherlands Food and Consumer Product Safety Authority (NVWA) to enable them to do a trace-back investigation for suspected food items.

Whole genome sequencing

In collaboration with the European Centre for Disease Prevention and Control (ECDC), all available Dutch isolates of *S. Bovismorbificans* from human cases in 2016 and those from the other affected countries were sequenced in January 2017, concurrently with the start of the outbreak investigation, using whole genome sequencing (WGS). The Dutch isolates from cases from January to April 2017 were sequenced at a later stage. Sequencing libraries were prepared using Nextera XT chemistry (Illumina Inc., San Diego, United States (US)) for a 250-bp paired-end sequencing run on an Illumina MiSeq sequencer. Samples were sequenced to aim for minimum coverage of 100-fold using Illumina's recommended standard protocols. The resulting FASTQ files de novo were assembled using the SPAdes assembler [3]. Core genome multilocus sequence typing (cgMLST) analysis was conducted using Ridom SeqSphere+ software (version 3.5.0, Ridom GmbH, Münster, Germany) [4]. A Spades assembly of *S. Bovismorbificans* SRR4099590 (<http://www.ebi.ac.uk/ena/data/view/SRR4099590>) was used as reference to construct a cgMLST gene set by the MLST+ target definer (version 1.1) function of SeqSphere+ with default parameters. Subsequently, the set of outbreak isolates were analysed using this cgMLST scheme and a minimum spanning tree was constructed from the allelic profile using the parameter 'pairwise ignore missing values' during distance calculation. One representative human

FIGURE 2

Minimum spanning tree of *Salmonella Bovismorbificans* strains based on core genome multilocus sequence typing analysis, the Netherlands, October 2016–March 2017



The tree was constructed from core-genome MLST allelic profile using the parameter 'pairwise ignore missing values' during distance calculation. Numbers correspond to the number of alleles difference between isolates/clusters. Isolates within five alleles difference (i.e. outbreak cluster) are within the blue circle.

outbreak isolate was uploaded to Enterobase (SAL_KA8933AA) (<https://enterobase.warwick.ac.uk/>). Persons who were included as a case in the study were excluded from the analysis if sequencing demonstrated the *S. Bovismorbificans* isolated was different (more than five alleles difference) than the outbreak strain.

To have more insight into the presence of the outbreak strain in historical isolates, we searched Enterobase for closely related *S. Bovismorbificans* strains, in both human and non-human isolates. To investigate the presence of the outbreak strain in non-human samples in the Netherlands, we also searched the RIVM surveillance database for all known isolates of *S. Bovismorbificans* in the Netherlands. Relevant strains underwent WGS and were included in the minimum spanning tree.

Results

Epidemiological investigation

From October 2016 to March 2017, 54 cases of *S. Bovismorbificans* were reported in the Netherlands (Figure 1). The cases were 5 to 90 years of age

(median 65 years; interquartile range (IQR): 49–73), and 29 were female. The cases were not equally distributed across the country as the majority of them were living in the less densely populated regions in the east and south of the country.

Case-control study

Twenty-nine cases were reported since December 2016 and consented to participate in the outbreak investigation. Of those, 24 cases responded and participated in the case-control study, together with 37 controls. The day of onset of symptoms for cases varied between 28 November 2016 and 4 February 2017. The cases were 5 to 89 years of age (median 66 years; IQR 45–76) and 14 were female, consistent with the profile of all identified cases. Most frequently reported symptoms were diarrhoea (17 cases) and stomach pain (15 cases), while fever was reported by 11 cases. Fifteen cases were admitted to the hospital due to the severity of their illness, hospitalised cases were 27 to 89 years of age (median 68 years; IQR 43–77).

Food items with the highest frequency of consumption among cases were ham and cheese products. In the univariable analysis, cases were more likely to have consumed raw ham (OR = 12.6; 95% CI: 2.2–125.5) and smoked ham (OR = 5.6; 95% CI: 1.1–36.5) than controls, but were not more likely to have consumed any of the individual cheese products. Furthermore, cases were also more likely to have shopped at a certain supermarket chain (chain 13, see Table). As the participants in both groups did not always remember what specific type of products they had consumed in the incubation period, we merged ham and cheese products into one pooled ham variable (raw, smoked and Coburg ham) and one pooled cheese variable (unsliced, sliced and grated), respectively. In the univariable analysis, cases were more likely to have consumed both ham products (OR = 7.1; 95% CI: 1.9–27.2) and cheese products (OR = 5.6; 95% CI: 1.4–24.7) than controls.

In the multivariable model, we also used the pooled ham and pooled cheese variables. Multivariable analysis confirmed that cases were more likely to have consumed ham products than controls and to have shopped at supermarket chain 13 (Table); consumption of cheese products was not significant and was omitted in the final model.

Based on the results of the case-control study, all cases that were willing to be contacted for further investigation were interviewed again for detailed information on the consumption of ham and cheese products. None of the cases had product leftovers from the exposure period, but three cases provided a photograph of a product as they reported always consuming that same ham product. The identification marks of these products together with supplier information from the suspected supermarket provided some direction in the trace-back investigation.

TABLE

Associations between *Salmonella Bovismorbificans* infection and food consumption or purchases at supermarkets, the Netherlands, September 2016 – March 2017.

Food consumption and supermarkets	Cases (n = 24)	Controls (n = 37)	OR	Adjusted OR	95% CI
Ham pooled ^a	15	7	7.1 ^b	12.5	2.0–76.6
Cheese pooled ^{c,d}	20	18	5.6 ^a	NA	NA
Supermarket chain 13	17	16	4.5 ^a	7.1	1.3–37.9

CI: confidence interval; NA: not applicable; OR: odds ratio.

^a Raw, smoked and Coburg ham.

^b p value < 0.05.

^c Unsliced, sliced and grated cheese.

^d Omitted in final model (not significant).

Trace-back investigation

The trace-back investigation by the NVWA led to a Belgian meat processor. In cooperation with the Belgian authorities, an inspection was performed by local authorities at the meat processor. No evidence was found for possible contamination during the production process. There were no positive samples of incoming meat and the production of raw ham was completely separated from other meat products to prevent cross contamination. The trace-back investigation in Belgium was not further extended to the farm level. The meat processor intensified its controls after the inspection. The NVWA then decided to focus further investigations on wholesalers and supermarkets in the Netherlands that were supplied by the Belgian meat producer. Samples of both half-finished products and finished (for retail) ham products were taken at several levels in the production chain to be analysed for the presence of *Salmonella*.

In April 2017, 4 weeks after the last case was reported in the Netherlands, one of the collected retail ham products (smoked Coburg ham, sliced at supermarket) tested positive for *Salmonella*. All ham products related to this batch were withdrawn from the Dutch market and the NVWA used the European Rapid Alert System for Food and Feed (RASFF) system to alert the authorities in other European countries. Further serotyping identified the *Salmonella* as serotype Bovismorbificans.

Whole genome sequencing

Sequencing of human *S. Bovismorbificans* isolates indicated that all isolates taken since October 2016 had less than five alleles difference and were therefore part of the same outbreak. All outbreak strains were ST142 (<http://www.genomicepidemiology.org>, accessed 18 August 2017). No acquired resistance genes were detected (ResFinder 2.1 <http://www.genomicepidemiology.org>, accessed 18 August 2017). Outbreak strains possessed the small *Escherichia coli* plasmid pIGJ156 (*col156*, NC_009781, 5,146 bp, 98% homology) with no predicted accessory genes that code for any resistance (PlasmidFinder 1.3, <http://www.genomicepidemiology.org>, accessed 18 August 2017). Next to the

Dutch isolates, seven isolates from Belgian patients and two isolates from French patients also matched the outbreak strain. The isolates from other countries and the Dutch patient isolates reported before October 2016 did not match the outbreak strain. WGS indicated that the strain of *S. Bovismorbificans* found in the ham product was identical to the outbreak strain (Figure 2).

In the Enterobase database, 178 hits for *S. Bovismorbificans* ST142 were found with isolation dates from 1970 to 2017. Of those, 26 were non-human isolates. The most closely related strains based on the Enterobase cgMLST V2 were strain SAL_KA7180AA (United Kingdom (UK), unknown source and year of isolation) and SAL_KA4075AA isolated from the Thames River (UK, 2009). In the RIVM surveillance database for the Netherlands, 11 isolates of *S. Bovismorbificans* from non-human sources were recorded. A pig faeces isolate from 2016 and a poultry food product isolate from 2015 (both ST142) underwent WGS. Neither of the strains matched the outbreak strain (Figure 2).

Discussion

The case–control study identified the consumption of ham products as the vehicle of the outbreak. This finding was confirmed by the identification of *S. Bovismorbificans* in a ham product from a Dutch supermarket, originating from a suspected meat processor, leading to a recall of related ham products to prevent further cases from occurring.

With 57 known cases (54 from the outbreak investigation and three cases that were reported later) over a period of 7 months, this outbreak was one of the smaller outbreaks of *Salmonella* detected in the Netherlands in the past 10 years. With 15 known hospitalisations among 24 interviewed cases, the hospitalisation rate in this outbreak was high compared with the estimated 3% hospitalisations (1,021 hospitalisations of 32,210 salmonella cases) in 2016 for all salmonellosis cases across the country [1]. This suggests the outbreak strain might be more pathogenic than other *Salmonella* strains, thus leading to a higher burden of disease for this specific strain.

However, as the number of cases was small, we expect the burden of disease from this outbreak to be limited as the total burden of disease from salmonellosis in the Netherlands is already low compared with some other food-borne pathogens [5,6]. Moreover, the burden of disease estimates of salmonellosis in the Netherlands is based on an overall under-reporting factor of 26 [7]. A more pathogenic strain, which we anticipated was the case in this outbreak, would probably lead to lower rate of under-reporting, suggesting that we may have seen a relatively high number of true cases compared with other *Salmonella* outbreaks.

Smaller and larger outbreaks of *Salmonella* are detected frequently through the Dutch surveillance network, but this was the first outbreak of *S. Bovismorbificans* ever reported in the Netherlands. With three to 14 cases per year (which is less than 1% of all reported cases per year), *S. Bovismorbificans* is one of the less-frequently observed serotypes of *Salmonella* in the Netherlands. This low incidence is comparable with the number of cases reported by other European countries [8]. Such a low background incidence facilitated detection of the increase and the subsequent start of an outbreak investigation. However, since the beginning of the investigation almost no new cases were reported for a period of 4 weeks. With the knowledge that several previous small outbreaks of rare serovars of *Salmonella* had stopped spontaneously, the recruitment of controls was postponed until enough new cases had been reported. Because of this, the start of the analytical study was delayed until the beginning of February.

The investigation led to (uncooked) ham products as the most likely vehicle of transmission in this outbreak. The consumption of raw pork is a known risk factor for salmonellosis; in the Netherlands an estimated 40% of salmonellosis cases are attributed to the consumption of pork [1]. Based on historical data and model-based source attribution analyses, pigs are the primary reservoir of *S. Bovismorbificans* [9]. However, vehicles of transmission detected in outbreaks from Finland, Australia the United States and Germany, include sprouts, hummus and lettuce [10-15]. Only one outbreak of *S. Bovismorbificans* in Germany from 2004 to 2005 was associated with the consumption of pork [16].

Only 15 of 24 cases enrolled in the study reported exposure to ham products in their incubation period. This could be the result of recall bias, as many cases were interviewed 4 to 6 weeks after the day of onset of gastro-intestinal symptoms, i.e. in some cases more than 7 weeks after the exposure. Another explanation could be cross-contamination of other products within individuals' homes. We assume it unlikely that other food products were involved in the outbreak, as no new cases were reported with the outbreak strain after the withdrawal of the contaminated batch. The low number of positively tested food samples from the incriminated batch of ham suggests that the infectious dose of the

contaminated batch was probably not very high. This could also be an explanation for the small number of cases in this outbreak.

WGS indicated that this outbreak was a multi-country outbreak, with a small number of isolates from Belgium and France being identical to the outbreak strain. The number of confirmed cases in these countries was too low to perform epidemiological studies. However, as the trace-back investigation led to a Belgian meat processor, it is likely that contaminated products were also available in Belgian stores and possibly in France.

This outbreak of a rare serotype of *Salmonella*, which led to a recall of suspected ham products, confirms once more the relevance of the Dutch laboratory surveillance system for non-(para)typhi *Salmonella* spp. infections outbreak detection, including that for rare serotypes. In the past 10 years, several outbreaks of *Salmonella* were detected by the system, including a large outbreak of *S. Thompson* in 2012, several smaller outbreaks of *S. Typhimurium* in 2014 and 2015, and a multi-country outbreak of *S. Enteritidis* in 2016 [1,17]. For smaller outbreaks it was not possible to find the source because in some instances the outbreak ended before an outbreak investigation could be initiated or the number of cases was too small to start a case-control study to find an association. In the presented outbreak, the number of cases was also relatively small, but the association with the contaminated food product in the case-control study was strong enough to start a trace-back investigation. The association could have been stronger with a matched analysis design, but the number of discordant pairs was too small.

Lessons learned

Two key factors in this outbreak investigation were the close collaboration between epidemiologists, microbiologists and food experts of the NVWA from the onset of the outbreak investigation and the use of WGS. Furthermore, the use of a broad trawling questionnaire in the beginning of the outbreak was found to be important. Pork products were not an unexpected source for salmonellosis, but the level of detail in the questionnaire pointed to certain types of ham used as sandwich filling as possible sources of interest already in an early phase.

Acknowledgements

We acknowledge the cases and controls, the officers from the public health services, and the physicians and microbiologists whose collaboration made this investigation possible. We thank Ingrid Friesema and Roan Pijnacker for their assistance in data management. Furthermore, we thank Aloys Tijsma, Stefanie Schoss and their colleagues of the NVWA involved in the trace-back investigation and recall of the batch, as well as the technicians of the Centre for Infectious Disease Diagnostics and Screening (IDS) of the RIVM.

This study was financed from the regular budget of the Centre for Infectious Disease Control of the RIVM, and we thank the

European Centre for Disease Prevention and Control (ECDC) for financially supporting the WGS of isolates.

Conflict of interest

None declared.

Authors' contributions

Study design: DB, CW, AH, EF

Epidemiological study: DB, CW, AT, AM, RJ, LM

Microbiological analysis: MH, AV, EF

Trace-back investigation: IF

Data analysis: DB, AT, AH, RJ, LM, EF

Manuscript writing: DB, CW, IF

Manuscript revision: AH, LM, HK, EF

References

1. National Institute for Public Health and the Environment (RIVM). Staat van Zoönosen 2016.[State of Zoonotic Diseases 2016]. Bilthoven: RIVM; 2017. Dutch. Available from: http://www.rivm.nl/Documenten_en_publicaties/Wetenschappelijk/Rapporten/2017/november/Staat_van_Zo_nosen_2016
2. van Pelt W, de Wit MA, Wannet WJ, Ligtvoet EJ, Widdowson MA, van Duynhoven YT. Laboratory surveillance of bacterial gastroenteric pathogens in The Netherlands, 1991-2001. *Epidemiol Infect.* 2003;130(3):431-41. PMID: 12825727
3. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol.* 2012;19(5):455-77. <https://doi.org/10.1089/cmb.2012.0021> PMID: 22506599
4. Jünemann S, Sedlazeck FJ, Prior K, Albersmeier A, John U, Kalinowski J, et al. Updating benchtop sequencing performance comparison. *Nat Biotechnol.* 2013;31(4):294-6. <https://doi.org/10.1038/nbt.2522> PMID: 23563421
5. Havelaar AH, Haagsma JA, Mangen MJ, Kemmeren JM, Verhoef LP, Vijgen SM, et al. Disease burden of foodborne pathogens in the Netherlands, 2009. *Int J Food Microbiol.* 2012;156(3):231-8. <https://doi.org/10.1016/j.ijfoodmicro.2012.03.029> PMID: 22541392
6. Mangen MJ, Friesema IHM, Bouwknegt M, van Pelt W. Disease burden of food-related pathogens in the Netherlands, 2015. Bilthoven: National Institute for Public Health and the Environment (RIVM); 2017. Available from: <http://www.rivm.nl/bibliotheek/rapporten/2017-0060.pdf>
7. Havelaar AH, Ivarsson S, Löfdahl M, Nauta MJ. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. *Epidemiol Infect.* 2013;141(2):293-302. <https://doi.org/10.1017/S0950268812000568> PMID: 22717051
8. European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control. (ECDC), 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal* 2017;15(12):5077, 228 pp. Available from: <https://doi.org/10.2903/j.efsa.2017.5077>
9. Mughini-Gras L, Enserink R, Friesema I, Heck M, van Duynhoven Y, van Pelt W. Risk factors for human salmonellosis originating from pigs, cattle, broiler chickens and egg laying hens: a combined case-control and source attribution analysis. *PLoS One.* 2014;9(2):e87933. <https://doi.org/10.1371/journal.pone.0087933> PMID: 24503703
10. Puohiniemi R, Heiskanen T, Siitonen A. Molecular epidemiology of two international sprout-borne Salmonella outbreaks. *J Clin Microbiol.* 1997;35(10):2487-91. PMID: 9316894
11. Mattila L, Leirisalo-Repo M, Pelkonen P, Koskimies S, Granfors K, Siitonen A. Reactive arthritis following an outbreak of Salmonella Bovismorbificans infection. *J Infect.* 1998;36(3):289-95. [https://doi.org/10.1016/S0163-4453\(98\)94243-8](https://doi.org/10.1016/S0163-4453(98)94243-8) PMID: 9661939

12. Stafford RJ, McCall BJ, Neill AS, Leon DS, Dorricott GJ, Towner CD, et al. A statewide outbreak of Salmonella bovisorbificans phage type 32 infection in Queensland. *Commun Dis Intell Q Rep.* 2002;26(4):568-73. PMID: 12549525
13. Rimhanen-Finne R, Niskanen T, Lienemann T, Johansson T, Sjöman M, Korhonen T, et al. A nationwide outbreak of Salmonella bovisorbificans associated with sprouted alfalfa seeds in Finland, 2009. *Zoonoses Public Health.* 2011;58(8):589-96. <https://doi.org/10.1111/j.1863-2378.2011.01408.x> PMID: 21824360
14. Centers for Disease Control and Prevention (CDC). Multistate outbreak of Salmonella serotype Bovismorbificans infections associated with hummus and tahini--United States, 2011. *MMWR Morb Mortal Wkly Rep.* 2012;61(46):944-7. PMID: 23169315
15. Knoblauch AM, Bratschi MW, Zuske MK, Althaus D, Stephan R, Hächler H, et al. Cross-border outbreak of Salmonella enterica ssp. enterica serovar Bovismorbificans: multiple approaches for an outbreak investigation in Germany and Switzerland. *Swiss Med Wkly.* 2015;145:w14182. PMID: 26430716
16. Gilsdorf A, Jansen A, Alpers K, Dieckmann H, van Treck U, Hauri AM, et al. A nationwide outbreak of Salmonella Bovismorbificans PT24, Germany, December 2004-March 2005. *Euro Surveill.* 2005;10(3):E050324.1. Available from: <https://doi.org/10.2807/esw.10.12.02667-en> PMID: 16702643
17. Friesema I, de Jong A, Hofhuis A, Heck M, van den Kerkhof H, de Jonge R, et al. Large outbreak of Salmonella Thompson related to smoked salmon in the Netherlands, August to December 2012. *Euro Surveill.* 2014;19(39):20918. <https://doi.org/10.2807/1560-7917.ES2014.19.39.20918> <https://doi.org/10.2807/1560-7917.ES2014.19.39.20918> PMID: 25306981

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2018.

Re-evaluation of a 2014 multi-country European outbreak of *Salmonella* Enteritidis phage type 14b using recent epidemiological and molecular data

Stefan Hörmansdorfer¹, Ute Messelhäußer¹, Albert Rampp¹, Katharina Schönberger¹, Tim Dallman², Franz Allerberger³, Christian Kornschober³, Andreas Sing¹, Peter Wallner¹, Andreas Zapf¹

1. Bavarian Health and Food Safety Authority, Oberschleißheim, Germany

2. Gastrointestinal Bacterial Reference Unit, London, United Kingdom

3. Austrian Agency for Health and Food Safety (AGES), NRC Salmonella, Graz, Austria

Correspondence: Stefan Hörmansdorfer (stefan.hoermansdorfer@lgl.bayern.de)

Citation style for this article:

Hörmansdorfer Stefan, Messelhäußer Ute, Rampp Albert, Schönberger Katharina, Dallman Tim, Allerberger Franz, Kornschober Christian, Sing Andreas, Wallner Peter, Zapf Andreas. Re-evaluation of a 2014 multi-country European outbreak of *Salmonella* Enteritidis phage type 14b using recent epidemiological and molecular data. *Euro Surveill.* 2017;22(50):pii=17-00196. <https://doi.org/10.2807/1560-7917.ES.2017.22.50.17-00196>

Article submitted on 13 Mar 2017 / accepted on 15 Aug 2017 / published on 14 Dec 2017

A European multi-country outbreak of *Salmonella* Enteritidis phage type (PT) 14b occurred from March to November 2014 associated with the consumption of eggs. The outbreak involved more than 400 human cases from France, Luxembourg, Austria and the United Kingdom. In 2016–2017, it has been re-evaluated combining recent epidemiological results with latest molecular data. The outbreak was traced back to one large Bavarian egg producer with four distinct premises, three located in Bavaria, one in the Czech Republic. The outbreak isolates of *S. Enteritidis* PT 14b were grouped into three closely related clades by whole genome sequencing. Two of these clades could be referred to two Bavarian premises of the egg producer on the basis of epidemiological and molecular data, while epidemiological data presumably linked the third clade to another premises of the egg producer. Interestingly and in contrast to the situation in other European countries where several outbreaks were documented, all notified 91 laboratory-confirmed cases of *S. Enteritidis* PT 14b from Bavaria were sporadic, singular cases not belonging to any epidemiological outbreaks. In conclusion, as demonstrated here, the resolution of food-related outbreaks with such a high discriminatory power is rare in outbreak investigation.

Introduction

Salmonella enterica ssp. *enterica* serovar Enteritidis (*S. Enteritidis*) is one of over 2,600 serovars of the genus *Salmonella* with zoonotic potential and often associated with food-borne human disease. Its reservoir is the intestinal tract of both humans and animals. *S. Enteritidis* forms a strongly clonal group with low genetic heterogeneity, whose members can be sufficiently discriminated from each other only by modern molecular techniques [1–6].

The incidence of human salmonellosis has decreased steadily in recent years. Nevertheless, in 2014, 88,175 confirmed cases of human salmonellosis causing 9,830 hospitalisations and 65 fatalities were reported across the European Union (EU). Among these, 16,000 cases of human salmonellosis were reported from Germany. As in previous years, *S. Enteritidis* was the predominant serovar (44.4% of all isolates) followed by *S. Typhimurium* (17.4%) and *S. Typhimurium*, monophasic variant (7.8%) [7].

Poultry meat and eggs have been shown as being potentially contaminated by *Salmonellae* [8,9] and consumers should be fully aware of that [10]. The EU has prescribed community targets for the reduction of *S. Enteritidis* and *S. Typhimurium* with Regulation (EC) 1168/2006, in adult laying hens of *Gallus gallus*, which are monitored by mandatory control programmes since 2009 [11]. These programmes have contributed to the decline of *Salmonella*-contaminated flocks of laying hens [2,12]. Only 2.54% of 34,757 flocks of laying hens were *Salmonella*-positive within the EU in 2014. *S. Enteritidis* was detected in 0.7% of all flocks. In Germany, only 1.39% of 5,256 flocks of laying hens were *Salmonella*-positive with *S. Enteritidis* detected in 0.44% of them [7]. Although *Salmonella* spp. in table egg units are only rarely detected (0.3% of single samples and 1% of batches in 2014), eggs and egg products were important sources of food-related *Salmonella* outbreaks in 2014 [5]. Phage type (PT) 14b of *S. Enteritidis* was reported in several food-related outbreaks in recent years, often with eggs or egg products as suspected or proven source [13–15].

The outbreak

From March to November 2014, a European multi-country outbreak occurred in France, Luxembourg,

TABLE 1Cases of *Salmonella* Enteritidis phage type 14b, Germany, 2014

Federal state of Germany	Number of cases	Inhabitants	Incidence per 100,000 inhabitants
Baden-Württemberg	6	10,879,618	0.06
Bavaria	107	12,843,514	0.83
Berlin	0	3,520,031	NA
Brandenburg	1	2,484,826	0.04
Bremen	1	671,489	0.15
Hamburg	3	1,787,408	0.17
Hesse	7	6,176,172	0.11
Lower Saxony	10	7,926,599	0.13
Mecklenburg-West Pomerania	1	1,612,362	0.06
North Rhine-Westphalia	12	17,865,516	0.07
Rhineland Palatinate	2	4,052,803	0.05
Saarland	0	995,597	NA
Saxony	17	4,084,851	0.42
Saxony-Anhalt	4	2,245,470	0.18
Schleswig Holstein	3	2,858,714	0.10
Thuringia	3	2,170,714	0.14
Total	177	82,175,684	0.22

NA: not applicable.

Source: Database of the National Reference Centre for *Salmonella* and other Bacterial Enteric Pathogens, Wernigerode, personal communication, Wolfgang Rabsch, October 2016 and [20].

Austria and the United Kingdom (UK) with more than 400 human cases. The outbreak strain was at that time identified as *S. Enteritidis*, PT 14b, multilocus variable-number tandem repeat analysis (MLVA) type 2–12–7–3–2 or 2–11–7–3–2 [1,5,16,17]. The outbreak was epidemiologically traced back to eggs from one large egg producer (company X) with three premises A, B and C in Bavaria and premises D in the Czech Republic. Besides that, company X owns two breeding units, one in Bavaria and one in the Czech Republic.

Following a Rapid Alert System for Food and Feed within the EU (RASFF) notification about a *S. Enteritidis* outbreak in France after consuming homemade ice cream prepared with raw eggs traced back to company X in early July 2014 [18], Bavarian authorities initiated extensive and integrated investigations at the three Bavarian premises A, B and C of company X. *S. Enteritidis* was cultured from eggs (surfaces and contents) and from faeces and stable dust at premises A and from egg surfaces at premises B. *S. Enteritidis* was not detected in the respective samples or materials obtained at the third Bavarian premises C. Here we report the re-evaluation of this multi-country European outbreak of *S. Enteritidis* PT 14b by combining recent epidemiological results with latest molecular data.

Methods

For the re-evaluation we used all relevant RASFF notifications and posts on the European Centre for Disease Prevention and Control (ECDC)'s Epidemic Intelligence Information System (EPIS), epidemiological outbreak

reports from ECDC / European Food Safety Authority (EFSA) [16] and from the concerned Austrian authorities [17] and we assessed and analysed published results of the UK outbreak investigation [5]. Moreover, data obtained from investigations performed by local Bavarian Health authorities together with the analysis of questionnaires from Bavarian cases as well as bilateral epidemiological information obtained directly from Austrian authorities [19] were included.

In a next step, we compared the obtained epidemiological data with the latest molecular results from whole genome sequencing (WGS) of European isolates, performed by Public Health England (PHE) [1]. All UK outbreak strains and further 45 isolates from France, Luxembourg, Austria and Germany were analysed by WGS. Bavarian environmental isolates and isolates from eggs of premises A and B of company X, which were obtained during official investigations, were also included.

Results

France

Six food-related outbreaks caused by homemade food prepared with raw eggs involving 45 human cases were reported in France between June and July 2014. *S. Enteritidis* was isolated from 16 human cases and from one egg sample taken in a private kitchen. The outbreaks were traced back epidemiologically to premises A and B of company X [16].

TABLE 2

Assignment of the European outbreak strains of Salmonella Enteritidis phage type 14b to three different clades and to single premises of company X

Clade 1, isolates from faeces, dust samples and eggs, directly sampled at premises A of company X (n=10)			
Country	Outbreak	Origin of isolates (number)	Comment
France	Outbreak after consumption of chocolate cream produced with raw eggs (RASFF-notification 2014.0938-info1)	Eggs (n=2)	Epidemiological link to company X
	Outbreak after consumption of ice cream produced with raw eggs (RASFF-notification 2014.0938-info1)	Human (n=2)	Epidemiological link to company X
United Kingdom	Sporadic cases	Human (n=3)	NA
Germany	Sporadic cases	Human (n=3)	No epidemiological link to company X
Clade 2 Isolates from eggs, directly sampled at premises B of company X (n=2)			
Country	Outbreak	Origin of isolates (number)	Comment
France	Outbreak after consumption of mayonnaise produced with raw eggs (RASFF-notification 2014.1072)	Human (n=3)	Epidemiological link to company X
	Sporadic cases	Human (n=2)	No epidemiological link to company X
Luxembourg	Sporadic case	Human (n=1)	Epidemiological link to company X
Austria	Outbreak in Tyrol in June/July 2014, caused by a caterer delivering food at homes for the elderly and “meals on wheels” to risk groups	Human (n=3)	Epidemiological link to company X through egg supply network
	Human isolates not belonging to the Tyrolian outbreak	Human (n=2)	Isolates from Upper Austria and Vienna ^a
United Kingdom	Outbreak in a kebab house	Human (n=9)	Epidemiological link to company X
	Sporadic cases	Human (n=23)	NA
Germany	Sporadic cases	Human (n=7)	No epidemiological link to company X
	Sporadic cases	Human (n=1)	Epidemiological link to company X (employee of company X, asymptomatic carrier)
Clade 3, no isolate from any premises of company X			
Country	Outbreak	Origin of isolates (number)	Comment
Austria	Human isolate not belonging to the Tyrolian outbreak	Human (n=1)	Isolate from Vienna ^a
	Sporadic cases	Human (n=1)	Isolate was cultured in 2013 and has no epidemiological link to the outbreak in 2014 ^a
Germany	Sporadic cases	Human (n=4)	No epidemiological link to company X
United Kingdom (UK)	Outbreaks in three Chinese restaurants and a UK hospital	Human (n=250)	Epidemiological link to company X
	Sporadic cases	Human (n=75)	NA

RASFF: Rapid Alert System for Food and Feed.

^a Source: personal communication: Tim Dallman, February 2016.

Source: [1] and personal communication: Wolfgang Rabsch, January 2017; Tim Dallman, February 2017.

Luxembourg

Luxembourg reported one human case infected with the outbreak strain in early June 2014. The patient residing in France close to the border to Luxembourg, consulted a hospital in Luxembourg and had consumed eggs possibly bought in a shop of the supermarket chain linked to the French cases [16].

Austria

Between June and October 2014, 151 *S. Enteritidis* PT 14b cases (90 confirmed, 38 probable and 23 possible cases) in Austria fulfilling the Austrian case definition, occurred with an accumulation of 69 cases in Tyrol as at 22 October 2014 [17,19]. A confirmed case was defined as an infection with *S. Enteritidis* PT14b (MLVA-type 2–12–7–3–2) occurring after week 23 (starting on 2 June) 2014 in a person living in Austria. A probable case was defined as an infection with *S. Enteritidis* PT14b without MLVA-typing occurring after week 23 in a person living in Austria. A possible case was defined as diarrhoea or vomiting occurring after week 23 in a person living in Austria and having an epidemiological link to a confirmed case [17].

In 51 of 69 Tyrolian cases (74% in total; 16 confirmed, 1 probable and 34 possible cases) the consumption of partly raw eggs-containing food was identified as common exposure. The food was prepared in a large-scale catering facility at Innsbruck. The food was delivered to ‘meals on wheels’ customers and residents of three nursing homes for the elderly, which were supplied by the above mentioned caterer. The large-scale catering facility bought eggs via a distributor in Innsbruck from an egg selling company in Bavaria which was supplied by company X. The Austrian authorities sampled 10 eggs delivered from premises B of company X on 4 July 2014 as well as 10 further eggs, beef soup powder, salt, corn starch, four different spices and one counter sample (vegetable casserole) from the large scale catering facility on 9 July 2014. *Salmonella* spp. could neither be isolated from the food nor from the egg samples. During the outbreak period, a member of the kitchen staff, a food distributor, was identified as carrying the outbreak strain [17,19]. They were probably infected by contaminated eggs or egg containing food.

United Kingdom

The UK reported an outbreak with a duration of 17 weeks from May to November 2014 [1]. Besides 101 sporadic cases there were five outbreaks with 259 cases, linked to a hospital, three Chinese restaurants and one kebab restaurant [1] and (personal communication, Tim Dallman, February 2017). Sixty-nine per cent (198/287) of confirmed cases could be epidemiologically linked to eggs supplied by company X [5]. *S. Enteritidis* PT 14b with the outbreak MLVA profile was isolated from environmental and food samples from the hospital and two restaurants, but not from eggs [5].

Germany

In Germany, 177 *S. Enteritidis* PT 14b cases were identified by phage typing in 2014 at the National Reference Centre (NRC) for Salmonella and other Bacterial Enteric Pathogens, Wernigerode, of which 107 were from Bavaria (personal communication, Wolfgang Rabsch, October 2016). Importantly, it should be noted that phage typing is not routinely performed in Germany. Only those isolates sent to the National Reference Centre for Salmonella and other Bacterial Enteric Pathogens are phage typed. Thus, the occurrence of different phage types among German *Salmonella* isolates is generally underestimated. The Bavarian Health and Food Safety Authority initiated phage typing since July 2014 for the Bavarian regions Upper Palatine and Lower Bavaria and since August 2014 for all Bavarian regions. Since August 2014, the national public health institute (Robert Koch Institute, Berlin) initiated phage typing for the other federal states on a voluntary basis. In Table 1 the number of cases and their incidence per 100,000 inhabitants in different Federal States of Germany in 2014 are described. Most of the cases and the highest incidence were observed in Bavaria, reflecting the intensified surveillance in Bavaria since July 2014. Ten or more *S. Enteritidis* PT 14b cases were observed in each of the federal states of Saxony, North Rhine-Westphalia and Lower Saxony.

In 2014, 91 Bavarian cases of *Salmonella Enteritidis* PT 14b were notified according to the German Infection Protection Act (SurvNet-Database of RKI, as at 31 March 2015). Ten of them were asymptomatic carriers, among whom one was an employee of company X. All Bavarian cases were sporadic, singular cases that did not belong to any epidemiological cluster or outbreak. All of them were asked to take part in an in depth questionnaire-based interview with a healthcare official on voluntary basis. Just over half of the cases (55%; n = 50) agreed to be interviewed. According to the questionnaire responses, 19 cases consumed eggs, but five of them had acquired their *Salmonella* infection abroad. The consumption of raw egg-containing food could be excluded for four persons. For the remaining 10 cases the origins of the consumed eggs were: unknown (n = 4), supermarket without further details (n = 2), eggs from free-range hens (n = 1), organic eggs without further details (n = 1), eggs directly bought at the farm (n = 1) and eggs from one’s own hens (n = 1). Taken together, no link to consumption of eggs of company X practicing cage production could be identified by these interviews.

Molecular investigation by whole genome sequencing

The outbreak strains formed a single five single nucleotide polymorphism (SNP) single-linkage cluster with a maximum distance between any two genomes of 23 SNPs. Three clades could be separated within the monophyletic cluster with a maximum difference of two SNPs [1]. The corresponding phylogenetic tree is published elsewhere [1]. The time to the most recent

common ancestor for the three clades was estimated to be 2.9 years (95% highest posterior density (HPD): 2.5–3.2 years) [1]. The analyses referred all three clades to company X or its egg supply network and postulated a common ancestor of all three clades [1].

All sequenced European strains can be assigned to the three clades as shown in Table 2 with the exception of two human strains from France and one human strain from Germany which could not be assigned to any of the three clades [1].

Combination of epidemiological and molecular results

The synoptic view of all epidemiological data with molecular typing data from PHE yielded the following scenario of the 2014 multi-country outbreak with epidemiological data confirming molecular results (Table 2).

Isolates of clade 1 were linked to premises A of company X based on molecular analysis of isolates from premises A. Two outbreaks in France were linked to premises A of company X due to molecular analysis of isolates from humans and eggs and the identification of premises A of company X as supplier of the eggs. Interventions at premises A, i.e. removal of the old flock between 20 June and 22 July 2014, immediate housing of a new flock, which never supplied table eggs, coincided with the cessation of the appearance of clade 1 isolates after the end of July 2014.

Isolates of clade 2 were linked to premises B of company X based on molecular analysis of isolates from premises B. One outbreak in France and one outbreak in a kebab house in the UK could be related to premises B of company X. Human isolates from France were linked to clade 2 by molecular analysis, while premises B of company X was identified as egg supplier. Isolates from the UK were traced back epidemiologically to company X through the egg supply network. After a delivery stop for eggs from premises B of company X to the United Kingdom on 17 July 2014 and the passing of the eggs' shelf life only a few singular isolates of clade 2 were detected in the UK.

Isolates of clade 2 and clade 3 were cultured from Austrian outbreak cases. Seven Austrian isolates were sequenced by WGS. Five of them belonged to clade 2, two of them to clade 3. One of the clade 3 isolates was cultured in 2013 (personal communication, Tim Dallman, February 2016) and could not be attributed to the 2014 outbreak.

Most of the UK cases could be linked to company X due to the egg supply network (Table 2) as reported elsewhere [5]. The major part of the UK isolates including the isolates from four of five outbreaks in the UK (one hospital and three Chinese restaurant outbreaks) belongs to clade 3. Isolates of clade 3 were continuously detected in the UK from March to November

2014. Interventions at premises A and B of company X did not influence the appearance of clade 3 isolates. However, after a delivery stop for eggs of premises D of company X from the Czech Republic to the UK on 1 September 2014 and the passing of the eggs' shelf life, only singular, sporadic cases of clade 3 isolates without further outbreaks were detected in the UK. Sixteen samples from premises D of company X (faeces (n=4), whole body (n=6), container (n=6)) were analysed by the Czech authorities between 3 March 2014 and 10 July 2014. *Salmonella* spp. were not detected as reported by the Czech government via the RASFF system.

Discussion and conclusions

Re-evaluating this large multi-country outbreak by combining the results of recent molecular and epidemiological data allowed us to clearly attribute some outbreaks to single premises of one great egg supplier, company X. The French outbreaks could be referred to premises A and B and the UK outbreak in a kebab house to premises B of company X.

In Austria, clade 2 and clade 3 strains were isolated during the outbreak. Although the isolation of *S. Enteritidis* from eggs or incriminated food failed, it can be supposed that premises B of company X was involved in the Tyrolian outbreak due to an epidemiological link between the large-scale catering facility at Innsbruck and company X via the egg supply network and the isolation of clade 2 strains from clinical cases. It is notable that 18 cases infected with a strain of *S. Enteritidis* PT 14b, indistinguishable from the outbreak strain 2014 by MLVA typing were detected both in Tyrol and other federal states of Austria already in October and November 2013 [17].

The appearance of clade 3 strains in Austria before and during the outbreak gives a hint that a further, yet unknown source might have been involved. For a more exact characterisation of the Austrian outbreak the sequencing of a greater number of Austrian outbreak strains would have been helpful. The fact that a human carrier of the outbreak strain worked as food distributor at the caterer as well as the distribution of raw egg containing food may have influenced the extent and the duration of the Austrian outbreak.

The source of clade 3 strains which were isolated from most of the UK cases remains obscure. There is the possibility that *S. Enteritidis* was merely not detected from eggs or environmental samples from premises C of company X in Bavaria, despite intensive sampling. In addition, the molecular relationship of all UK outbreak strains provides some epidemiological evidence that the source for clade 3 strains could be premises D of company X in the Czech Republic. This hypothesis is supported by the fact that UK clade 3 cases strongly decreased and only few sporadic cases were detected after the delivery stop on 1 September 2014 for eggs from the Czech premises D of company X and after the expiration of their shelf life.

As molecular data postulate a common ancestor strain of all three clades ca 2.9 years ago, we suppose that the ancestor strain was introduced from an external parent or grandparent herd to the premises of company X in the past and started to develop independently at each premises finally forming the three clades. This hypothesis is supported by the fact that *S. Enteritidis* PT 14b clade 2 strains were isolated during a *S. Enteritidis* hospital outbreak in Bavaria in summer 2015 and from the laying hens of the hospital's egg supplier. In this outbreak *S. Enteritidis* PT 14b clade 2 was isolated from 11 persons who were directly or indirectly linked to a Bavarian hospital in Swabia (patients (n=6), relatives (n=2), trainee (n=1), cook (n=1) and cook mate (n=1)). The route of transmission inside the hospital could not be clarified, but it was supposed that *S. Enteritidis* was transmitted via cross-contaminated food from the kitchen. The most likely primary source was identified as eggs from a local egg supplier about two hundred kilometres away from the premises of company X (data not shown).

An official sampling of the five laying hen herds (separate faecal/dust samples from every herd and 120 eggs from the egg packing centre) of the egg supplier was done and *S. Enteritidis* PT 14b clade 2 was isolated from one of the five laying hen herds (faecal/dust samples). The egg samples taken in the egg packing centre of the company were negative for *Salmonella* spp. In a routine self-check of the egg supplier done incidentally one week before the official sampling, *S. Enteritidis* was also isolated from a second laying hen herd. Summarising the results, two of the five laying hen herds of the local egg supplier tested positive for *S. Enteritidis* PT 14b clade 2 (data not shown).

In contrast to company X, the egg supplier received *Salmonella*-immunised young hens from a local breeding farm in the north of Bavaria and kept them in deep litter. A connection between the local egg supplier and company X (e. g. feed, water supply, litter) could not be identified. Besides, the whole flock of premises B of company X shedding clade 2 strains had been slaughtered in September 2014, 11 months before the hospital outbreak.

Conclusions

Comprehensive outbreak investigations require the combination of epidemiological data and molecular typing techniques, especially if bacteria with a very low natural mutation rate such as *Salmonella* spp. are concerned. Low discriminatory typing techniques like phage typing or MLVA typing may be well suited for investigating small, circumscribed, local outbreaks. However, the investigation of large-scale, long-lasting, cross-border outbreaks calls for highly discriminatory techniques such as WGS to allow distinct and unambiguous assignment and classification of isolates. The collection of epidemiological data from different sources together with molecular typing data enabled us to retrospectively evaluate the 2014

multi-country *S. Enteritidis* PT 14b outbreak tracing back the incriminated food across country borders to its source at the farm. We were even able to link different parts of the outbreak to distinct premises of one large egg supplier. A resolution of food-related outbreaks with such a high discrimination is a very rare result in outbreak investigations. The close collaboration of the participating authorities of all countries was crucial in achieving this.

Acknowledgements

We thank Wolfgang Rabsch from the German National Reference Centre for Salmonella and other Bacterial Enteric Pathogens for phage typing of *S. Enteritidis* strains from Germany.

FASTQ sequences were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under the BioProject PRJNA248792 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA248792>).

Supplementary material is available at the following git repository (https://github.com/timdallman/sent_14b) [1].

Conflict of interest

None declared.

Authors' contributions

Stefan Hörmansdorfer: bacteriological examination of feces and environmental samples from premises A, B and C of company X in Bavaria, serological differentiation of salmonella isolates from animal and human sources in Bavaria.

Ute Messelhäußer: Bacteriological examination of egg samples from premises A, B and C of company X in Bavaria, serological differentiation of salmonella isolates from eggs in Bavaria.

Albert Rampp: outbreak investigation and coordination in Bavaria.

Katharina Schönberger: epidemiology of human salmonellosis in Bavaria.

Tim Dallman: NGS typing of European outbreak isolates.

Franz Allerberger: outbreak investigation and coordination in Austria, MLVA typing of outbreak isolates.

Christian Kornschober: outbreak investigation and coordination in Austria, MLVA typing of outbreak isolates.

Andreas Sing: outbreak investigation and coordination in Bavaria.

Peter Wallner: outbreak investigation and coordination in Bavaria.

Andreas Zapf: outbreak investigation and coordination in Bavaria.

References

1. Dallman T, Inns T, Jombart T, Ashton P, Loman N, Chatt C, et al. Phylogenetic structure of European wide Salmonella

- Enteritidis outbreak correlates with national and international egg distribution network. *Microbial Genomics*, 2016. Available from: <http://mgen.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.o.000070>
- EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA J.* 2014;12(2):3547.
 - Grimont P A D, Weill F-X. *Antigenic Formulae of the Salmonella Serovars*. WHO Collaborating Centre for Reference and Research on Salmonella, 9th edition, 2007.
 - Hopkins KL, Peters TM, de Pinna E, Wain J. Standardisation of multilocus variable-number tandem-repeat analysis (MLVA) for subtyping of *Salmonella enterica* serovar Enteritidis. *Euro Surveill.* 2011;16(32):19942. Available from: <https://doi.org/https://10.2807/es.e16.32.19942-en> PMID: 21871223
 - Inns T, Lane C, Peters T, Dallman T, Chatt C, McFarland N, et al. A multi-country *Salmonella* Enteritidis phage type 14b outbreak associated with eggs from a German producer: 'near real-time' application of whole genome sequencing and food chain investigations, United Kingdom, May to September 2014. *Euro Surveill.* 2015;20(16):21098. <https://doi.org/10.2807/1560-7917.ES2015.20.16.21098> PMID: 25953273
 - Malorny B, Junker E, Helmuth R. Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. *BMC Microbiol.* 2008;8(1):84. <https://doi.org/10.1186/1471-2180-8-84> PMID: 18513386
 - EFSA (European Food Safety Authority) and ECDC. (European Centre for Disease Prevention and Control). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2014. *EFSA J.* 2015;13(12):4329.
 - European Food Safety Authority (EFSA) / European Centre for Disease Prevention and Control (ECDC). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. *EFSA Journal* 2017;15(2):4694, 212 pp. doi: <https://doi.org/10.2903/j.efsa.2017.4694>
 - Whitley H, Ross K. *Salmonella* and eggs: from production to plate. *Int J Environ Res Public Health.* 2015;12(3):2543-56. <https://doi.org/10.3390/ijerph120302543> PMID: 25730295
 - Lievonen S, Havulinna AS, Maijala R. Egg consumption patterns and *Salmonella* risk in Finland. *J Food Prot.* 2004;67(11):2416-23. <https://doi.org/10.4315/0362-028X-67.11.2416> PMID: 15553622
 - Commission Regulation (EC) No 1168/2006 of 31 July 2006 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in laying hens of *Gallus gallus* and amending Regulation (EC) No 1003/2005. Luxembourg: Official Journal of the European Union. Available from: <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1168&qid=1513003372935&from=EN>
 - EFSA (European Food Safety Authority) and European Centre for Disease Prevention and Control. (ECDC). Multi-country outbreak of *Salmonella* Enteritidis phage type 8, MLVA type 2-9-7-3-2 infections. 17 March 2016, Stockholm, 2016. <http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2017.EN-1188/epdf>
 - Nygård K, de Jong B, Guerin PJ, Andersson Y, Olsson A, Giesecke J. Emergence of new *Salmonella* Enteritidis phage types in Europe? Surveillance of infections in returning travellers. *BMC Med.* 2004;2(1):32. <https://doi.org/10.1186/1741-7015-2-32> PMID: 15345058
 - Mossong J, Ragimbeau C, Schuh J, Weicherding P, Peetso R, Wildemaue C, et al. Investigation of an excess of *Salmonella* enteritidis phage type 14b and MLVA type 4-7-3-13-10-2-2 in Luxembourg, Belgium and Germany during 2010. *Bull Soc Sci Med Grand Duche Luxemb.* 2012; (2):49-62. PMID: 23362566
 - Janmohamed K, Zenner D, Little C, Lane C, Wain J, Charlett A, et al. National outbreak of *Salmonella* Enteritidis phage type 14b in England, September to December 2009: case-control study. *Euro Surveill.* 2011;16(15):19840. Available from: <https://doi.org/10.2807/es.e16.15.19840-en> PMID: 21507321
 - European Centre for Disease Prevention and Control (ECDC) / European Food Safety Authority (EFSA). Multi-country outbreak of *Salmonella* Enteritidis infections associated with consumption of eggs from Germany. 25 August 2014. Stockholm and Parma: ECDC/EFSA; 2014. Available from: <http://www.efsa.europa.eu/de/supporting/pub/646e>
 - Schmid D, Kanitz E. Bericht zu einem bundesländerübergreifenden lebensmittelbedingten Krankheitsausbruch (BL-LMbKA). BKZoon-ID: 2/2014 – *Salmonella* Enteritidis 14 b/Tirol. [Report on a food related outbreak concerning several federal states of Austria]. AGES, Vienna, 2014.
 - European Commission. Rapid Alert System for Food and Feed (RASFF). RASFF notification 2014.0938 of 09.07.2014. Brussels: RASFF. [Accessed 8 Dec 2017]. Available from: https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationDetail&NOTIF_REFERENCE=2014.0938
 - Herzog U. Schreiben BMG-74000/0031-II/B/2016 vom 07.03.2016. Bundesministerium für Gesundheit [Letter of the Austrian Federal Ministry of Health], Vienna, 2016.
 - Statistisches Bundesamt. The Federal Statistical Office of Germany. [Internet]. Wiesbaden: Statistisches Bundesamt. [Accessed 9 Jun 2017]. German. Available from: <https://www.destatis.de/DE/ZahlenFakten/LaenderRegionen/Regionales/Regionaldaten.html;jsessionid=9AB7FA88369D6AECF6BED0404EBD2AB.cae4>

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2017.

Investigation using whole genome sequencing of a prolonged restaurant outbreak of *Salmonella* Typhimurium linked to the building drainage system, England, February 2015 to March 2016

John Mair-Jenkins^{1,2,3}, Roberta Borges-Stewart⁴, Caroline Harbour⁵, Judith Cox-Rogers⁵, Tim Dallman⁶, Philip Ashton⁶, Robert Johnston⁷, Deborah Modha⁸, Philip Monk⁴, Richard Puleston^{3,9}

1. Field Epidemiology Training Programme, Public Health England, United Kingdom

2. European Programme for Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden

3. Field Epidemiology Service, National Infection Service, Public Health England, United Kingdom

4. East Midlands Health Protection Team, Public Health England, United Kingdom

5. Environmental Health, Blaby District Council, Blaby, United Kingdom

6. Gastrointestinal Bacteria Reference Unit, National Infection Service, Public Health England, United Kingdom

7. Food Water and Environment Laboratory, National Infection Service, Public Health England, United Kingdom

8. Clinical Microbiology, Leicester Royal Infirmary, University Hospitals of Leicester NHS Trust, United Kingdom

9. University of Nottingham, School of Medicine, Division of Epidemiology and Public Health, United Kingdom

Correspondence: John Mair-Jenkins (john.mairjenkins@phe.gov.uk)

Citation style for this article:

Mair-Jenkins John, Borges-Stewart Roberta, Harbour Caroline, Cox-Rogers Judith, Dallman Tim, Ashton Philip, Johnston Robert, Modha Deborah, Monk Philip, Puleston Richard. Investigation using whole genome sequencing of a prolonged restaurant outbreak of *Salmonella* Typhimurium linked to the building drainage system, England, February 2015 to March 2016. *Euro Surveill.* 2017;22(49):pii=17-00037. <https://doi.org/10.2807/1560-7917.ES.2017.22.49.17-00037>

Article submitted on 11 Jan 2017 / accepted on 04 Jun 2017 / published on 07 Dec 2017

Following notification of a *Salmonella enterica* serovar Typhimurium gastroenteritis outbreak, we identified 82 cases linked to a restaurant with symptom onset from 12 February 2015 to 8 March 2016. Seventy-two cases had an isolate matching the nationally unique whole genome sequencing profile (single nucleotide polymorphism (SNP) address: 1.1.1.124.395.395). Interviews established exposure to the restaurant and subsequent case-control analysis identified an association with eating carvery buffet food (adjusted odds ratios (AOR): 20.9; 95% confidence interval (CI): 2.2 – ∞). Environmental inspections, food/water testing, and a food trace-back investigation were inconclusive. Repeated cycles of cleaning were undertaken, including hydrogen peroxide fogging, however, transmission continued. After 7 months of investigation, environmental swabbing identified 106 isolates from kitchen surfaces and restaurant drains matching the outbreak profile. We found structural faults with the drainage system and hypothesised that a reservoir of bacteria in drain biofilm and under-floor flooded areas may have sustained this outbreak. Ineffective drain water-traps (U-bends) may have also contributed by allowing transmission of contaminated aerosols into the kitchen environment. These findings suggest that routine swabbing of sink drain points and inspection of drainage systems should be considered in future outbreak scenarios.

Introduction

It is estimated that over 38,000 community cases of salmonellosis occur annually within the United Kingdom (UK) [1,2]. Salmonellosis often results from consumption of contaminated food or water [3], however, transmission via asymptomatic shedding by food handlers and exposure to contaminated environments where conditions are favourable for pathogen survival have also been implicated [3,4]. Here we report the findings of an investigation of an outbreak of salmonellosis where the environment was pivotal in continued transmission.

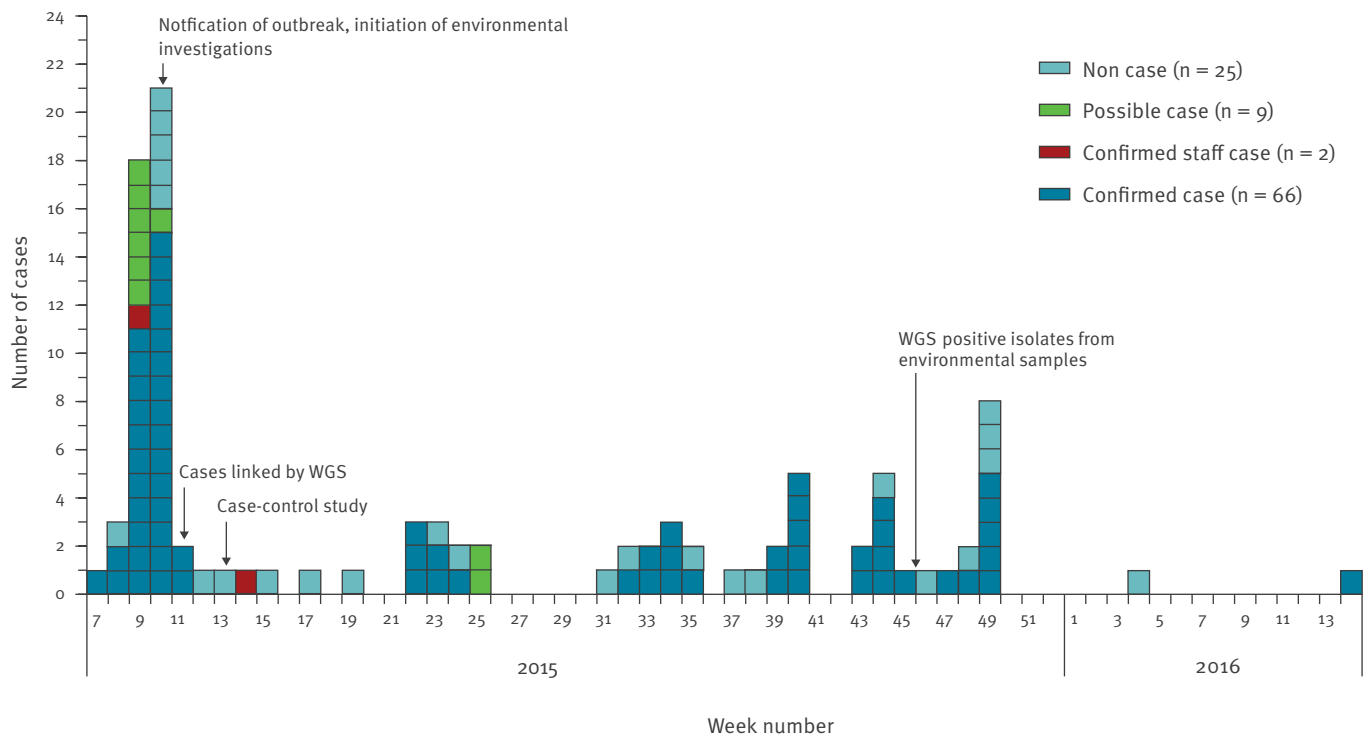
The event

On 7 March 2015, Public Health England (PHE) East Midlands was alerted by the clinical microbiology laboratory of a local hospital to 21 cases of *Salmonella enterica* serovar Typhimurium gastroenteritis, with onset in February 2015. Seven cases in this initial phase of the outbreak required hospitalisation. Following this notification we suspected there was a community outbreak of *S. Typhimurium*; investigations and attempts to control the outbreak followed.

Hypothesis-generating interviews at the outset of the investigation identified that several cases had eaten at the same restaurant during the incubation period for their illness. Descriptive epidemiological analyses including subsequent cases pointed to the restaurant being the likely source. This popular, purpose (newly

FIGURE 1

Reported date of symptom onset of cases of a *Salmonella* outbreak and people not meeting case definitions with matching whole genome sequencing of clinical isolates, United Kingdom, Week 7 (February) 2015–Week 14 (March) 2016 (n = 102^a)



WGS: whole genome sequencing.

^a Onset dates were not available for two confirmed asymptomatic staff cases, two other confirmed cases, one possible case, and six non cases with matching WGS results (n = 11).

built restaurant had opened only 18 months before the outbreak. The restaurant offered a full table-service menu, self-service salad bar and hot self-service carvery buffet serving roasted meats (turkey, beef, gammon and pork at weekends) and vegetables and condiments. Despite interventions to control the initial outbreak, cases continued to emerge followed by a prolonged period of transmission until 2016. The evolution of the investigation into this community outbreak and subsequent control measures is described, with specific reference to the use of whole genome sequencing (WGS) to link isolates and the role of the drains in continued pathogen transmission.

Methods

Epidemiological investigations

Case finding used information from existing cases, local primary care practitioners and pre-existing surveillance systems (statutory disease and routine laboratory notifications, including routine WGS) [5]. Active case finding using restaurant booking information was not possible as most customers did not pre-book.

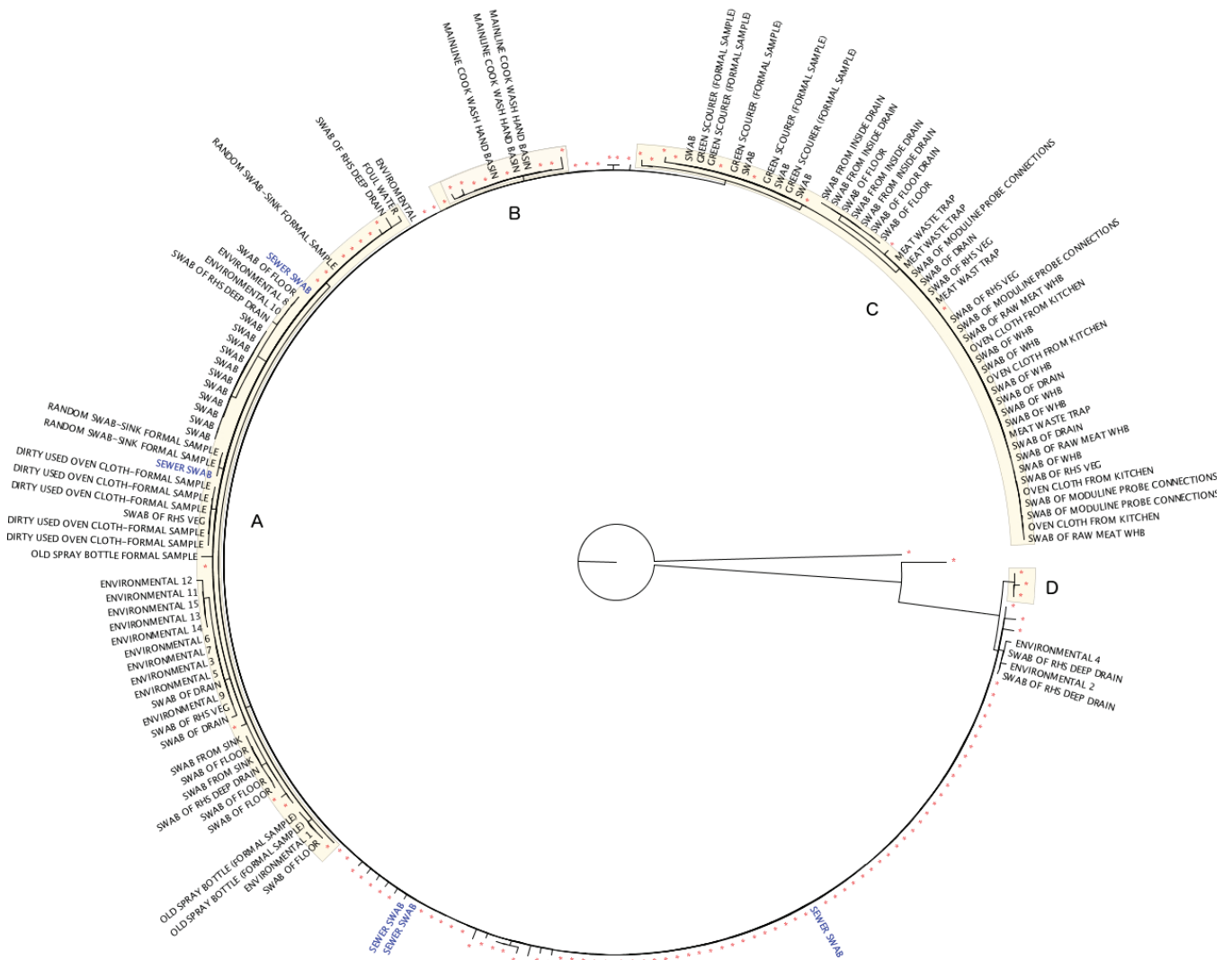
Hypothesis-generating interviews and descriptive analyses informed a case-control study in March

2015 where we defined confirmed cases as UK residents with *S. Typhimurium* infection (with or without gastroenteritis) with an isolate matching the nationally unique WGS outbreak profile (five single nucleotide polymorphisms (SNP) single linkage cluster 1.1.1.124.395.395) [6] with symptom onset or a positive sample taken within 12 days of visiting the restaurant. Possible cases were defined as having gastroenteritis (diarrhoea – three loose stools within 24 hours – or any two of abdominal pain, fever or nausea) within 12 days of visiting the restaurant [7,8].

As part of the outbreak investigations a case-control study was undertaken, to understand the association between consuming restaurant food or drink and illness, with the assumption that a food associated source was the most likely vector. Cases were recruited and were requested to nominate people who had eaten with them as controls (1:1 ratio). No other criteria were set for control selection. After data collection any controls who met case definitions were reassigned as cases. We asked about exposure to food and drink and potential confounding exposures: immunosuppression, recent use of antibiotics or antacids, age and sex. We calculated univariate odds ratios (OR), and adjusted ORs (aOR) using multivariable exact logistic regression.

FIGURE 2

Maximum likelihood phylogenetic tree of clinical (red), sewer (blue) and other environmental samples matching the SNP address 1.1.1.124.395.395 of a *Salmonella* outbreak strain, United Kingdom, February 2015–March 2016 (n = 220 outbreak sequences^a)



SNP: single nt polymorphism.

^a 114 sequences from clinical isolates (cases and non-cases), this includes sequences where there were multiple samples from cases. 106 sequences from environmental isolates are shown.

An asterisk (*) denotes the position of a clinical isolate in the tree. The first two asterisks at the base of the tree denote the root of the tree and nearest neighbouring sequence.

Due to continued case occurrences after the implementation of outbreak control measures (as informed by the findings of the case–control study), further investigations ensued, including increased scrutiny of the environment.

Clinical microbiological investigations

Stool samples were collected for culture and characterisation following clinical investigations and voluntary sampling of restaurant staff in March 2015 and again following further case occurrences in November 2015. Positive isolates were submitted to the PHE

TABLE 1

Results of univariate analysis of food items eaten at a restaurant, implicated in a *Salmonella* outbreak, United Kingdom, March 2015 (n = 30 persons investigated)

Food eaten	Cases			Controls			Crude OR (95% CI)	Exact p value
	Total	Exposed	%	Total	Exposed	%		
Carvery	20	20	100	10	5	50	Ind ^a (4.21–Ind)	0.002
Any sharing platter	19	0	0	10	3	30	0 (0.00–0.56)	0.05
Side dish	20	0	0	10	2	20	0 (0.00–0.89)	0.1
Any burger	20	0	0	10	1	10	0 (0.00–Ind)	0.3
Any pie	20	0	0	10	1	10	0 (0.00–Ind)	0.3
Any fish	19	0	0	10	1	10	0 (0.00–Ind)	0.3
Any starter	19	0	0	10	1	10	0 (0.00–Ind)	0.3
Any pudding	20	3	15	10	3	30	0.41 (0.04–3.97)	0.3
Any drink	20	18	90	10	9	90	1 (0.02–21.72)	1

CI: confidence interval; ind: indeterminate; OR: odds ratio.

No cases or controls ate items from the following menu sections: jacket potatoes, light bites, salads, steaks, hero dishes, classic dishes.

^a Exact logistic regression, median unbiased estimate univariate OR: 21.37 (95% CI: 2.52–∞).

Gastrointestinal Bacteria Reference Unit (GBRU) for serological confirmation, phage typing and multilocus variable-number tandem repeat analysis (MLVA), until April 2015. Extracted DNA from isolates was also sequenced using Nextera library preparation (Illumina, Inc. San Diego, California, United States) on an Illumina HiSeq 2500 machine. High quality Illumina reads were mapped to the *S. Typhimurium* reference genome (GenBank: AE006468) as previously described [9]. Core genome positions that had a high quality SNP (>90% consensus, minimum depth 10x, genotype quality ≥ 30) in at least one strain were extracted and RaxMLv8.17 (Stamatakis, 2014) used to derive the maximum likelihood phylogeny of the isolates under the general time-reversible (GTR)-CAT model of evolution. Single linkage SNP clustering was performed [9]. FASTQ reads from all sequences in this study can be found at the PHE Pathogens BioProject at the National Center for Biotechnology Information (Accession PRJNA248792). Isolates within the five SNP single linkage cluster 1.1.1.124.395.395 were defined as part of the outbreak.

Environmental investigations and site visits

Environmental Health Officers (EHOs) carried out a routine inspection at the restaurant and reviewed staff sickness records when the outbreak was first identified. Surface swabs using Dacron tipped swabs and sponge swabs, along with food and water samples were collected using standard techniques [10]. Repeat site visits and inspections continued throughout the outbreak. A food trace-back investigation was conducted to identify the suppliers of meat, eggs, stuffing and gravy to establish if local suppliers were being used or whether larger national/international suppliers had links to other reported cases or outbreaks of *S. Typhimurium*.

In November 2015, following failure to control the outbreak, we considered potential environmental sources

that could provide an ongoing intermittent exposure which had not previously been investigated. Repeated cycles of cleaning were undertaken at the restaurant, including deep cleans using hydrogen peroxide fogging, however these still failed to control the incident. Further investigations including a Water Fittings Inspection [11], sewer sampling using a non-validated method similar to a Moore swab [12] and an inspection of the restaurant drainage systems were undertaken. The drains were visually inspected and microbiological sampling of the kitchen floor drains and sink drains was undertaken. Expert advice was sought on the movement of bio-aerosols along drainage systems [13]. Drainage systems were inspected internally using remote cameras, swabbing was conducted and smoke testing was used to establish air flows and the integrity of the drainage. Repeat inspections and environmental swabbing were undertaken after remedial works.

Environmental samples were submitted to PHE Food, Water and Environmental Microbiology Laboratories. These were tested for *Salmonella* sp. using routine detection methods for surface swabs and food [14]. Testing methods were adapted for non-routine samples including kitchen cloths and spray bottles. Isolates from positive samples were referred to GBRU for WGS and SNP analysis allowing comparison with clinical samples.

Results

Eighty-two cases (72 confirmed and 10 possible) reported visiting the restaurant. The onset of symptoms (mainly diarrhoea) ranged from 12 February 2015 to 8 March 2016 with apparent point source outbreaks in February and June 2015, followed by a prolonged period of transmission (Figure 1). Four cases (all confirmed) worked in the restaurant kitchen or served food at the restaurant, two of whom were asymptomatic (identified through staff sampling).

TABLE 2

Final exact logistic regression multivariable model of carvery food items eaten at a restaurant implicated in a *Salmonella* outbreak, United Kingdom, March 2015 (n = 29a)

Exposure	AOR (95% confidence interval)	P value
Carvery	20.9 (2.2–∞)	< 0.007
Sex (male)	2.7 (0.2–148.6)	> 0.7
Recent antibiotic use	1.0 (0.7–63.6)	> 0.99

AOR: adjusted odds ratio.

^a One case had no record of antibiotic use and was excluded from the model (n = 29), model p < 0.005.

Cases had a median age of 28 years (range: 5 months–85 years), 65% (53/82) were female and 99% (81/82) lived in the same county as the restaurant. Symptoms were mainly self-limiting although 19 cases were hospitalised (1 to intensive care with invasive disease). We also identified 31 people during case finding whose isolates matched the nationally unique WGS outbreak clade but reported not visiting the restaurant, and had no apparent link to cases or takeaway food (n = 13) or declined interview (n = 18). These people did not meet the case definitions, but otherwise did not appear different from cases; median age: 28 years (range: 10 months – 85 years), 52% (16/31) female, with similar symptoms and onset dates, however 19% (6/31) lived in other areas of England or Northern Ireland.

Case–control study

At the time of the case–control study, 44 cases reported having eaten at the restaurant. Twenty (17 confirmed, 3 possible) responded to the case–control questionnaire (response rate: 20/44). Cases only nominated 10 controls; failing to achieve a 1:1 ratio.

A range of foods, including uncooked salad items were investigated in the case–control study (Table 1). Eating food from the carvery was the only significant (p < 0.01) harmful exposure identified by univariate analysis (OR: 21.37; 95% confidence interval (CI): 2.52 – ∞). All cases had consumed carvery food. Multivariable analysis identified sex and recent antibiotic use, as confounders. The adjusted odds of becoming a case remained over 20 times greater after eating any carvery food (aOR: 20.9; 95% CI: 2.2 – ∞, Table 2). Effect modification was not identified between covariates in the final model. These results were confirmed by sensitivity analyses (data not shown). Throughout the investigation, 94% (60/64) of cases with a known food history reported eating food from the carvery; no cases reported eating salads.

Clinical microbiological investigations

S. Typhimurium was isolated in 75 case samples (72 confirmed, 3 possible); stool samples were not available for seven cases. Phage-type DT193 was identified

for 16 isolates, 58 isolates were untypable, and one isolate was not phage-typed. A single MLVA profile was identified (3–14–9–0–0211) in 34 cases (one additional case had a single locus variant). This profile had been associated with human cases and pigs in several regions of England (data not shown). In addition WGS was available for 96% (72/75) of isolates with the first results available 7 days after the start of the investigation, providing increased discrimination over MLVA results.

All the isolates clustered within a nationally unique five SNP cluster (which is further referred to as the outbreak clade), 71% (53/72) of isolates were identical across their core genome with no SNP differences observed (outbreak profile). The remaining 19 case isolates varied from the most frequent genotype by between one and five SNPs. An overview of the sequences associated with this nationally unique outbreak profile is shown in the dendrogram (Figure 2) with sequences from environmental sampling, and the additional 31 sequences identified from people during case finding who did not meet case definitions (i.e. non cases). Eight of 13 sequences from non-cases that reported not visiting the restaurant had no SNP difference from the outbreak sequence. Three sub-lineages (Figure 2, marked A–C) which emerged from November 2015 were apparent. Prior to this, genetic variation had been observed, but distinct sub-lineages had not been identified, with the exception of three cases in March 2015 which appeared distinct from the main outbreak lineage (Figure 2, marked D).

Environmental investigations

All routine environmental inspections at the restaurant had been satisfactory before the outbreak. Eighteen inspections were carried out during the investigation; until November 2015 these were inconclusive and therefore the restaurant remained open. There were no reports of staff illness before the outbreak. Carvery food was not available for sampling due to the high food turnover, however, raw meat and fresh water samples were taken at the beginning of each cluster and in early 2016 (n = 21, all negative). The food trace-back investigations were inconclusive and did not identify any suppliers linked with other cases or outbreaks.

Environmental sampling in the kitchen, restaurant, staff rooms, toilets and flats above the restaurant was conducted throughout the investigation, with 354 samples collected (Table 3). These were negative throughout the early stages of the investigation, but from November 2015 onwards *S. Typhimurium* matching the outbreak clade was isolated from 106 environmental samples (Table 3). Isolates from three sewer swabs matched the outbreak profile. From November 2015 samples taken from cleaning materials, the pot wash area and wash hand basins were also found to have isolates that matched the outbreak profile. From January 2016 further areas of the kitchen were implicated along with drains (Table 3). The majority of environmental isolates

TABLE 3

Summary of environmental samples (n = 375) with isolates implicated in a Salmonella outbreak (n = 106) by whole genome sequencing, United Kingdom, March 2015–May 2016

Sample date	Water samples (n)	Food samples (n)	Environmental samples (n)	WGS positive isolates within five SNP clade(n)	Positive sample source
Mar 2015	1	0	26	0	NA
Jun 2015	0	1	16	0	NA
Sep 2015	1	2	27	0	NA
Nov 2015	0	0	19	3	Sinks/wash hand basins
				7	Cleaning materials
				3 ^a	Sewer swabs
Dec 2015	0	0	67	5	Kitchen cloths
				2 ^b	Sinks/wash hand basins
				2 ^b	Cleaning materials
Jan 2016	6	9	47	15	Surface and deep drain swabs
				4	Kitchen cloths
				12 ^a	Sinks/wash hand basins
				4	Meat sink waste trap
				7	Floor swabs
				10	Kitchen surface swabs
Feb 2016	1	0	42	19	Surface and deep drain swabs
				1	Sinks/wash hand basins
Mar 2016	0	0	41	12	Surface and deep drain swabs
Apr 2016	0	0	59	0	NA
May 2016	0	0	10	0	NA
Total	9	12	354	106	NA

NA: not applicable; SNP: single nucleotide polymorphism; WGS: whole genome sequencing.

^a Three isolates matched outbreak sequence (0 SNP difference).

^b One isolate matched outbreak sequence (0 SNP difference).

fell into sub-lineages A–C, however several deep drain swabs were identical to the outbreak sequence (Figure 2).

Mapping and visual inspection of the drainage systems identified significant issues. Water filled traps (u-bends) designed to prevent foul air flow from the drainage system into the building had failed and smoke testing revealed some ineffective drain seals, potentially allowing contaminated bio-aerosol to be disseminated into the kitchen. One sink drain was not connected to any drainage system with waste water pooling under the floor. Other larger drains had failed after leaking waste-water washed away the supporting substrate forming a cavity under the kitchen area. It transpired at that point that drainage water had, on occasion, risen into the kitchen area, although this had not been previously reported. Substantial remedial works were undertaken, however, these were found to have failed on re-inspection and so these drains were later decommissioned.

Interventions

The restaurant responded to the outbreak by rotating staff away from carvery duties in case they were unsuspecting carriers, retraining staff on hygiene procedures, facilitating voluntary staff sampling, installing and monitoring additional hand washbasins, moving raw meat storage outside of the kitchen, and conducting deep cleans of the indoor environment, which over the outbreak increased in frequency and progressed to include weekly steam cleaning and hydrogen peroxide fogging. Cameras were also installed to monitor the carvery buffet, following concerns that deliberate contamination may have occurred.

The restaurant voluntarily closed for 2 days for cleaning following positive environmental swab results in November 2015 and again for several weeks after further positive sampling to allow deep cleaning, kitchen refitting, and restaurant refurbishment. Remedial work was undertaken on the drains in February 2016. Capping of all kitchen floor drains and hydrogen peroxide fogging followed in March 2016. This appeared to resolve issues of contamination. Throughout the

investigation EHOs and PHE staff remained in close contact with the restaurant and provided advice.

Discussion

Our investigation identified a prolonged outbreak of *S. Typhimurium* linked to a restaurant where 82 cases (72 confirmed, 10 possible) had eaten food. There was strong epidemiological evidence of a link to the restaurant early in the investigation, supported by timely WGS results which provided a highly discriminatory microbiological link between cases and confirmed an initial point source outbreak. In total, 72 case isolates and 106 environmental isolates from the restaurant clustered into a unique 5 SNP cluster with 61 isolates (53 from cases and eight from environmental samples) identical at the core genome level. However, the environmental link with the restaurant was not established until 7 months after the start of the outbreak when WGS positive isolates were obtained from the kitchen and drainage system.

We found the drains had failed in several places and hypothesised that a reservoir of bacteria in biofilm [15] and flooded areas in underfloor cavities may have sustained this outbreak, after repeated environmental cleaning failed. Drainage problems in one area of the kitchen led to liquid from the drains seeping into the kitchen suggesting a contamination pathway. We found isolates matching the outbreak strain on kitchen cloths, swabs from kitchen sinks, and pot wash areas suggesting contact with sinks may have provided a second contamination pathway. We also identified ineffective drain water-traps potentially allowing the movement of contaminated bio-aerosols [13]. Smoke tests demonstrated the potential for dissemination of foul air into the kitchen.

While aerosolised drain contamination has not been previously described in *S. Typhimurium* outbreaks, there is supporting evidence for this transmission pathway for Gram-negative bacteria in hospital drainage systems (modelled using *Pseudomonas putida*) [13]. Bio-aerosol transmission in building drains was implicated in a large outbreak of severe acute respiratory syndrome suggesting this as a feasible hypothesis [16]. Previous outbreak investigations have also identified the prolonged survival of food-borne pathogens such as *Listeria monocytogenes* in drainage systems of food production plants [15,17]. Sinks and drains have also been linked with prolonged bacterial outbreaks in hospital settings [18,19]. Occult environmental contamination can therefore be difficult to identify and control in diverse settings.

There was strong epidemiological evidence that eating carvery food was associated with illness, suggesting it may have been an important transmission vehicle. However, there was no other evidence to confirm contamination of food or utensils, and temperature logs suggested food was cooked appropriately therefore the cooked food must have been contaminated in the

kitchen environment if this hypothesis is correct. Direct transmission from asymptomatic staff could also not be ruled out as not all staff complied with the first round of screening. We could not rule out the effect of potential lapses in cleaning and kitchen hygiene practices that may also have sustained this outbreak. Eating at busy weekend periods appeared to be linked to cases in the descriptive analysis, suggesting working practices may have been less rigorously applied during busy periods, even after staff received additional hygiene training during the outbreak.

Many of the isolates sampled from drains belonged to one of several genetic sub-lineages which had evolved from the main outbreak lineage. Several *Salmonella* isolates sampled from the drains also matched the main outbreak sequence supporting the hypothesis that the drains were potentially an important reservoir. The kitchen drains and sewer system were unlikely to be the original source and may have become contaminated by asymptomatic staff, or a diner [20]. It is also possible that a one-off contamination event from raw or undercooked food was responsible for seeding this outbreak as raw meats such as pork cannot be guaranteed to be *Salmonella* free, although our food trace-back did not find a supply chain problem.

We were unable to identify sources of infection or transmission routes for the 31 people infected with the outbreak strain (13 reported no restaurant exposure). We considered that some of these people may have been unknowing secondary cases, given the national unique WGS results, which had not been observed before or since this outbreak, and also that asymptomatic carriage was observed during the outbreak, however no epidemiological links to outbreak cases with restaurant exposure were identified.

Strengths and limitations

A key strength of this investigation was the use of routine WGS which strengthened the epidemiological evidence of a point-source outbreak enabling rapid implementation of control measures before a holiday weekend in March 2015. However, there were also a number of limitations. Early cases noted their suspicions of the restaurant on social media which may have introduced recall bias in our case-control study, inflating measures of association. The analytical study had a poor response rate and a small sample size. This possibly introduced type II error for other food exposures or potential confounders which may partially explain why other foods were not identified as potential sources of infection even though contamination in the kitchen was possible. Our analysis plan excluded protective food exposures potentially increasing residual confounding in our final model.

Other information biases may have been present in this investigation as many cases were identified after the hypothesis had been established, potentially leading

to over ascertainment of exposure at the restaurant. However, the investigation did not identify a common source other than the restaurant, no other outbreaks with the unique profile have been identified and since the drainage issues have been resolved, no further cases have been identified.

Evidence of environmental contamination was lacking before November 2015. This was possibly due to the lower density and frequency of sampling or the techniques used, although it is unclear if these or other factors resulted in inconclusive findings.

Implications for public health

Salmonella outbreaks are commonly linked to food, restaurants and water supplies [21-26]. Biofilm may harbour *Salmonella* sp. in drains and long-term environmental contamination is possible [3], but rarely reported nor is the potential for bio-aerosol related contamination. Our findings suggest greater consideration should be given to undertaking drain swabbing at an early stage of restaurant and food related outbreak investigations. This will enable identification of similarities between environmental and clinical isolates that may have previously not been possible before the routine use of highly discriminatory WGS.

This outbreak was unexpected in a newly built restaurant managed by a national chain. Defective drains may have been a one-off incident. However, repeated failures of drains and continued contamination of the kitchen identified here suggests the design and/or installation of drainage systems was suboptimal. Increased inspections in the building process may be required. Consideration should be given to drainage systems which facilitate inspection of water-traps or use of multiple traps should be considered to reduce the impact of failure.

Conclusions

This outbreak of a nationally unique strain of *S. Typhimurium* was linked to a single restaurant. The defective drainage system in the restaurant may have acted as an environmental reservoir and dissemination of bio-aerosol from the drains into the kitchen was plausible. This linked with possible lapses in kitchen hygiene may have enabled intermittent contamination of food during the period of the outbreak. While the original source remains unclear, this protracted outbreak was controlled after remedial work on the drains, a kitchen and restaurant refurbishment and a deep clean involving hydrogen peroxide fogging.

Public health professionals should consider drainage systems and bio-aerosols as potential sources in any outbreak of salmonellosis and environmental investigations should include swabbing drains early in outbreak scenarios. Investigators should work towards accessing timely WGS analysis, which in this investigation was essential for case finding, establishing a single most probable source and underpinning the

epidemiological evidence used to demonstrate a need for actions to prevent further cases.

Acknowledgements

We are grateful to all restaurant staff and customers who aided this investigation along with Srilaxmi Degala, Lesley Larkin, Dr Jeremy Hawker (Public Health England), Deb Morgan, Drazenka Tubin-Delic, Tracy Bishop (Food Standards Agency) for contributions to the Outbreak Control Team. We thank Dr Sam Bracebridge (Public Health England) for her critique of this manuscript.

Conflict of interest

None declared.

Authors' contributions

Incident Director: PM. Conceived and designed the case-control study protocol: JMJ, RP. Data collection, processing, analysis or interpretation: JMJ, RBS, PM, CH, JCR, TD, PA, RJ, DM, RP. Drafting the manuscript: JMJ, RBS, TD, PM, RP.

References

1. Roberts JA, Cumberland P, Sockett PN, Wheeler J, Rodrigues LC, Sethi D, et al. Infectious Intestinal Disease Study Executive. The study of infectious intestinal disease in England: socio-economic impact. *Epidemiol Infect.* 2003;130(1):1-11. <https://doi.org/10.1017/S0950268802007690> PMID: 12613740
2. Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, et al. IID2 Study Executive Committee. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. *Gut.* 2012;61(1):69-77. <https://doi.org/10.1136/gut.2011.238386> PMID: 21708822
3. Silva C, Calva E, Maloy S. One Health and Food-Borne Disease: *Salmonella* Transmission between Humans, Animals, and Plants. *Microbiol Spectr.* 2014;2(1):OH-0020-2013. 10.1128/microbiolspec.OH-0020-2013 PMID: 26082128
4. Holman EJ, Allen KS, Holguin JR, Torno M, Lachica M. A community outbreak of *Salmonella enterica* serotype Typhimurium associated with an asymptomatic food handler in two local restaurants. *J Environ Health.* 2014;77(2):18-20. PMID: 25226780
5. Ashton PM, Nair S, Peters TM, Bale JA, Powell DG, Painset A, et al. *Salmonella* Whole Genome Sequencing Implementation Group. Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ.* 2016;4:e1752. <https://doi.org/10.7717/peerj.1752> PMID: 27069781
6. Ashton P, Nair S, Peters T, Tewolde R, Day M, Doumith M, et al. Revolutionising Public Health Reference Microbiology using Whole Genome Sequencing: *Salmonella* as an exemplar. *bioRxiv.* 2015.
7. Brooks JT, Matyas BT, Fontana J, DeGroot MA, Beuchat LR, Hoekstra M, et al. An outbreak of *Salmonella* serotype Typhimurium infections with an unusually long incubation period. *Foodborne Pathog Dis.* 2012;9(3):245-8. <https://doi.org/10.1089/fpd.2011.0992> PMID: 22283668
8. Ethelberg S, Lisby M, Torpdahl M, Sørensen G, Neimann J, Rasmussen P, et al. Prolonged restaurant-associated outbreak of multidrug-resistant *Salmonella* Typhimurium among patients from several European countries. *Clin Microbiol Infect.* 2004;10(10):904-10. <https://doi.org/10.1111/j.1469-0691.2004.00990.x> PMID: 15373885
9. Ashton PM, Peters T, Ameh L, McAleer R, Petrie S, Nair S, et al. Whole Genome Sequencing for the Retrospective Investigation of an Outbreak of *Salmonella* Typhimurium DT 8. *PLoS Curr.* 2015;7:ecurrents.outbreaks.2c05a47d292f376afc5a6fcd88a7a3b6. <https://doi.org/10.1371/currents.outbreaks.2c05a47d292f376afc5a6fcd88a7a3b6> PMID: 25713745
10. Public Health England Microbiology Services. Detection and enumeration of bacteria in swabs and other environmental samples. Version 2. London: Public Health England; 2014.

11. The Water Supply (Water Fittings) Regulations. 1999. Statutory Instrument: 1999 No. 1148 (1999). Available from: [http://www.dwi.gov.uk/stakeholders/legislation/ws\(fittings\)regs1999.pdf](http://www.dwi.gov.uk/stakeholders/legislation/ws(fittings)regs1999.pdf)
12. Moore B, Perry EL, Chard ST. A survey by the sewage swab method of latent enteric infection in an urban area. *J Hyg (Lond)*. 1952;50(2):137-56. <https://doi.org/10.1017/S0022172400019501> PMID: 14938529
13. Gormley M, Aspray TJ, Kelly DA, Rodriguez-Gil C. Pathogen cross-transmission via building sanitary plumbing systems in a full scale pilot test-rig. *PLoS One*. 2017;12(2):e0171556. <https://doi.org/10.1371/journal.pone.0171556> PMID: 28187135
14. Public Health England Microbiology Services. Detection of Salmonella species, Microbiology Services Food Water and Environmental Microbiology, Standard Method. Version 2. London: Public Health England; 2014.
15. Coughlan LM, Cotter PD, Hill C, Alvarez-Ordóñez A. New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry. *Front Microbiol*. 2016;7:1641. <https://doi.org/10.3389/fmicb.2016.01641> PMID: 27803696
16. McKinney KR, Gong YY, Lewis TG. Environmental transmission of SARS at Amoy Gardens. *J Environ Health*. 2006;68(9):26-30, quiz 51-2. PMID: 16696450
17. Berrang ME, Meinersmann RJ, Frank JF, Ladely SR. Colonization of a newly constructed commercial chicken further processing plant with *Listeria monocytogenes*. *J Food Prot*. 2010;73(2):286-91. <https://doi.org/10.4315/0362-028X-73.2.286> PMID: 20132673
18. Vergara-López S, Domínguez MC, Conejo MC, Pascual Á, Rodríguez-Baño J. Wastewater drainage system as an occult reservoir in a protracted clonal outbreak due to metallo- β -lactamase-producing *Klebsiella oxytoca*. *Clin Microbiol Infect*. 2013;19(11):E490-8. <https://doi.org/10.1111/1469-0691.12288> PMID: 23829434
19. Hota S, Hirji Z, Stockton K, Lemieux C, Dedier H, Wolfaardt G, et al. Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. *Infect Control Hosp Epidemiol*. 2009;30(1):25-33. <https://doi.org/10.1086/592700> PMID: 19046054
20. Sánchez-Vargas FM, Abu-El-Haija MA, Gómez-Duarte OG. Salmonella infections: an update on epidemiology, management, and prevention. *Travel Med Infect Dis*. 2011;9(6):263-77. <https://doi.org/10.1016/j.tmaid.2011.11.001> PMID: 22118951
21. Moffatt CRM, Combs BG, Mwanri L, Holland R, Delroy B, Cameron S, et al. An outbreak of *Salmonella* Typhimurium phage type 64 gastroenteritis linked to catered luncheons in Adelaide, South Australia, June 2005. *Commun Dis Intell Q Rep*. 2006;30(4):443-8. PMID: 17330386
22. Ethelberg S, Sørensen G, Kristensen B, Christensen K, Krusell L, Hempel-Jørgensen A, et al. Outbreak with multi-resistant *Salmonella* Typhimurium DT104 linked to carpaccio, Denmark, 2005. *Epidemiol Infect*. 2007;135(6):900-7. <https://doi.org/10.1017/S0950268807008047> PMID: 17335629
23. Silk BJ, Foltz JL, Ngamsnga K, Brown E, Muñoz MG, Hampton LM, et al. Legionnaires' disease case-finding algorithm, attack rates, and risk factors during a residential outbreak among older adults: an environmental and cohort study. *BMC Infect Dis*. 2013;13(1):291. <https://doi.org/10.1186/1471-2334-13-291> PMID: 23806063
24. Ethelberg S, Lisby M, Torpdahl M, Sørensen G, Neimann J, Rasmussen P, et al. Prolonged restaurant-associated outbreak of multidrug-resistant *Salmonella* Typhimurium among patients from several European countries. *Clin Microbiol Infect*. 2004;10(10):904-10. <https://doi.org/10.1111/j.1469-0691.2004.00990.x> PMID: 15373885
25. Ailes E, Budge P, Shankar M, Collier S, Brinton W, Cronquist A, et al. Economic and health impacts associated with a *Salmonella* Typhimurium drinking water outbreak-Alamosa, CO, 2008. *PLoS One*. 2013;8(3):e57439. <https://doi.org/10.1371/journal.pone.0057439> PMID: 23526942
26. O'Reilly CE, Bowen AB, Perez NE, Sarisky JP, Shepherd CA, Miller MD, et al. Outbreak Working Group. A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. *Clin Infect Dis*. 2007;44(4):506-12. <https://doi.org/10.1086/511043> PMID: 17243052

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2017.

Outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* 1/2a in sliced cold beef ham, Italy, May 2016

Cristiana Maurella¹, Silvia Gallina¹, Giuseppe Ru¹, Daniela Adriano¹, Alberto Bellio¹, Daniela Manila Bianchi¹, Laura Chiavacci¹, Maria Ines Crescio¹, Margherita Croce², Valeria D'Errico¹, Maria Franca Dupont², Alessandro Marra¹, Ubaldo Natangelo¹, Francesco Pomilio³, Angelo Romano¹, Stefano Stanzione², Teresa Zaccaria⁴, Fabio Zuccon¹, Maria Caramelli¹, Lucia Decastelli¹

1. Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta, Turin, Italy
2. Igiene degli Alimenti e della Nutrizione (Food Hygiene and Nutrition Service) Local Health Unit TO4, Ciriè, Italy
3. Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, National Reference Centre for *Listeria monocytogenes*, Teramo, Italy
4. Laboratorio Microbiologia e Virologia U Città della Salute di Torino, Turin, Italy

Correspondence: Cristiana Maurella (cristiana.maurella@izsto.it)

Citation style for this article:

Maurella Cristiana, Gallina Silvia, Ru Giuseppe, Adriano Daniela, Bellio Alberto, Bianchi Daniela Manila, Chiavacci Laura, Crescio Maria Ines, Croce Margherita, D'Errico Valeria, Dupont Maria Franca, Marra Alessandro, Natangelo Ubaldo, Pomilio Francesco, Romano Angelo, Stanzione Stefano, Zaccaria Teresa, Zuccon Fabio, Caramelli Maria, Decastelli Lucia. Outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* 1/2a in sliced cold beef ham, Italy, May 2016. Euro Surveill. 2018;23(10):pii=17-00155. <https://doi.org/10.2807/1560-7917.ES.2018.23.10.17-00155>

Article submitted on 01 Mar 2017 / accepted on 24 May 2017 / published on 08 Mar 2018

In May 2016, two separate clusters of febrile gastroenteritis caused by *Listeria monocytogenes* were detected by the local health authority in Piedmont, in northern Italy. We carried out epidemiological, microbiological and traceback investigations to identify the source. The people affected were students and staff members from two different schools in two different villages located in the Province of Turin; five of them were hospitalised. The epidemiological investigation identified a cooked beef ham served at the school canteens as the source of the food-borne outbreak. *L. monocytogenes* was isolated from the food, the stools of the hospitalised pupils and the environment of the factory producing the cooked beef ham. All isolates except one were serotype 1/2a, shared an indistinguishable PFGE pattern and were 100% identical by whole genome sequencing (WGS). By combining a classical epidemiological approach with both molecular subtyping and WGS techniques, we were able to identify and confirm a *Listeria* gastroenteritis outbreak associated with consumption of sliced cold beef ham.

Introduction

Listeria monocytogenes is a ubiquitous Gram-positive food-borne pathogen that causes listeriosis both in humans and in several animal species. In some groups (people with weakened immune systems, older adults, newborns, pregnant women and their unborn babies), the disease can be an important cause of life-threatening septicaemia and meningoencephalitis [1]. Food-borne transmission of *L. monocytogenes* can also cause a self-limited acute febrile gastroenteritis, primarily reported among healthy people.

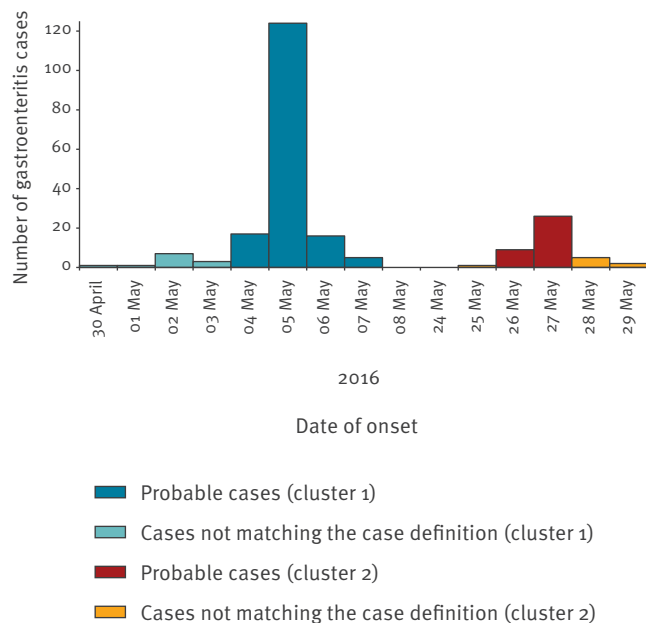
L. monocytogenes is widely distributed in natural environments and has been isolated from soil, decaying vegetation, stream water, sewage, urban environments and human and animal faeces [2]. Its ubiquity and ability to adapt and to survive under environmentally stressed conditions make it challenging to control and eradicate *L. monocytogenes* in food-processing environments and constitute a concern for the food industry [2]. The role of *L. monocytogenes* in food-borne outbreaks has been clearly recognised in the last decades [3-6]. Since 1993, listeriosis has been a reportable disease in Italy.

Outbreak description

On 5 May 2016, five children from School A, an institution that includes two scholastic levels, a nursery with pupils aged 3–6 years and a primary school with pupils aged 6–10, and located in village A, a municipality of the province of Turin, were taken to the paediatric emergency room for symptoms of acute febrile gastroenteritis. On the same day, 26 of 162 (16%) pupils from the nursery and 25 of 400 (6%) pupils from the primary school returned home early (some of them before lunch time) because of fever, vomiting and abdominal pain. A report of a suspected food-borne outbreak was sent by the paediatric emergency room to the local health authority. In the following 3 days, additional pupils and staff from School A became ill including one hospitalised. On 6 May, the inspectors from the local health authority went to the mass catering food service (Caterer A) that provides meals to School A and collected retention samples (i.e. representative samples of the full meal stored by the mass catering food service) from the meals served on 3, 4 and 5 May 2016.

FIGURE 1

Epidemic curve of gastrointestinal illness in two schools, *Listeria monocytogenes* outbreak, Italy, May 2016 (n = 217)



The plot shows a classical point-source epidemic. Bars indicate probable cases by date of onset.

From 25 to 29 May 2016, 20 days after this first cluster was identified, children from School B, located in village B in the same Province, reported gastrointestinal symptoms similar to those in the first cluster. Once again, the local health authority inspectors collected retention samples from the mass catering food service (Caterer B, different from the previous one) that provided meals to School B.

We present the results of epidemiological, microbiological and traceback investigations for source attribution.

Methods

Case finding and hypothesis generation

An investigation team was created involving personnel from the local health authority, the hospital microbiology laboratory of Turin (Città della Salute) and the regional branch of the Istituto Zooprofilattico Sperimentale (IZS) in Turin as coordinating unit, dealing with both epidemiology and food analysis.

A standardised trawling questionnaire for gastrointestinal illness including questions on clinical symptoms, canteen attendance and foods eaten, was distributed by the local health authority to all the pupils' parents and to staff of Schools A and school B. Administration of the questionnaire was postponed 20 days for the cluster associated with School B because of school holidays. At the IZS, questionnaire data were entered

in an ad hoc database and analysed using Stata 14.1 [7] software.

Foods and environmental samples were submitted to the IZS laboratories for pathogen detection and quantification, and faecal samples were sent to the microbiology laboratory of the local hospital.

Case definition

We initially defined a probable case as an attendee (pupils or staff) of School A or School B presenting with at least two of the following symptoms: headache, nausea, vomiting, diarrhoea, abdominal pain, temperature above 38°C and an onset date starting from the day before the peak of the associated cluster. This definition served as the basis to build the epidemic curve for both clusters of the outbreak.

Individuals who reported symptom onset 2 days before the peak of the associated cluster were excluded from the case definition to favour specificity over sensitivity, and because information on foods eaten at this time was not available.

After a preliminary cohort analysis in School A, and to further increase specificity, we amended the case definition for the first cluster and defined a probable case as an attendee of School A presenting with at least three of the above-mentioned symptoms. This narrower operational case definition was not applied to the second cluster as there could have been a recall bias on the number of symptoms because of the time lag between the illness and the interviews.

We also used a final case definition for a confirmed case: an attendee of School A or School B presenting clinical symptoms and whose diagnostic test on stool samples confirmed the presence of *L. monocytogenes*.

Cohort study and statistical analysis

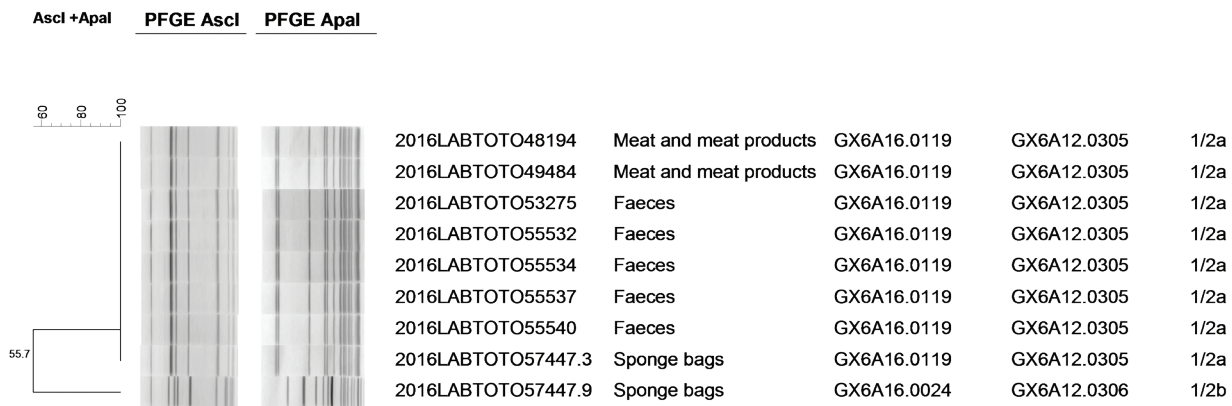
We conducted a retrospective cohort study and calculated the attack rate (AR) and the relative risk (RR) for each food item. Confidence interval was set at 95% (95% CI).

For the first cluster, statistical analysis was performed both on the overall School A dataset and at the nursery and primary school level, used as a proxy for age. Only exposure data regarding 3 and 4 May were taken into account as the epidemic peak occurred on 5 May 2016 and it seemed unlikely that the meal served on that date, or later, could be the source of infection.

For the second cluster, the same exclusion criteria were applied and the data analysis referred to the meals consumed on 25 and 26 May.

FIGURE 2

Dendrogram showing genetic similarity and genomic clusters of *Listeria monocytogenes* strains isolated from sliced beef ham, stools and environmental samples, Italy, May 2016 (n = 9)



Clusters are indicated on the side of the figure. The identification numbers of isolates, origin of the samples, Ascl and Apal profiles and serotypes are listed in the columns.

Microbiological investigation and traceback

Retention samples, other food samples and environmental samples

Retention samples were sent to the food control laboratory of the IZS for quantification of *Bacillus cereus*, beta-glucuronidase-positive *Escherichia coli*, *Clostridium perfringens*, *Enterobacteriaceae*, coagulase-positive staphylococci, *Listeria monocytogenes* and sulfite-reducing anaerobic bacteria by routine methods. Samples were also tested for *B. cereus* diarrhoeal and emetic toxins, norovirus, *Salmonella* spp., Shiga toxin-producing *E. coli*, and staphylococcal enterotoxins.

Regarding the first cluster and following preliminary analysis of the data, a specific food item was suspected as the source of the outbreak. Hence the local health authority was requested to trace-back the retailer and the producer to collect additional samples of the suspected food. Therefore, two weeks after the peak, one more food sample, an unopened package from a different batch, was collected from the producer (a cured meat factory) who had supplied the suspected food to the mass catering food service.

Regarding the second cluster, only retention samples from the meal served on 26 May 2016 were available at the mass catering food service, and an unopened package of the suspected food item from the same batch as the one served was retrieved from the producer. The same producer supplied the two mass catering food services involved in the two clusters. Environmental samples were collected pre-moistened sampling cellulose sponge bags (Solar-Cult, Solar Biologicals Inc, Vancouver, Canada) from surfaces that were not in contact with food at the mass catering food service that

served School B and at the producer who had supplied food to both mass catering services.

Stool samples

Stool specimens were collected from the five hospitalised children (four from the first cluster and one from second cluster) and cultured using specific culture media for pathogenic bacteria (*B. cereus*, *Campylobacter* spp., *L. monocytogenes*, *Salmonella* spp., Shiga toxin-producing *E. coli*, *Shigella* spp., *Staphylococcus aureus*, and *Yersinia enterocolitica*). Characteristic colonies were only on *Listeria* medium (PalcamAgar, Liofilchem, Italy). *Listeria* strains were identified by MALDI-TOF technology.

Identification of the *Listeria monocytogenes* strains

All *L. monocytogenes* strains isolated from stool, food and environmental samples were identified according to the ISO 11290 method using the Vitek MS system (bioMérieux, Marcy l'Etoile, France) and serotyped based on agglutination reactions with antisera for *L. monocytogenes* (Denka Seiken Co., Tokyo, Japan).

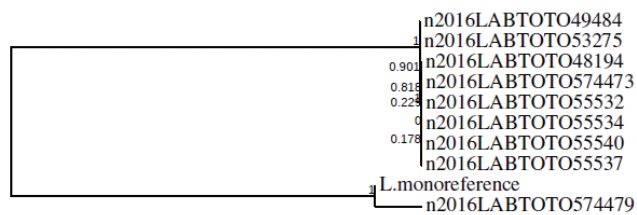
Pulsed-field gel electrophoresis (PFGE) was performed by the National Reference Laboratory for *L. monocytogenes* in Teramo, according to the United States Centres for Disease Control and Prevention PulseNet protocol [8], and analysed with Applied Maths BioNumerics software package (Version 7.5, Applied Maths, Saint-Martins-Latem, Belgium).

Whole genome sequencing

Whole Genome Sequencing (WGS) of the DNA extracted from *L. monocytogenes* isolates was performed on the MiSeq platform (Illumina, San Diego, United States),

FIGURE 3

Single-nucleotide polymorphism analysis of *Listeria monocytogenes* isolates, Italy, May 2016 (n = 9)



The maximum likelihood tree done with the CSI Phylogeny 1.2 tool highlights two distinct clusters, one that includes eight ST₁₁ isolates and one that includes the ST₅ environmental isolate and the reference strain “*L. monoreference*” (GenBank accession number: AL591824.1).

using paired-end libraries which were prepared by following the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, United States), with 200-bp read length. The reads were first subjected to FastQC Read Quality reports (Galaxy Version 0.65, accessed via the Galaxy public server at <https://usegalaxy.org>) [9] to provide the quality control checks on raw sequence data. We used Assembler 1.2 (accessed via Technical University of Denmark, DTU Center; <https://cge.cbs.dtu.dk/services/Assembler/>) to assemble genomes [10]. All samples were processed for multilocus sequence typing (MLST) with MLST 1.8 (accessed via <https://cge.cbs.dtu.dk/services/MLST/>) [10]. A phylogenetic comparison was made with CSI Phylogeny 1.2 (accessed via <https://cge.cbs.dtu.dk/services/CSIPhylogeny/>) [11]. The single-nucleotide polymorphism (SNP)-typing phylogenetic method was used because of its high discriminatory power that allows distinguishing very closely related isolates to a degree not achievable by other typing methods [12]. SNP was performed with the following parameters: 10 × minimum depth at SNP position, 10% minimum relative depth at SNP position, 100 bp minimum distance between SNPs, 20 for minimum SNP quality, 20 for minimum read mapping quality, 1.98 minimum Z-score for each SNP, and including *L. monocytogenes* (GenBank accession number: AL591824.1) as reference.

Results

Epidemiological investigations

The epidemic curve of the whole outbreak is shown in Figure 1. For each cluster, the pattern was compatible with a point-source epidemic.

In the investigation of the first cluster, 484 completed questionnaires (response rate: 91.7%) were collected and used to build the cohort study. Symptoms were reported by 174 persons; 162 of 484 (33.7%) matched the probable case definition, while 11 people were excluded from the analysis. Reported symptoms among the 162 were: abdominal pain (n = 122; 75%),

fever (n = 110; 68%), headache (n = 102; 63%), nausea (n = 85; 52%), diarrhoea (n = 79; 49%) and vomiting (n = 47; 29%). Two probable cases were staff members. All five probable cases who went to the hospital were afterwards confirmed.

Eight retention samples were analysed. As the microbiological analysis did not give any positive results, the output of the first cluster cohort analysis were used to hypothesise the most likely source of infection. ARs and RRs (Table 1) showed a positive association between consumption of a ‘beef ham’ (sliced cold beef meat, cured-cooked and dressed with oil and lemon) and the disease (RR=2.23; 95% CI: 1.30–3.84). The association between the beef ham and the disease was still significant when stratifying the analysis by school level (nursery vs primary school) (Table 1). Moreover, the strength of the association further increased by using the narrower operational case definition (RR=3.5; 95% CI: 1.5–8.3).

A significant RR > 1 was found between the disease and the consumption of halibut (RR=1.48; 95% CI: 1.02–2.14) for the initial case definition. However, this association disappeared after stratification by school (Table 1). When using the narrower case definition, halibut consumption again did not have a statistically significant association with the cases (RR=1.18; 95% CI: 0.76–1.84), hence we considered it a chance finding and did not proceed to investigate it further.

In the investigation of the second cluster, 382 questionnaires were collected (response rate: 86.8%), although the questionnaires were administered with a delay of 20 days because of school holidays. Of these, 30 were excluded because data were not complete, and 352 were used for the cohort study analysis. Of those 352, 43 (12%) matched the probable case definition, with a peak of the outbreak on 27 May 2016 (Figure 1). The frequency of symptoms among those 43 was similar to the first cluster: abdominal pain (n = 34) headache (n = 25) fever (n = 23), nausea (n = 22), diarrhoea (n = 17) and vomiting (n = 13). We did not find any statistically significant associations, although the beef ham was the food item with the highest RR (RR=1.4; 95% CI: 0.62–3.15; total responses: 268; 34 cases among 215 exposed and six cases among 53 unexposed).

Microbiological investigations

Based on the results of the epidemiological analysis of the first cluster, a microbiological analysis was carried on both the retention sample and on the additional unopened package of the beef ham from the same batch as the one served in school A (batch no. 127529). Moreover, a retention sample and an unopened package belonging to the same batch as the one served in School B (batch no. 130578) from the second cluster were analysed, along with stools specimens from both the clusters and environmental samples from the shared producer (Table 2).

TABLE 1

 Attack rates among exposed and unexposed groups, and relative risks by food item consumed, by school level and day, *Listeria monocytogenes* outbreak, Italy, May 2016 (n =484)

Food	Exposed			Unexposed			RR	95% CI
	Total	Cases	% AR	Total	Cases	% AR		
Two days before peaking								
School A								
Pasta with tomato sauce	357	128	35.85	76	22	28.95	1.24	0.85-1.81
Halibut	338	126	37.28	95	24	25.26	1.48	1.02-2.14
Courgettes	202	74	36.63	231	76	32.90	1.11	0.86-1.44
Bread	328	112	34.15	105	38	36.19	0.94	0.70-1.27
Fresh fruit	277	105	37.91	156	45	28.85	1.31	0.98-1.75
Water	409	143	34.96	24	7	29.17	1.20	0.63-2.27
Nursery (pupils aged 3-6)								
Pasta with tomato sauce	293	93	31.74	62	15	24.19	1.31	0.82-2.10
Halibut	270	87	32.22	85	21	24.71	1.30	0.87-1.96
Courgettes	165	55	33.33	190	53	27.89	1.19	0.92-1.67
Bread	259	77	29.73	96	31	32.29	0.92	0.65-1.30
Fresh fruit	216	74	34.26	139	34	24.46	1.40	0.99-1.98
Water	336	104	30.95	19	4	21.05	1.47	0.61-3.56
Primary school (pupils aged 6-10)								
Pasta with tomato sauce	64	35	54.69	14	7	50.0	1.09	0.62-1.93
Halibut	68	39	57.35	10	3	30.0	1.91	0.73-5.04
Courgettes	37	19	51.35	41	23	56.10	0.92	0.60-1.39
Bread	69	35	50.72	9	7	77.78	0.65	0.43-0.99
Fresh fruit	61	31	50.82	17	11	64.71	0.79	0.51-1.2
Water	73	39	53.42	5	3	60.00	0.89	0.42-1.88
One day before peaking								
School A								
Vegetables soup	272	119	43.75	72	24	33.33	1.31	0.92-1.87
Beef ham	290	132	45.52	54	11	20.37	2.23	1.30-3.84
Roasted potatoes	286	114	39.86	58	29	50.00	0.8	0.59-1.07
Bread	267	107	40.07	77	36	46.75	0.86	0.65-1.13
Fresh fruit	242	103	42.56	102	40	39.22	1.09	0.82-1.44
Water	333	139	41.74	11	4	36.36	1.15	0.52-2.53
Nursery (pupils aged 3-6)								
Vegetables soup	205	85	41.46	59	17	28.81	1.44	0.93-2.22
Beef ham	224	93	41.52	40	9	22.50	1.85	1.02-3.35
Roasted potatoes	224	84	37.50	40	18	45.00	0.83	0.57-1.22
Bread	194	70	36.08	70	32	45.71	0.79	0.58-1.08
Fresh fruit	176	69	39.20	88	33	37.50	1.05	0.75-1.45
Water	257	99	38.52	7	3	42.86	0.9	0.38-2.14
Primary school (pupils aged 6-10)								
Vegetables soup	67	34	50.75	13	7	53.85	0.94	0.54-1.64
Beef ham	66	39	59.09	14	2	14.29	4.14	1.13-15.16
Roasted potatoes	62	30	48.39	18	11	61.11	0.79	0.51-1.24
Bread	73	37	50.68	7	4	57.14	0.89	0.45-1.75
Fresh fruit	66	34	51.52	14	7	50.00	1.03	0.58-1.83
Water	76	40	52.63	4	1	25.00	2.11	0.38-11.65

AR: attack rate; CI: confidence interval; RR: relative risk.
Significant associations are shown in bold.

TABLE 2Microbiological results of stool, food and environmental samples, *Listeria monocytogenes* outbreak, Italy, May 2016 (n = 23)

Cluster	Number of tested samples	Material sampled	Sampling place	Qualitative analysis (in 25 g)	Quantitative analysis	Serotype	PFGE profile (Ascl/Apal)	MLST
1	1	Beef ham/retention sample (batch no. 127529)	Mass catering food service school A	Absence	<10 cfu/g	ND	ND	ND
1	1	Beef ham/unopened batch (no. 127529)	Producer	Presence	>15,000 cfu/g	1/2a	GX6A16.0119 / GX6A12.0305	ST11
1	4	Stool specimens	Hospital	Presence	ND	1/2a	GX6A16.0119 / GX6A12.0305	ST11
2	1	Beef ham/retention sample (batch no. 130578)	Mass catering food service school B	Presence	15,000 cfu/g	1/2a	GX6A16.0119 / GX6A12.0305	ST11
2	1	Beef ham/unopened batch (no. 130578)	Producer	Absence	<10 cfu/g	ND	ND	ND
2	5	Environmental samples	Mass catering food service school B	Absence	ND	ND	ND	ND
2	1	Stool specimen	Hospital	Presence	ND	1/2a	GX6A16.0119 / GX6A12.0305	ST11
1 and 2	7	Environmental samples	Producer	Absence	ND	ND	ND	ND
1 and 2	1	Environmental sample (cutter machine)	Producer	Presence	ND	1/2a	GX6A16.0119 / GX6A12.0305	ST11
1 and 2	1	Environmental sample (fridge handle)	Producer	Presence	ND	1/2b	GX6A16.0024 / GX6A12.0306	ST5

cfu: colony-forming units; MLST: multilocus sequence type; ND: not done; PFGE: pulsed-field gel electrophoresis.

As a result, nine of 23 samples tested positive for *L. monocytogenes*. These were five stool samples (four from the first cluster and one from the second cluster), two food samples (one unopened package from a different batch obtained during the investigation of the first cluster and one retention sample from the second cluster, both with a count exceeding 15,000 colony-forming units (cfu)/g) and two environmental samples from the incriminated producer's premise (one from the cutter machine and one from the fridge handle).

Identification of the *Listeria* strain

Both the unopened batch (no. 127529) of beef ham collected during the investigation of the first cluster and the beef ham from the retention samples collected during the investigation of the second cluster tested positive for *L. monocytogenes*, with >15,000 cfu/g and 15,000 cfu/g, respectively. In addition, seven *L. monocytogenes* isolates were obtained from the stool specimens from five children and from two environmental samples from the producer. Agglutination reactions assigned eight isolates to serotype 1/2a, while the remaining one (from the producer) was serotype 1/2b. In addition, all serotype 1/2a isolates showed indistinguishable PFGE pattern whereas the serotype

1/2b strain showed a different PFGE profile (Figure 2 and Table 2).

Whole genome sequencing data analysis

In silico MLST showed that all serotype 1/2a isolates belonged to ST11 and the 1/2b isolate was ST5 (Table 2). The maximum likelihood tree obtained through the SNPs analysis showed two distinct clusters. The first cluster included eight ST11 isolates and the second cluster included the reference genome and the environmental ST5 isolate (Figure 3).

The maximum likelihood tree done with the CSI Phylogeny 1.2 tool highlights two distinct clusters, one that includes eight ST11 isolates and one that includes the ST5 environmental isolate and the reference strain "L. monoreference" (GenBank accession number: AL591824.1).

Discussion

By combining an epidemiological approach and molecular typing including WGS techniques, we were able to identify and confirm a *Listeria* outbreak associated with the consumption of one food item, sliced cold beef ham.

The outbreak consisted of two school-associated clusters of non-invasive listeriosis cases linked to one *L. monocytogenes* strain. Cases experienced mostly abdominal pain, fever, headache and nausea. Gastrointestinal symptoms occurred rapidly after consumption of the incriminated meals, probably because a large dose of *L. monocytogenes* was ingested. A short incubation period for *L. monocytogenes*-associated gastroenteritis varying from 6 to 240 hours has already been reported elsewhere [13].

This outbreak was characterised by a high level of contamination of the suspected food. Unfortunately, it was not possible to determinate the exact number of *L. monocytogenes* in the beef ham sampled during the investigation of the first cluster because the laboratory in charge of official control had not expected a very high level of contamination and therefore only performed the dilutions needed in compliance with the Commission Regulation (EC) no. 2073/2005 to determine the threshold of *L. monocytogenes* < 100 cfu/g. It was not possible to repeat the analysis because it is mandatory to discard the samples after microbiological investigation. Isolation of a matching strain from the cutter machine suggests that the food contamination probably occurred in the production plant.

Another remarkable feature of the outbreak was the quite long persistence of the contamination. Norton et al. showed that specific *L. monocytogenes* ribotypes persisted over time in the environments of two of three processing plants for smoked fish [14]. Similar findings have been reported by a variety of groups that showed persistence of specific *L. monocytogenes* subtypes in different processing plants for smoked fish, poultry, meat or dairy [15-17]. In our investigation, the time span was 20 days from the first isolation of *Listeria* in the beef ham (unfortunately the date of production of the contaminated batch was not available) and the isolation from the environment of the cured meat factory where the beef ham was produced. This further confirms that *L. monocytogenes* can persist in the environment.

Among the 13 known serotypes of *L. monocytogenes*, the ones most frequently associated with human listeriosis are 1/2a, 1/2b and 4b, representing more than 95% of the infections. Our results are consistent with previous reports suggesting that serotype 1/2a is predominant in *L. monocytogenes*-associated gastroenteritis outbreaks [18], whereas serotype 4b was the most frequent up to 2010 and 4b is still the most reported serotype in invasive listeriosis outbreaks [19,20].

Although *L. monocytogenes* was not found in the retention sample of the first cluster, the epidemiological investigation was helpful in identifying a strong association between the consumption of beef ham and the disease. This association triggered the targeting and retrieval of a specific food for further microbiological analysis. A possible but weak association with another

food, halibut, was seen in a first analysis based on the initial case definition, but not in stratified analysis. When using the narrowed case definition, it was considered a chance finding and no traceback was performed for halibut. Findings from the cohort study of the second cluster, although not statistically significant, were consistent and supported the same hypothesis. As shown by the inconclusive results from the epidemiological investigation of the second cluster, a stand-alone epidemiological approach may suffer from problems associated with the data collection or from a small sample size when the affected individuals come from a small community. In the second cluster, distribution and collection of questionnaires had to be carried out with some delay (20 days after the peak of the outbreak) leading to incomplete answers and probably recall problems. Consequently, there was the potential for non-differential misclassification of the exposure. That, combined with the small sample size, may explain our failure to obtain a statistically significant association.

However, microbiological, molecular typing and WGS data helped confirm the link between the clusters that appeared to be independent based on the spatial distance (two different municipalities), the 20-day interval between them and the different catering suppliers. Historically, food-borne disease outbreaks of local scale, often linked to a single restaurant or social event, are caused by pathogens other than *Listeria* (e.g. *Salmonella*), even if *Listeria* have also been reported in connection with local outbreaks [21]. This is one example of a short *Listeria* outbreak that can also be responsible for scattered epidemics. Today, outbreaks often involve food products that are centrally produced and widely distributed geographically. In our case, two different mass catering food suppliers that served a number of schools scattered over many municipalities were supplied by a unique cured meat factory.

The problems in identifying the epidemiological links were successfully addressed through an integrated approach including questionnaire-based studies, trace-back investigations and biomolecular techniques. In the current study, the use of WGS made it possible to link human cases that occurred over a period of 20 days, brought about the understanding that there was in fact a single continuous outbreak source, and established links between two different mass catering food suppliers and one producer.

The correlation between food, stool and environmental specimens found in WGS, MLST and the phylogenetic analysis based on SNPs provided sufficient evidence for the local authorities to act accordingly and recall the contaminated food to prevent more outbreak-related cases. The authorities were also advised to monitor the follow-up of people exposed to the incriminated beef ham as it has been reported that severe listeriosis can have a long incubation period.

The output of the analysis from the first cluster highlighted a heavier impact in 6–10 year-old pupils than in younger ones, associated with a modification of effect between the exposure to *L. monocytogenes* and the age of the children. This may be explained by larger servings offered to the older pupils who probably experienced the highest exposure.

Conclusion

The current study stresses the importance of an integrated approach when dealing with public health issues and suggests the usefulness of diagnostic techniques that enable clustering of isolates from different episodes.

Acknowledgements

The authors gratefully acknowledge the Prevention Operator/Technician (TPALL) and the veterinary officers involved in tracing back the food and in performing the environmental samplings and Dr Marion M Simmons for her valuable suggestions in improving the English spelling of the text.

Conflict of interest

None declared.

Authors' contributions

Cristiana Maurella and Giuseppe Ru analysed the epidemiological data and wrote the article. Margherita Croce, Maria Franca Dupont and Stefano Stanzione administered and collected the interviews. Valeria D'Errico, Alessandro Marra, Ubaldo Natangelo entered and managed the epidemiological data. Silvia Gallina, Daniela Adriano and Fabio Zuccon carried out the laboratory analyses on food and environmental samples. Alberto Bellio, Angelo Romano, and Lucia Decastelli typified bacteria isolates from faecal, food and environmental samples. Teresa Zaccaria collected and analysed the faecal samples. Francesco Pomilio performed the strain identification and critically revised the article. Maria Ines Crescio and Laura Chiavacci internally revised the paper. Maria Caramelli and Daniela Manila Bianchi coordinated the communication between the involved public health authorities. All the authors contributed to the discussion of the article.

References

1. Jordan K, Leong D, Ordóñez Alvarez A. *Listeria monocytogenes* in the food processing Environment. SpringerBriefs in food, health and nutrition. Springer; 2015. p. 3-4.
2. Ryser ET, Marth EH, editors. *Listeria, listeriosis and food safety*. 3rd ed. New York: CRC Press; 2007.
3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis*. 2011;17(1):7-15. <https://doi.org/10.3201/eid1701.P11101>
4. Goulet V, Hebert M, Hedberg C, Laurent E, Vaillant V, De Valk H, et al. Incidence of listeriosis and related mortality among groups at risk of acquiring listeriosis. *Clin Infect Dis*. 2012;54(5):652-60. <https://doi.org/10.1093/cid/cir902>
5. Sauders BD, D'Amico DJ. *Listeria monocytogenes* cross-contamination of cheese: risk throughout the food supply chain. *Epidemiol Infect*. 2016;144(13):2693-7. <https://doi.org/10.1017/S0950268816001503>

6. Goulet V, Lepoutre A, Rocourt J, Courtieu A-L, Dehaumont P, Veit P. (Listeriosis epidemic in France. Final assessment and results of the epidemiological survey). *Epidemie de listeriose en France. Bilan final et résultats de l'enquête épidémiologique*. *Bull Epidemiol Hebdomadaire*. 1993;4:13-4.
7. StataCorp. Stata/SE version 14.1. College Station: StataCorp LP; 2016. Available from: www.stata.com
8. Centers for Disease Control and Prevention (CDC). Standard operating procedure for PulseNet PFGE of *Listeria monocytogenes*. Atlanta: CDC; 2013. Available from: <https://www.cdc.gov/pulsenet/pdf/listeria-pfge-protocol-508c.pdf>
9. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Cech M, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res*. 2016;44(W1):W3-10. <https://doi.org/10.1093/nar/gkw343>
10. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol*. 2012;50(4):1355-61. <https://doi.org/10.1128/JCM.06094-11>
11. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One*. 2014;9(8):e104984. <https://doi.org/10.1371/journal.pone.0104984>
12. Leekitcharoenphon P, Kaas RS, Thomsen MCF, Friis C, Rasmussen S, Aarestrup FM. snpTree—a web-server to identify and construct SNP trees from whole genome sequence data. *BMC Genomics*. 2012;13(Suppl 7):S6. <https://doi.org/10.1186/1471-2164-13-S7-S6>
13. Goulet V, King LA, Vaillant V, de Valk H. What is the incubation period for listeriosis? *BMC Infect Dis*. 2013;13(1):11. <https://doi.org/10.1186/1471-2334-13-11>
14. Norton DM, McCamey MA, Gall KL, Scarlett JM, Boor KJ, Wiedmann M. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl Environ Microbiol*. 2001;67(1):198-205. <https://doi.org/10.1128/AEM.67.1.198-205.2001>
15. Nesbakken T, Kapperud G, Caugant DA. Pathways of *Listeria monocytogenes* contamination in the meat processing industry. *Int J Food Microbiol*. 1996;31(1-3):161-71. [https://doi.org/10.1016/0168-1605\(96\)00978-6](https://doi.org/10.1016/0168-1605(96)00978-6)
16. Rørvik LM, Caugant DA, Yndestad M. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. *Int J Food Microbiol*. 1995;25(1):19-27. [https://doi.org/10.1016/0168-1605\(94\)00080-P](https://doi.org/10.1016/0168-1605(94)00080-P)
17. Senczek D, Stephan R, Untermann F. Pulsed-field gel electrophoresis (PFGE) typing of *Listeria* strains isolated from a meat processing plant over a 2-year period. *Int J Food Microbiol*. 2000;62(1-2):155-9. [https://doi.org/10.1016/S0168-1605\(00\)00395-0](https://doi.org/10.1016/S0168-1605(00)00395-0)
18. Althaus D, Lehner A, Brisse S, Maury M, Tasara T, Stephan R. Characterization of *Listeria monocytogenes* strains isolated during 2011-2013 from human infections in Switzerland. *Foodborne Pathog Dis*. 2014;11(10):753-8. <https://doi.org/10.1089/fpd.2014.1747>
19. Stephan R, Althaus D, Kiefer S, Lehner A, Hatz C, Schmutz C, et al. Foodborne transmission of *Listeria monocytogenes* via ready-to-eat salad: A nationwide outbreak in Switzerland, 2013-2014. *Food Control*. 2015;57:14-7. <https://doi.org/10.1016/j.foodcont.2015.03.034>
20. Swaminathan B, Gerner-Smidt P. The epidemiology of human listeriosis. *Microbes Infect*. 2007;9(10):1236-43. <https://doi.org/10.1016/j.micinf.2007.05.011>
21. Pichler J, Much P, Kasper S, Fretz R, Auer B, Kathan J, et al. An outbreak of febrile gastroenteritis associated with jellied pork contaminated with *Listeria monocytogenes*. *Wien Klin Wochenschr*. 2009;121(3-4):149-56. <https://doi.org/10.1007/s00508-009-1137-3>

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2018.

Rise and fall of outbreak-specific clone inside endemic pulsotype of *Salmonella* 4,[5],12:i:-; insights from high-resolution molecular surveillance in Emilia-Romagna, Italy, 2012 to 2015

Marina Morganti¹, Luca Bolzoni², Erika Scaltriti¹, Gabriele Casadei¹, Elena Carra³, Laura Rossi⁴, Paola Gherardi⁴, Fabio Faccini⁴, Norma Arrigoni⁵, Anna Rita Sacchi⁴, Marco Delle Donne⁴, Stefano Pongolini^{4,2}

1. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Sezione di Parma, Parma, Italy

2. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Risk Analysis Unit, Parma, Italy

3. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Sezione di Modena, Modena, Italy

4. Local Health Unit of Piacenza, Department of Public Health, Piacenza, Italy

5. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Sezione di Piacenza, Gariga-Podenzano, Italy

Correspondence: Stefano Pongolini (stefano.pongolini@izsler.it)

Citation style for this article:

Morganti Marina, Bolzoni Luca, Scaltriti Erika, Casadei Gabriele, Carra Elena, Rossi Laura, Gherardi Paola, Faccini Fabio, Arrigoni Norma, Sacchi Anna Rita, Delle Donne Marco, Pongolini Stefano. Rise and fall of outbreak-specific clone inside endemic pulsotype of *Salmonella* 4,[5],12:i:-; insights from high-resolution molecular surveillance in Emilia-Romagna, Italy, 2012 to 2015. *Euro Surveill.* 2018;23(13):pii=17-00375. <https://doi.org/10.2807/1560-7917.ES.2018.23.13.17-00375>

Article submitted on 12 Jun 2017 / accepted on 05 Nov 2017 / published on 29 Mar 2018

Background and aim: Epidemiology of human nontyphoid salmonellosis is characterised by recurrent emergence of new clones of the pathogen over time. Some clonal lines of *Salmonella* have shaped epidemiology of the disease at global level, as happened for serotype Enteritidis or, more recently, for *Salmonella* 4,[5],12:i:-, a monophasic variant of serotype Typhimurium. The same clonal behaviour is recognisable at sub-serotype level where single outbreaks or more generalised epidemics are attributable to defined clones. The aim of this study was to understand the dynamics of a clone of *Salmonella* 4,[5],12:i:- over a 3-year period (2012–15) in a province of Northern Italy where the clone caused a large outbreak in 2013. Furthermore, the role of candidate outbreak sources was investigated and the accuracy of multilocus variable-number tandem repeat analysis (MLVA) was evaluated. **Methods:** we retrospectively investigated the outbreak through whole genome sequencing (WGS) and further monitored the outbreak clone for 2 years after its conclusion. **Results:** The study showed the transient nature of the clone in the population, possibly as a consequence of its occasional expansion in a food-processing facility. We demonstrated that important weaknesses characterise conventional typing methods applied to clonal pathogens such as *Salmonella* 4,[5],12:i:-, namely lack of accuracy for MLVA and inadequate resolution power for PFGE to be reliably used for clone tracking. **Conclusions:** The study provided evidence for the remarkable prevention potential of whole genome sequencing used as a routine tool in systems that integrate human, food and animal surveillance.

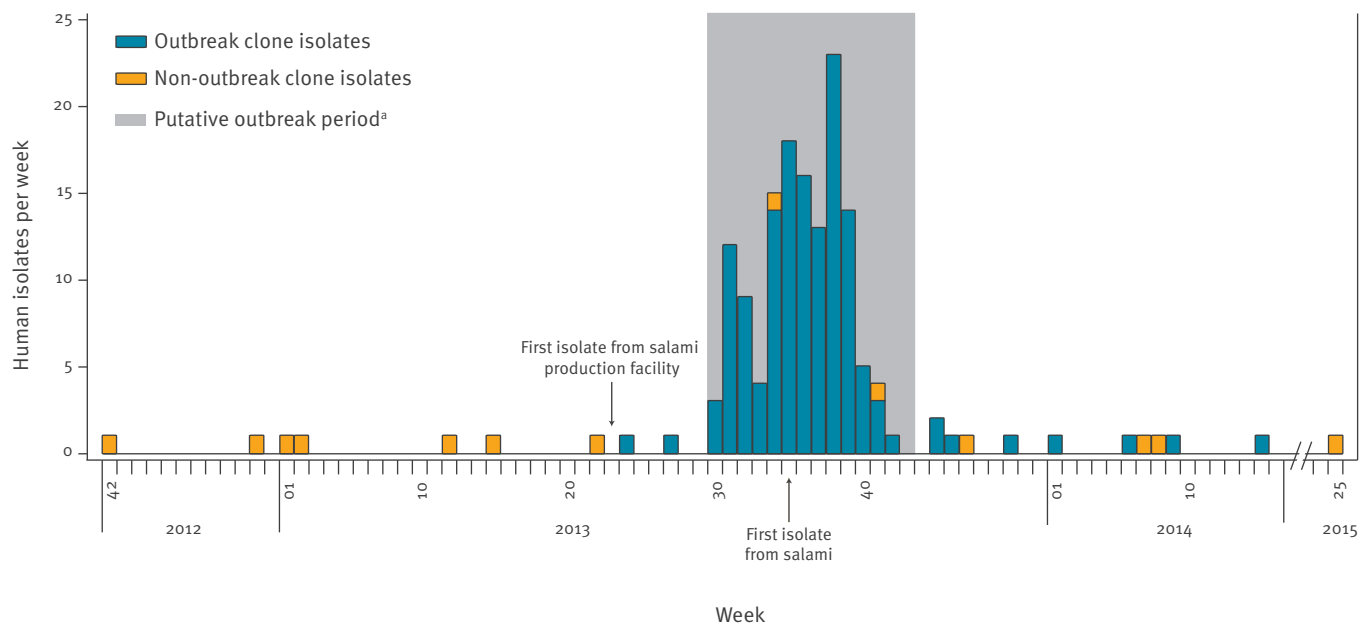
Introduction

Salmonellosis is among the most frequently reported food-borne diseases worldwide [1] and the second most-reported in the European Union (EU) [2]. In the past few decades, the epidemiology of salmonellosis has seen the emergence and/or expansion of specific types of *Salmonella* both at serotype and sub-serotype level [3]. In some instances, these newly-emerged types have gained wide and long-lasting geographic diffusion, as in the case of serotype Enteritidis, which gained worldwide epidemic diffusion in the 1980s and 1990s [4]. More recently, *Salmonella* 4,[5],12:i:- has been among the most frequently isolated serotypes from human cases in the EU [5-8]. The clonality of these emerging *Salmonella* has challenged the routinely-used typing methods and the effectiveness of laboratory-based surveillance [9,10].

In this study, we retrospectively investigated a large outbreak by *Salmonella* 4,[5],12:i:- through whole genome sequencing (WGS) after it had become evident that conventional epidemiology and routine molecular methods, namely PFGE and multilocus variable-number tandem repeat analysis (MLVA), could not elucidate some critical aspects of the infectious episode. Furthermore, to accurately understand the temporal dynamics of the outbreak clone we extended WGS monitoring of the clone from 1 year before the outbreak onset to 2 years after its conclusion, i.e. from June 2012 to December 2015. The outbreak, which occurred in the Emilia-Romagna region of Italy in 2013, was detected by the routine regional surveillance system, based on PFGE. The epidemiological investigation

FIGURE 1

Number of isolates per week of *Salmonella* 4,[5],12:i:- pulsotype STYMX.0131 in Piacenza province, Italy, 2012–2015 (n = 158)



^aThe putative outbreak period was defined based on the epidemiological evidence available.

rapidly identified the food involved (fermented dry-cured salami made from pork) and the facility implicated, but was not able to attribute several individuals infected with the implicated strain to the outbreak and could not confirm or exclude the role of suspect sources at abattoir and farm level. This was mainly the consequence of the outbreak pulsotype (STYMXB.0131) being the most common in the surveillance database and endemic in the area since at least 2008 [8,11]. More specifically, in the monitored pre-outbreak period (June 2012 to June 2013) the pulsotype represented 8.9% of all *Salmonella* isolates from humans and 19.2% of *Salmonella* 4,[5],12:i:-, in line with previous reports of this pulsotype in several European countries since the mid-2000s [12,13]. In particular, an extended Swiss study reported 37% prevalence of the pulsotype among human isolates of *Salmonella* 4,[5],12:i:- over the period 2007–11 [14]. Additional uncertainty during the outbreak investigation originated from the finding of variant types, although similar, among outbreak-related isolates, by PFGE (one variant) and, even more, by MLVA (five variants). The study was designed to address the unresolved issues and to answer specifically the following questions: (i) was there an outbreak-specific clone inside the endemic pulsotype STYMXB.0131 of *Salmonella* 4,[5],12:i:- and was it limited to the outbreak or circulating before and after it?; (ii) what was the exact responsibility of different candidate sources of the outbreak?; (iii) to what extent did the many observed MLVA variants belong to the outbreak clone? In other words, how accurate is MLVA in tracking *Salmonella* Typhimurium and variants in

field conditions?; and (iv) what lessons can be learnt about the benefit of WGS as a surveillance tool in field conditions?

Methods

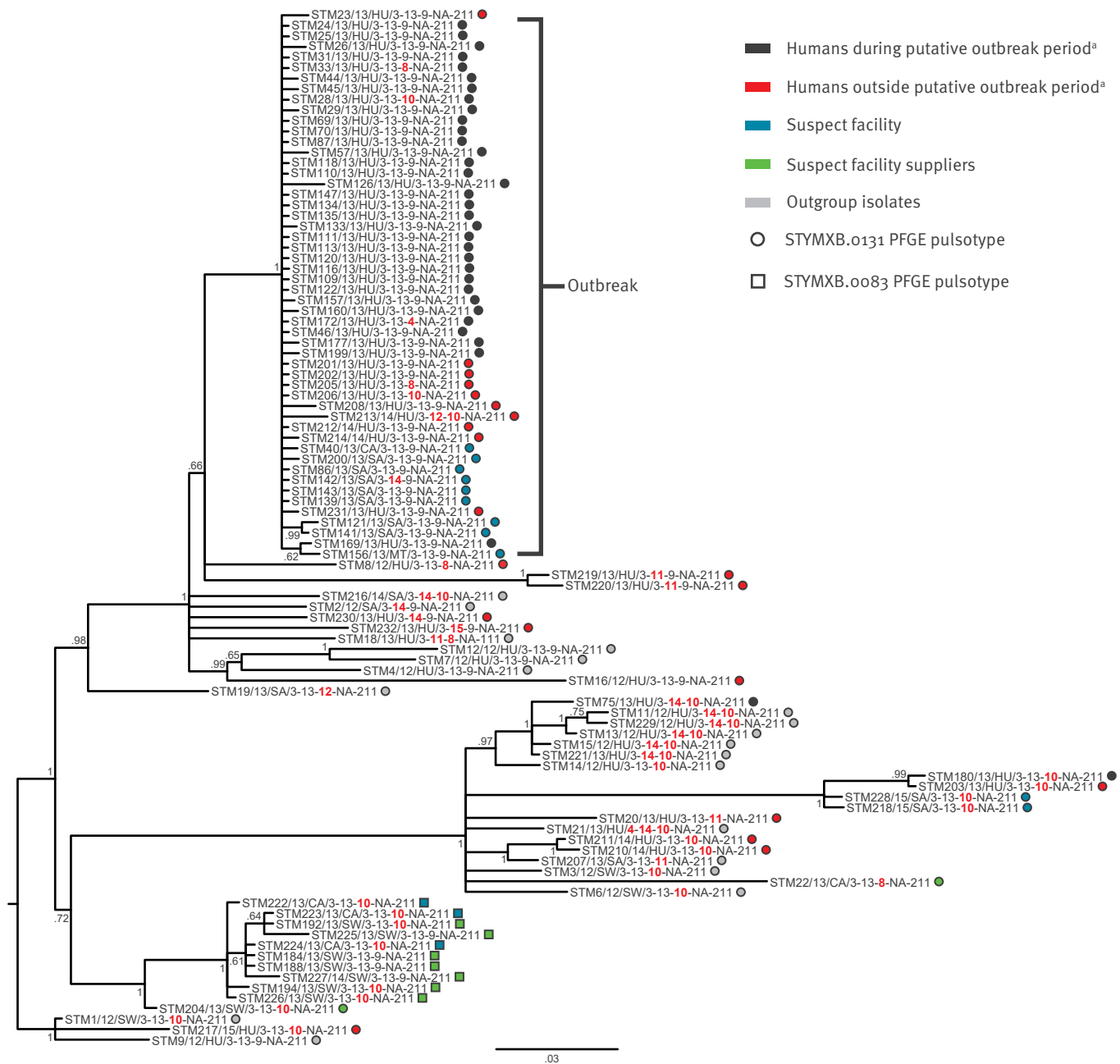
Laboratory surveillance system for salmonellosis

The laboratory surveillance system for human salmonellosis for Emilia-Romagna, an administrative region in Northern Italy with a population of ca 4.5 million residents [15], is a routine system based on serotyping and PFGE typing of all isolates recovered weekly from the regional network of medical microbiology laboratories. MLVA is performed on all isolates of *S. Typhimurium* and *Salmonella* 4,[5],12:i:- at a later stage (1–3 weeks later), as supplementary information to PFGE. To promptly identify potential outbreaks, a weekly analysis of the surveillance database is performed to assess whether any of the pulsotypes shows clustering in time beyond the expected threshold as previously described [16,17]. The outbreak alarm statistic is calculated with the OutbreakP algorithm from the ‘surveillance’ package in the R statistical environment [18]. The algorithm uses a weekly rolling window to evaluate whether the number of cases belonging to a given pulsotype is significantly greater than the expected number of cases during that period, based on the database of isolates starting from June 2012.

The system is integrated with the regional animal and foodstuff surveillance, which includes testing of

FIGURE 2

Phylogenetic Bayesian tree of *Salmonella* 4,[5],12:i:- isolates sequenced in this study, Emilia-Romagna, Italy, 2012–2015



CA: carcass; HU: human; MLVA: multilocus variable-number tandem repeat analysis ; MT: meat; SA: salami; SW: swine.

^a The putative outbreak period was defined based on the epidemiological evidence available.

The string identifying each isolate reports the study ID of the isolate, the year of isolation, the origin and MLVA profile (3 variant loci to the outbreak profile are in red).

official samples, private diagnostic activity on animals and some self-control testing by the food industry.

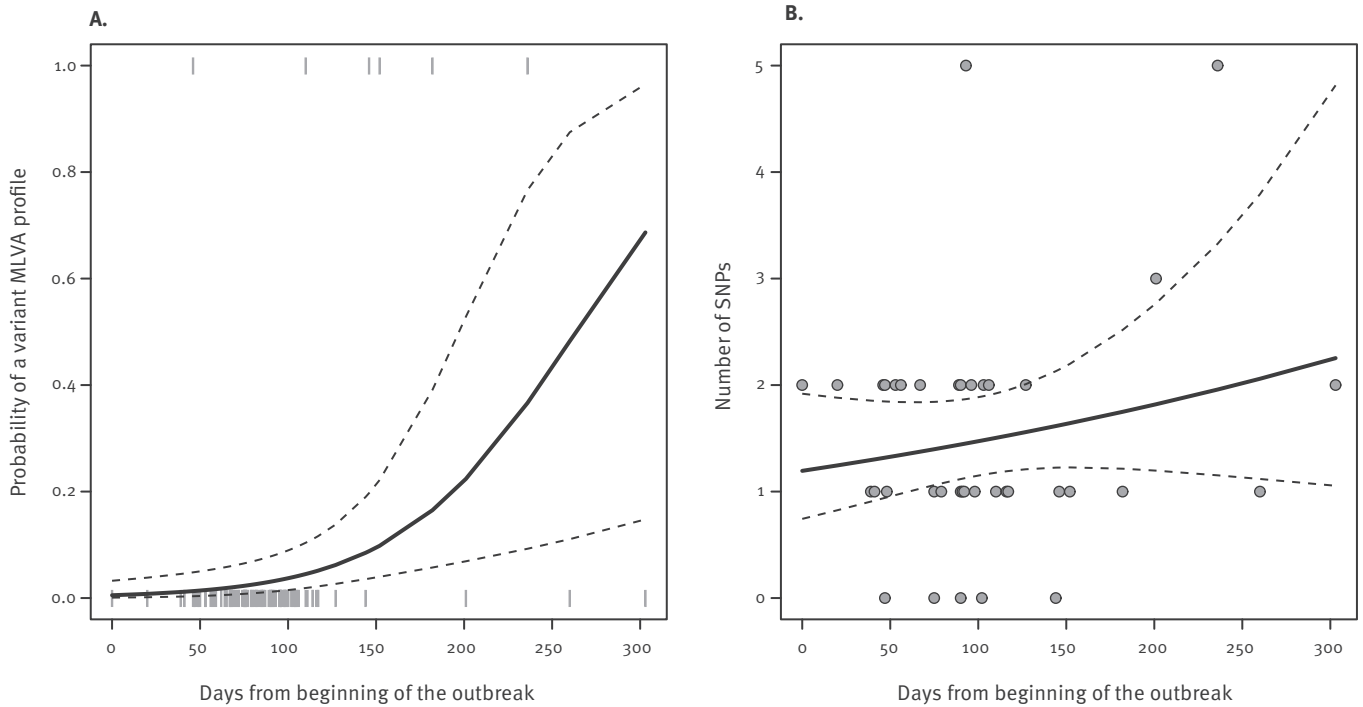
Epidemiological investigation

Case definition: following the alert for a possible outbreak of salmonellosis, a case was initially defined as any culture-confirmed human infection by *Salmonella* 4,[5],12:i:- with pulsotype STYMXB.o131,

detected in the province of Piacenza after 22 July 2013. For the purpose of this study, the case definition included cases up to the epidemiologically defined end of the outbreak set at 29 October 2013. No cases other than laboratory-confirmed ones were considered within the scope of the study.

FIGURE 3

Probability of occurrence of variant multilocus variable-number tandem repeat analysis profiles (panel A) and single nucleotide polymorphisms to the consensus (panel B) in outbreak isolates as a function of the isolation date



MLVA: multilocus variable-number tandem repeat analysis; SNPs: single nucleotide polymorphisms.

Generalised linear models with binomial distribution (panel A) or Poisson distribution (panel B). Solid black lines represent the best fit, dashed black lines represent 95% confidence intervals. In panel A, grey bars represent the occurrence (top bars) or not (bottom bars) of a variant MLVA profile in the isolate. In panel B, grey dots represent the number of SNPs to the consensus in the outbreak isolates.

Case interviews: the cases were interviewed by staff from the Local Health Unit of Piacenza using a standard format from the food-borne outbreaks reporting manual in accordance with directive 2003/99/EC from 2011 [19].

Microbiological investigation

Various types of samples were collected from the suspected sources during the outbreak investigation and tested for *Salmonella*. Sampling included salami, pork, the surface of pig carcasses, swine faeces, environmental swabs and faeces from the workers at the implicated salami-production facility. Foodstuffs were tested according to Regulation 2073/2005 [20]; for carcasses, four different sites of 100 cm² each (hind limb medial, back, belly and cheek) were sampled using the abrasive sponge method. Swine faeces and boot swabs were tested with ISO 6579:2002/Amd.1:2007 [21]. After the end of the outbreak, intensified monitoring of the salami-production facility was implemented within its hazard analysis and critical control points (HACCP) plan. The post-outbreak monitoring, from November 2013 to December 2015, included 123 batches of salami, 87 batches of raw meat, and 83 environmental swabs.

Typing of isolates and phylogenetic analysis

After serotyping, the monophasic character of the isolates was confirmed by PCR as previously described [22]. PFGE was performed according to the PulseNet protocol with *Xba*I digestion of DNA [23] and restriction profiles were analysed by BioNumerics 7.5 (Applied Maths, Saint-Martens – Latem, Belgium). MLVA was performed as previously described [24] with a CEQ 8000 Genetic Analysis System (Beckman Coulter, US) and profiles were assigned according to the MLVA nomenclature suggested by Larsson et al. (2009) [25].

Ninety-eight isolates of the outbreak-related pulsotypes underwent WGS (Table 1), comprising a set of 19 diverse STYMXB.0131 isolates presumed to be unrelated to the outbreak and included in the analysis as outgroup isolates. To maximise the probability of these isolates not being outbreak-related, and therefore constituting adequate outgroup isolates, they were selected from human, animal and food isolates recovered from other parts of the region excluding Piacenza. They included a cluster of six isolates representing a small known outbreak that occurred in a nursery school 200 km from Piacenza in November 2012.

TABLE 1Isolates of *Salmonella* 4,[5],12:i:-, study in Emilia-Romagna, Italy, 2012–2015 (n = 198)

Source	Recovered isolates	WGS-analysed isolates	MLVA-variant isolates ^a		PFGE-variant isolates ^b	
			Number of isolates	Number of types	Number of isolates	Number of types
Human (during putative outbreak period)	137	35 ^c	5	4	0	0
Human (outside putative outbreak period)	21	21	13	7	0	0
Salami production facility and salami	14	14	6	2	3	1
Suppliers of salami production facility	26	9 ^d	5	2	7	1
Outgroup isolates	NA	19	15	7	0	0
Total	198	98	44	NA	10	NA

MLVA: multilocus variable-number tandem repeat analysis; NA: not applicable; WGS: whole genome sequencing.

^a With reference to outbreak type 3–13–9-NA-211; all variant isolates were WGS analysed.

^b With reference to outbreak pulsotype STYMXB.0131; all variant isolates were WGS analysed.

^c Isolates selected for WGS analysis were evenly distributed throughout the putative outbreak period.

^d Including seven randomly selected STYMXB.0083 isolates of the 24 recovered from pig farm and the two STYMXB.0131 isolates recovered from pig farm and external slaughterhouse.

For WGS, sequencing libraries were prepared from genomic bacterial DNA with the Nextera XT sample preparation kit and run on an Illumina MiSeq (Illumina, San Diego, US) with a 2x250 paired-end run. The reads of the 98 genomes of *Salmonella* 4,[5],12:i:- were deposited at the European Bioinformatics Institute under Project Number PRJEB7560. The average sequencing coverage was 194X with >75% of bases having Q value equal to or greater than 30. The genome of outbreak isolate STM45, used as reference for phylogenetic analysis, was assembled with MIRA 4.0 [26] using ‘accurate settings for de novo assembly mode’ and discarding small contigs (size < 1,000 bp). The average assembled-genome characteristics were: 5,010,485 nt length, 158X coverage, 144 contigs > 5,000 nt and N50 of 77,805.

For phylogenetic analysis, the single nucleotide polymorphisms (SNPs) of each genome were extracted using a reference-based United States Food and Drug Administration SNP-pipeline (version 0.6.1), based on Bowtie2 and VarScan [27]. The default settings of the pipeline were used for SNP calling, i.e. a minimum coverage of 8x and a minimum variant allele frequency of 0.90. A Bayesian tree was generated with MRBAYES [28] from the SNP matrix of the analysed genomes. The Bayesian analysis was run using the GTR substitution model for 2,000,000 generations with chain sampled every 1,000th generation. The final parameter values and trees were summarised after discarding 25% of the posterior sample.

We assessed whether the occurrence of outbreak isolates with variant MLVA increased as a function of the isolation date by fitting a generalised linear model (GLM) with binomial error distribution. Similarly, we assessed whether the number of SNPs to the consensus, in the polymorphic positions shared by all

outbreak-related human isolates, increased as a function of the isolation date by fitting a GLM with Poisson error distribution.

Antimicrobial resistance and strain characterisation

WGS-analysed isolates were tested for resistance to ampicillin, streptomycin, sulphonamides, tetracycline, gentamicin, chloramphenicol, enrofloxacin and cefotaxime by the disk diffusion susceptibility test [29]. The presence of antimicrobial resistance genes (ARG) was investigated in silico using the ResFinder database [30]. Mutations of *gyrA-gyrB* genes conferring resistance to quinolones were investigated by mapping the reads of the *gyrA* and *gyrB* genes of all sequenced isolates on *gyrA* and *gyrB* nt sequences of sensitive *S. Typhimurium* LT2 [31]. The presence of Insertion Sequence 26 (IS26), known to drive genomic evolution linked to the monophasic character of 4,[5],12:i:- strains [32], was investigated in the study genomes. To this end, sequencing reads were mapped on the biphasic *S. Typhimurium* LT2 reference genome (accession number AE006468) and on the IS26 composite transposon regions of the previously characterised monophasic strains VAR-2009/08643/1 (accession number K999732) [32], 07–2006 (accession number KR856283) [11] and 105/7/03 (accession number HQ331538) [33]. To further explore the genomic differences between pulsotype STYMXB.0131 and its single-band variant STYMXB.0083, the SNPs exclusive to the STYMXB.0083 pulsotype were extracted and annotated using Prokka 1.7 [34]. Exclusive SNPs were defined as those present in all the genomes of a pulsotype and absent in all other genomes of the study.

TABLE 2

Single nucleotide polymorphisms exclusive to the outbreak clone (detected in all the outbreak isolates and in no other isolate of the study), Emilia-Romagna, Italy, 2012–2015

Position in reference	Non-outbreak Nt	Outbreak Nt	Codon change	Aminoacid change	Strand	Type of SNP	Gene	Product name
1005978	A	G	ATT → ATC	I → I	-	Synonymous	-	Short-chain dehydrogenase
1226252	A	G	TTG → CTG	L → L	-	Synonymous	mdtH	Multidrug resistance protein
2145307	C	T	ACT → ATT	T → I	+	Non-synonymous	pduX	L-threonine kinase
2265999	T	C	AAT → GAT	N → D	-	Non-synonymous	yehU	Sensor histidine kinase
3526676	A	G	TAC → TGC	Y → C	+	Non-synonymous	acul	Putative acrylyl-CoA reductase
3912784	T	C	TGC → CGC	C → G	+	Non-synonymous	recG	ATP-dependent DNA helicase
4510357	A	C	TGG → GGG	W → G	-	non-synonymous	fdhF 1	Formate dehydrogenase

ATP: adenosine triphosphate; Nt: nucleotide; SNP: single nucleotide polymorphism.

Results

Descriptive epidemiology

The regional outbreak detection system signalled a potential outbreak by *Salmonella* 4,[5],12:i:- with pulso-type STYMXB.0131 on 14 August 2013. The first isolates with the outbreak pulso-type (OP) that were included in the alert by the signalling algorithm dated back to 22 July 2013. A total of 29 isolates with OP (each isolate corresponding to a different patient) had been recovered from the region between 22 July and 14 August. All but four of the isolates were from the province of Piacenza; therefore, it was considered that the potential outbreak concerned only that province, where the OP was increasing sharply while its incidence remained at the endemic level in the rest of the region where, overall, the OP was the most frequently reported pulso-type. The epidemic curve based on the case definition was steeply increasing, confirming the existence of the outbreak (Figure 1).

In particular, the average weekly number of isolates with the OP from Piacenza changed from 0.2 before the 22 July to 7.6 in the period 22 July to 14 August. The health authority was informed of the potential outbreak on 16 August. The interviews to the cases and their geographic restriction to a limited area of the province (in the north) rapidly led to the identification of a local facility producing fermented dry-cured salami made from pork as the possible source of the outbreak. The retail network of the producer overlapped with the location of the cases and the shop at the facility was mentioned repeatedly in case interviews. Microbiological confirmation of the facility as the source of the outbreak, interruption of distribution and recall of contaminated salami led to the reversion to the normal incidence of isolation of *Salmonella* 4,[5],12:i:- with OP

and the outbreak was declared over on 29 October. Overall, 137 human isolates with OP were recovered from the outbreak territory between 22 July and 29 October. Based on the epidemiological evidence available, this time interval was identified as the putative outbreak period for the purpose of this study.

Trace-back and environmental investigation

Inspection and microbiological investigation of the salami production facility demonstrated *Salmonella* 4,[5],12:i:- with OP in five of 21 tested batches of salami produced from 30 April to 10 September 2013 (representative isolates were: STM86; STM121; STM139; STM142; STM143; STM200). No batches processed before 30 April were still available for microbiology at the time of first inspection on 30 August. All environmental swabs (n=50) were negative for *Salmonella*. The supply-chain information of the facility showed that some of the pork for salami production came from a small internal slaughterhouse at the facility and some was purchased from external industrial slaughterhouses. The pigs for the internal slaughterhouse originated predominantly from a single farm and to a lesser extent from other farms which supplied pigs occasionally.

Twenty-five faecal pools of 58 tested from the pen floors of the main pig-supplying farm were positive for *Salmonella* 4,[5],12:i:-, one had OP (STM204), all others had pulso-type STYMXB.0083, highly similar to the OP (representative isolates were: STM184; STM188; STM192; STM194; STM225; STM226). Pulso-type STYMXB.0083 was also detected on carcasses tested at the internal slaughterhouse and derived from pigs from that same farm (STM222; STM223; STM224). The facility's HACCP records reported that a carcass swab from the internal slaughterhouse had been

found positive for *Salmonella* before outbreak onset, on 3 June 2013. Upon typing, the isolate showed a *Salmonella* 4,[5],12:i:- with OP (STM40). The carcass was from one of the farms which supplied pigs occasionally. The microbiological sampling of this farm (six environmental boot swabs) remained negative. As a result of the investigation and follow-up monitoring, *Salmonella* with OP was further detected. It was isolated from four of 22 healthy workers at the facility (STM133; STM134; STM135; STM172), from a batch of meat that had not yet entered salami processing in the internal slaughterhouse on 23 September (STM156) and from two batches of salami from the facility in January and March 2015 (STM218; STM228). Remarkably, one of the industrial slaughterhouses supplying meat to the facility had reported the presence of *Salmonella* 4,[5],12:i:- with OP (STM22) during its internal routine testing of carcass surfaces, before outbreak onset, on 28 June 2013.

Molecular epidemiology

All isolates included in the study had pulsotype STYMXB.0131 (part of the case definition) or its highly similar variant STYMXB.0083. As regards MLVA types, profile 3–13–9-NA-211 was detected in 132 isolates of 137 human isolates from the putative outbreak period, and was thus the most represented, but four other profiles were present among the remaining five isolates (STM28; STM33; STM75; STM172; STM180). Considering all human isolates eventually assigned to the outbreak cluster by WGS, four MLVA variants to 3–13–9-NA-211 were detected, representing six isolates (STM28; STM33; STM172; STM205; STM206; STM213) (Figure 2).

The main outbreak profile (3–13–9-NA-211) was also detected among the isolates from salami, the internal slaughterhouse, the main pig-supplying farm and some of the outgroups of the phylogenetic analysis. Similarly to the human isolates, the isolates from the food chain showed a variety of MLVA profiles, generally closely related to profile 3–13–9-NA-211, consisting of single-locus variants most of the time (Figure 2).

Whole genome sequencing

WGS analysis was performed to elucidate the individual responsibility of suspect sources along the food chain and to determine whether an outbreak-specific clone existed within the endemic STYMXB.0131 and what its time extension was. This high-resolution approach was required, given the existence of MLVA and PFGE variants among the outbreak isolates.

The outcome of the phylogenetic analysis is represented in Figure 2. Thirty-three of the 35 human isolates of the putative outbreak period, included in the analysis, belonged to the same clade that included the isolates from the four positive workers, all the isolates from salami collected during the investigation and the isolates from the internal slaughterhouse, STM40 (carcass before outbreak onset) and STM156 (meat for salami processing). No other isolates belonged to that

clade except 10 humans out of the 21 recovered from Piacenza outside the putative outbreak period; the remaining 11 belonged to various different clades and were clearly not part of the outbreak. Seven SNPs were exclusive to and shared by all the outbreak isolates, supporting their cluster (Table 2).

Inside the outbreak clade, the number of SNPs to the consensus ranged from none to five (median value: one SNP), while the closest non-outbreak isolate (STM8) differed by 19 SNPs. As regards the responsibility of the food-chain operators, WGS confirmed the role of the salami producer, while both the main pig-supplying farm and the external slaughterhouse appeared to have no role in the contamination, different to what PFGE and MLVA testing had seemed to point to. In fact, all isolates with pulsotype STYMXB.0083 which came from the farm belonged to a well-segregated cluster in the phylogeny, distant from the outbreak clade (114 SNPs differed between the outbreak and the STYMXB.0083 consensus sequences), regardless of their PFGE and MLVA relatedness to the outbreak type. Similarly, the STYMXB.0131 isolates from the farm (STM204) and the external slaughterhouse (STM22) were shown to be distant from the outbreak clade. Remarkably, the isolates with OP from the salami facility of the follow-up monitoring were not related to the outbreak clone.

The phylogenetic analysis highlighted the clonality of the outgroup isolates from the 2012 nursery school outbreak, confirming the resolution power and accuracy of the analysis.

Multilocus variable-number tandem repeat analysis variant analysis

Statistical analyses revealed that the probability of an outbreak isolate being an MLVA variant increased at the end of the outbreak (GLM output: chi-squared=8.04, DF=1, p=0.0046), while the number of SNPs to the consensus did not significantly increase throughout the outbreak (GLM output: chi-squared=1.65, DF=1, p=0.199), see Figure 3.

Antimicrobial resistance and strain characterisation

All sequenced isolates were tested for antimicrobial resistance and all but four were multidrug-resistant with R-type ASSuT (i.e. resistant to ampicillin, streptomycin, sulphonamides and tetracycline). The four exceptions did not belong to the outbreak clade and had ASSu R-type (STM2, STM3, STM232) or SuT R-type (STM8). The following ARGs were identified in all ASSuT isolates: *bla*TEM (ampicillin), *str*A and *str*B (streptomycin), *sul*2 (sulphonamides), *tet*(B) (tetracyclines). The three ASSu isolates lacked *tet*(B) and the SuT isolate lacked *bla*TEM and *str*B. No mutations associated with quinolone resistance were detected in *gyr*A and *gyr*B genes of the sequenced isolates. Similarly to other 4,[5],12:i:- strains [11,32,33], both

STYMXB.0131 and STYMXB.0083 isolates of the study possessed IS26 composite transposon insertions. Mapping of the sequencing reads on the LT2 reference genome revealed the deletion of STM2760-STM2772 chromosomal region in all sequenced genomes. This deletion, associated with IS26 insertions, corresponds to the loss of *fljAB* operon and *hin* gene (STM2770–2772 loci of LT2), the genetic determinants of biphasic phenotype. The loss was consistent, in all study isolates, with the negative *fljB*-targeted PCR [35]. Further analysis of IS26 transposon insertions was done through comparison with the IS26 composite transposon regions of the previously characterised monophasic strains VAR-2009/08643/1 [32], 07–2006 [11] and 105/7/03 [33]. The comparison confirmed STM2760-STM2772 deletion, as already observed in 07–2006, a strain with STYMXB.0131 pulsotype, where it was associated with the IS26-driven insertion of the RR3 element, a chromosomal module comprising various plasmid-derived genes and the ARGs *bla*TEM, *strA*, *strB*, *sul2* and *tet(B)*. Conversely, the study genomes did not have the STM2753-STM2759 deletion observed in 105/7/03, and they did not present the insertion of RR1 and RR2 elements around the *fljAB* operon described in VAR-2009/08643/1. Furthermore, all genes reported in the RR3 element of strain 07–2006 were detected in the study genomes with only five exceptions. These were represented by non-outbreak isolates lacking some of the RR3 genes, namely from *tniAΔ*, to *methΔ* genes (STM2, STM3, STM232); from *tniAΔ* to *merR* genes (STM20); from *tnp2RΔ* to *tnpB* and *strB* genes (STM8). Overall, these findings support the hypothesis that an RR3-like resistance element was present in STYMXB.0131 and STYMXB.0083 of the study in place of the STM2760-STM2772 region, similarly to strain 07–2006. This analysis showed that STYMXB.0131 and STYMXB.0083 from this study did not differ from one another as regards IS26-related genomic architecture and antibiotic resistance genes. Conversely, five synonymous SNPs located on a single gene, *dus3*, a putative tRNA-dihydrouridine synthase, were found to be exclusive to STYMXB.0083.

Discussion

Antimicrobial resistance and strain characterisation

The ASSuT R-type and resistance genes of the outbreak (OB) clone indicate its belonging to the clonal lineage of *Salmonella* 4,[5],12:i:- with pulsotype STYMXB.0131 and chromosomal multidrug resistance to ampicillin, streptomycin, sulphonamides and tetracycline commonly circulating in Europe [12]. The two related pulsotypes of the study showed the same genomic structure as regards the insertions of IS26, involved in their monophasic phenotype. That structure was already reported in Italian strains of pulsotype STYMXB.0131 collected between 2008 and 2012 [11], confirming the long-lasting circulation of this lineage in Italy. Nevertheless, according to WGS phylogeny, in our scenario, the minimum difference between the two PFGE

profiles did correspond to great phylogenetic distance between the OB isolates (having STYMXB.0131 pulsotype) and the STYMXB.0083 isolates from the suspected farm. This distance is apparent from the tree topology of the study isolates and is substantiated by 114 SNPs of difference between the OB and the STYMXB.0083 consensus sequences.

Outcome of the investigation

WGS analysis accurately identified the existence of an outbreak-specific clone within the endemic pulsotype STYMXB.0131 of *Salmonella* 4,[5],12:i:- in the region affected. The 3-year long WGS monitoring extended the time boundaries of the outbreak beyond the originally identified (putative) outbreak period. Its duration was widened by 6 weeks before the epidemiologically-defined onset as a consequence of the identification of two early OB isolates and by 6 months after the apparent conclusion with the identification of eight late OB isolates (Figure 1). While the suspected salami was confirmed as the vehicle of infection, similarly to previous reports [36], the origin of the contamination upstream from the salami-processing facility was not demonstrated. The responsibility of the facility suppliers, initially suspected based on PFGE and MLVA, was not confirmed by WGS. In the absence of a demonstrated external origin of the contamination, this could be speculatively attributed to the positive healthy workers of the facility, although their positive status could be either the cause or the consequence of the salami contamination, with no possibility of solving the ambiguity. An alternative possible cause of the outbreak could have been persistent contamination inside the facility by the outbreak strain that could have entered the facility at some time before the outbreak, established itself and eventually contaminated the food products, reaching a level high enough to generate the observed high incidence of infections. The hypothesis of persistence is consistent with the sporadic isolation of the outbreak clone inside the facility before (STM40) and during the outbreak (STM156). Regardless of the origin of the contamination, the very limited number of SNPs differentiating the isolates of the OB cluster and the lack of an evolutionary structure inside the cluster are indicative of a single, time-restricted source of the OB. This would be consistent with a sudden expansion of the contaminating clone inside the production premises, regardless of its origin (e.g. the facility environment, the raw meat or carrier workers). The in-depth cleaning of the facility, following the demonstration of its implication, probably led to the eradication of the strain from the processing plant, as confirmed by the negative results of the 2-year long post-outbreak monitoring on hundreds of samples. These results coincided with the rapid decline and the disappearance of the clone from the human population within 6 months of the official outbreak closure.

Performance of typing methods

Genomic investigation of the study outbreak through WGS confirmed, in a field scenario, that the high

resolution of this approach allowed for the accurate assignment of the isolates to the outbreak clone, unlike current routine methods, PFGE and MLVA, which showed inadequate resolution and doubtful stability, respectively. This advantage of WGS, observed with a clonal pathogen like *Salmonella* 4,[5],12:i:- on a local geographical scale, could likely be helpful with clonal pathogens on larger geographical and temporal scales as well, where a greater number of variants can be expected with traditional typing methods. The use of WGS in widespread outbreaks, e.g. internationally, would imply sharing of standardised WGS data and analysis. In this study, MLVA showed a diversity of variants among the outbreak isolates that would potentially lead to incorrect assignment of some of the cases to the outbreak and to identify wrong links with suspected sources actually not related to the outbreak. This was the case for profiles 3-13-8-NA-211 and 3-13-10-NA-211 shared by some cases and suspected sources, which were eventually cleared of any responsibility. Even more misleading was the finding in the suspected pig farm of isolates having the main outbreak profile 3-13-9-NA-211 (STM184; STM188; STM225) which were eventually demonstrated not to belong to the outbreak clone. The results of other studies, conducted in recent years [8], and references thereof [37-39], are consistent with the ambiguity of MLVA evidenced by our data, despite the efforts of these authors to establish interpretation cut-offs for MLVA in terms of acceptable numbers of intra-outbreak repeats or variable loci. The high number of isolates analysed in our study and the complexity of the outbreak setting have shown limits of MLVA usage that those previous studies could not fully demonstrate. Interestingly, in our scenario the probability of emergence of MLVA variants was higher in the late phase of the outbreak, possibly reflecting mutation of the assayed loci with passing time at a rate higher than the rest of the genome, as measured through the accumulation of SNPs. While this is most likely not a limit in small and time-focused outbreaks, it could constitute a problem in lengthy ones.

Lessons learnt

The primary origin of the outbreak clone or its introduction into the region remained unexplained. However, the observed appearance and disappearance in the human population of the outbreak clone during a 3-year monitoring, its association with a specific food plant and the absence of identified primary sources, tell us that the emergence of a clone of *Salmonella* capable of generating a significant outbreak can indeed be a time-limited, transient event, possibly caused by extemporary clonal expansions inside food processing facilities, not necessarily with substantial quantitative contributions by upstream primary production.

From the methodological point of view, the study case showed that, based on WGS data, the first isolates unambiguously linking the salami facility (STM40) and the infection of humans (STM231) were available

on 3 June and 13 June, respectively. This was more than a month in advance of the outbreak onset based on incidence (22 July) and more than 2 months in advance of the identification of the salami facility as the source of contamination (beginning of September). Retrospectively speaking, the availability of such genomic evidence in real-time or within the few days needed to produce it, would have favoured the adoption of mitigation measures with enough advance to prevent dozens of infections, indicating the preventive value of WGS if used routinely and promptly in surveillance systems combining human and food isolates. Although the identification of epidemiological links between clinical and food isolates will continue to be central in the attribution of outbreaks to their sources, the availability to the food control authorities of 'routine' food-human matching information can contribute to better planning and prioritisation of the control activity on facilities and food chains. These results contribute to a better understanding of the dynamics of *Salmonella* contamination at the food-human interface and highlighted the potential of WGS to improve the procedures of surveillance, investigation and trace-back of *Salmonella* as observed by others [10,37,40-42].

Acknowledgements

The authors acknowledge the contribution of the Regional Health Service of Emilia-Romagna.

The study was partially funded by the Ministry of Health 'Ricerca Corrente' Grant N. IZSLER-PRC2013011 and N. IZSLER-PRC2014006.

Conflict of interest

None declared.

Authors' contributions

MM, LB, ES, GC and SP designed the study; MM performed the PFGE and laboratory surveillance; LB analysed the epidemiological data, did the statistical analyses and contributed to manuscript drafting; ES did the WGS, bioinformatic and phylogenetic analyses; GC contributed to bioinformatic and phylogenetic analyses, data collection and revision of results; EC performed MLVA and contributed to the revision of results; LR did the epidemiological data collection and contributed to the epidemiological investigation; PG performed data collection in support of microbiological investigation; FF performed epidemiological investigation and contributed to epidemiological data collection; NA performed the food and environmental microbiological investigations; ARS managed the public health surveillance system; MDD supervised the epidemiological investigation; SP coordinated the study and wrote the manuscript.

References

1. MajowiczSEMustoJ, ScallanE, AnguloFJ, KirkM, O'BrienSJ The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis*. 2010;50(6):882-9. DOI: 10.1086/650733 PMID: 20158401

2. European Food Safety Authority (EFSA) / European Centre for Disease Prevention and Control (ECDC). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA J.* 2015;13(1):3991. DOI: 10.2903/j.efsa.2015.3991
3. O'Brien SJ. The 'decline and fall' of nontyphoidal salmonella in the United Kingdom. *Clin Infect Dis.* 2013;56(5):705-10. DOI: 10.1093/cid/cis967 PMID: 23166188
4. Ward LR, Threlfall J, Smith HR, O'Brien SJ. Salmonella enteritidis epidemic. *Science.* 2000;287(5459):1753-4, author reply 1755-6. DOI: 10.1126/science.287.5459.1753c PMID: 10755925
5. European Food Safety Authority (EFSA) / European Centre for Disease Prevention and Control (ECDC). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA J.* 2014;12(2):3547. DOI: 10.2903/j.efsa.2014.3547
6. European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA J.* 2015;13(12):4329. DOI: 10.2903/j.efsa.2015.4329
7. Cito FB, Baldinelli F, Calistri P, Di Giannatale E, Scavia G, Orsini M. Outbreak of unusual Salmonella enterica serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014. *Euro Surveill.* 2016;21(15):30194. DOI: 10.2807/1560-7917.ES.2016.21.15.30194 PMID: 27105170
8. Barco L, Ramon E, Cortini E, Longo A, Dalla Pozza MC, Lettini AA. Molecular characterization of Salmonella enterica serovar 4,[5],12:i:- DT193 ASSuT strains from two outbreaks in Italy. *Foodborne Pathog Dis.* 2014;11(2):138-44. DOI: 10.1089/fpd.2013.1626 PMID: 24328499
9. Olson AB, Andrysiak AK, Tracz DM, Guard-Bouldin J, Demczuk W, Ng LK. Limited genetic diversity in Salmonella enterica serovar Enteritidis PT13. *BMC Microbiol.* 2007;7(1):87. DOI: 10.1186/1471-2180-7-87 PMID: 17908316
10. Allard MW, Luo Y, Strain E, Pettengill J, Timmer R, Wang C. On the evolutionary history, population genetics and diversity among isolates of Salmonella Enteritidis PFGE pattern JEGX01.0004. *PLoS One.* 2013;8(1):e55254. DOI: 10.1371/journal.pone.0055254 PMID: 23383127
11. García P, Malorny B, Rodicio MR, Stephan R, Hächler H, Guerra B. Horizontal Acquisition of a Multidrug-Resistance Module (R-type ASSuT) Is Responsible for the Monophasic Phenotype in a Widespread Clone of Salmonella Serovar 4,[5],12:i:-. *Front Microbiol.* 2016;7:680. DOI: 10.3389/fmicb.2016.00680 PMID: 27242707
12. Hopkins KL, Kirchner M, Guerra B, Granier SA, Lucarelli C, Porrero MC. Multiresistant Salmonella enterica serovar 4,[5],12:i:- in Europe: a new pandemic strain? *Euro Surveill.* 2010;15(22):19580. PMID: 20546690
13. Hauser E, Tietze E, Helmuth R, Junker E, Blank K, Prager R. Pork contaminated with Salmonella enterica serovar 4,[5],12:i:-, an emerging health risk for humans. *Appl Environ Microbiol.* 2010;76(14):4601-10. DOI: 10.1128/AEM.02991-09 PMID: 20472721
14. Gallati C, Stephan R, Hächler H, Malorny B, Schroeter A, Nüesch-Inderbinen M. Characterization of Salmonella enterica subsp. enterica serovar 4,[5],12:i:- clones isolated from human and other sources in Switzerland between 2007 and 2011. *Foodborne Pathog Dis.* 2013;10(6):549-54. DOI: 10.1089/fpd.2012.1407 PMID: 23614800
15. Italian National Institute of Statistics (Istat). Resident population on 1st January. Rome: Istat. [Accessed 27 Sep 2017]. Available from: <http://dati.istat.it/Index.aspx?lang=en&SubSessionId=0579eb-ed-fa5c-4c91-bc67-1ec73751e213&themetreeid=21>
16. Frisén M, Andersson E, Pettersson K. Semiparametric estimation of outbreak regression. *Statistics.* 2010;44(2):107-17. DOI: 10.1080/02331880903021484
17. Taure V. Molecular subtyping and the transformation of public health. *Foodborne Pathog Dis.* 2006;3(1):4-8. DOI: 10.1089/fpd.2006.3.4 PMID: 16602974
18. Höhle M, Meyer S, Paul M. Surveillance: Temporal and Spatio-Temporal Modelling and Monitoring of Epidemic Phenomena. 2015; R package version 1.9-1. [Accessed 9 Jun 2017]. Available from: <http://CRAN.R-project.org/package=surveillance>
19. European Food Safety Authority (EFSA). Manual for reporting on food-borne outbreaks in accordance with Directive 2003/99/EC for information derived from the year 2012. Parma: EFSA; 2012. Available from: <http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2012.EN-265/epdf>
20. European Commission. Regulation (EC) n. 2073/2005 on Microbiological criteria for foodstuffs. Luxembourg: Publications Office of the European Union. 22.12.2005; L 338/1. Available from: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0001:0026:EN:PDF>
21. International Organization for Standardization (ISO). Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production stage. ISO 6579:2002/Amd 1:2007. Geneva: ISO; July 2007. Available from: <https://www.iso.org/standard/42109.html>
22. Barco L, Lettini AA, Ramon E, Longo A, Saccardin C, Pozza MC. A rapid and sensitive method to identify and differentiate Salmonella enterica serotype Typhimurium and Salmonella enterica serotype 4,[5],12:i:- by combining traditional serotyping and multiplex polymerase chain reaction. *Foodborne Pathog Dis.* 2011;8(6):741-3. DOI: 10.1089/fpd.2010.0776 PMID: 21247297
23. Centre for Disease Prevention and Control (CDC). Standard Operating Procedure for PulseNet PFGE of Escherichia coli O157:H7, Escherichia coli non-O157 (STEC), Salmonella serotypes, Shigella sonnei and Shigella flexneri; Atlanta: CDC; 2013. Available from: <http://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf>
24. Lindstedt B, Avarund T, Aas L, Kapperud G. Multiple-locus variable-number tandem-repeat analysis of Salmonella enterica subsp. enterica serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. *J Microbiol Methods.* 2004;59(2):163-72. DOI: 10.1016/j.mimet.2004.06.014 PMID: 15369852
25. Larsson J, Torpdahl M, Petersen R, Sorensen G, Lindstedt B, Nielsen E. Development of a new nomenclature for Salmonella typhimurium multilocus variable number of tandem repeats analysis (MLVA). *Euro Surveill.* 2009;14(15):14. PMID: 19371515
26. Chevreux B, Wetter T, Suhai S. Genome Sequence Assembly Using Trace Signals and Additional Sequence Information. Computer science and biology: Proceedings of German conference on bioinformatics (GCB); 1999.
27. Pettengill B, Luo Y, Davis S, Chen Y, Gonzalez-Escalona N, Ottesen A. An evaluation of alternative methods for constructing phylogenies from whole genome sequence data: a case study with Salmonella. *PeerJ.* 2014;2:e620. DOI: 10.7717/peerj.620 PMID: 25332847
28. Huelsenbeck J, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics.* 2001;17(8):754-5. DOI: 10.1093/bioinformatics/17.8.754 PMID: 11524383
29. Bauer A, Kirby W, Sherris J, Tenckhoff M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol.* 1966;45(4):493-6. DOI: 10.1093/ajcp/45.4_ts.493 PMID: 5325707
30. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother.* 2012;67(11):2640-4. DOI: 10.1093/jac/dks261 PMID: 22782487
31. Hopkins KL, Davies RH, Threlfall E. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. *Int J Antimicrob Agents.* 2005;25(5):358-73. DOI: 10.1016/j.ijantimicag.2005.02.006 PMID: 15848289
32. Boland C, Bertrand S, Mattheus W, Dierick K, Jasson V, Rosseel T. Extensive genetic variability linked to IS26 insertions in the fljB promoter region of atypical monophasic variants of Salmonella enterica serovar Typhimurium. *Appl Environ Microbiol.* 2015;81(9):3169-75. DOI: 10.1128/AEM.00270-15 PMID: 25724958
33. Lucarelli C, Dionisi A, Filetici E, Owczarek S, Luzzi L, Villa L. Nucleotide sequence of the chromosomal region conferring multidrug resistance (R-type ASSuT) in Salmonella Typhimurium and monophasic Salmonella Typhimurium strains. *J Antimicrob Chemother.* 2012;67(1):111-4. DOI: 10.1093/jac/dkr391 PMID: 21990047
34. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics.* 2014;30(14):2068-9. DOI: 10.1093/bioinformatics/btu153 PMID: 24642063
35. Tennant S, Diallo S, Levy H, Livio S, Sow S, Tapia M. Identification by PCR of non-typhoidal Salmonella enterica serovars associated with invasive infections among febrile patients in Mali. *PLoS Negl Trop Dis.* 2010;4(3):e621. DOI: 10.1371/journal.pntd.0000621 PMID: 20231882
36. Scaltriti E, Sasser A, Comandatore F, Morganti M, Mandalari C, Gaiarsa S. Differential single nucleotide polymorphism-based analysis of an outbreak caused by Salmonella enterica serovar Manhattan reveals epidemiological details missed by standard pulsed-field gel electrophoresis. *J Clin Microbiol.* 2015;53(4):1227-38. DOI: 10.1128/JCM.02930-14 PMID: 25653407
37. Dimovskik Cao H, Wijburg O, Strugnelli R, Mantena R, Whipp M. Analysis of Salmonella enterica serovar Typhimurium variable-number tandem-repeat data for public health investigation based on measured mutation rates and whole-genome sequence comparisons. *J Bacteriol.* 2014;196(16):3036-44. DOI: 10.1128/JB.01820-14 PMID: 24957617

38. Petersen RFL, Istrup E, Larsson JT, Torpdahl M, Sørensen G, Müller L. Molecular characterization of *Salmonella* Typhimurium highly successful outbreak strains. *Foodborne Pathog Dis.* 2011;8(6):655-61. DOI: 10.1089/fpd.2010.0683 PMID: 21381921
39. Wuyts V, Mattheus W, De Laminne de Bex G, Wildemauwe C, Roosens NH, Marchal K. MLVA as a tool for public health surveillance of human *Salmonella* Typhimurium: prospective study in Belgium and evaluation of MLVA loci stability. *PLoS One.* 2013;8(12):e84055. DOI: 10.1371/journal.pone.0084055 PMID: 24391880
40. Lienau EK, Strain E, Wang C, Zheng J, Ottesen AR, Keys CE. Identification of a salmonellosis outbreak by means of molecular sequencing. *N Engl J Med.* 2011;364(10):981-2. DOI: 10.1056/NEJMc1100443 PMID: 21345093
41. Octavia S, Wang Q, Tanaka MM, Kaur S, Sintchenko V, Lan R. Delineating community outbreaks of *Salmonella enterica* serovar Typhimurium by use of whole-genome sequencing: insights into genomic variability within an outbreak. *J Clin Microbiol.* 2015;53(4):1063-71. DOI: 10.1128/JCM.03235-14 PMID: 25609719
42. Wuyts V, Denayer S, Roosens NH, Mattheus W, Bertrand S, Marchal K. Whole Genome Sequence Analysis of *Salmonella* Enteritidis PT4 Outbreaks from a National Reference Laboratory's Viewpoint. *PLoS Curr.* 2015;7:10. PMID: 26468422

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2018.

National Bulletins

AUSTRIA

Mitteilungen der Sanitätsverwaltung
Bundesministerium für Gesundheit Familie und Jugend, Vienna
Monthly, print only. In German.
<http://www.bmgfj.gv.at/cms/site/thema.html?channel=CHo951>

BELGIUM

Vlaams Infectieziektebulletin
Department of Infectious Diseases Control, Flanders
Quarterly, print and online. In Dutch, summaries in English.
<http://www.infectieziektebulletin.be>

Bulletin d'information de la section d'Epidémiologie
Institut Scientifique de la Santé Publique, Brussels
Monthly, online. In French.
<http://www.iph.fgov.be/epidemio/epifr/episcoop/episcoop.htm>

BULGARIA

Bulletin of the National Centre of Infectious and Parasitic Diseases, Sofia
Print version. In Bulgarian.
<http://www.ncipd.org/>

CYPRUS

Newsletter of the Network for Surveillance and Control of Communicable Diseases in Cyprus
Medical and Public Health Services, Ministry of Health, Nicosia
Biannual, print and online. In Greek.
<http://www.moh.gov.cy>

CZECH REPUBLIC

Zpravy CEM (Bulletin of the Centre of Epidemiology and Microbiology)
Centrum Epidemiologie a Mikrobiologie Státního Zdravotního Ústavu, Prague
Monthly, print and online. In Czech, titles in English.
<http://www.szu.cz/cema/adefaultt.htm>

EPIDAT (Notifications of infectious diseases in the Czech Republic)
<http://www.szu.cz/cema/epidat/epidat.htm>

DENMARK

EPI-NEWS
Department of Epidemiology, Statens Serum Institut, Copenhagen
Weekly, print and online. In Danish and English.
<http://www.ssi.dk>

FINLAND

Kansanterveyslaitos
Department of Infectious Disease Epidemiology, National Public Health Institute, Helsinki
Monthly, print and online. In Finnish.
http://www.ktl.fi/portal/suomi/osastot/infe/tutkimus/tartuntatautien_seuranta/tartuntatautilaakarin_komentit/

FRANCE

Bulletin épidémiologique hebdomadaire
Institut de veille sanitaire, Saint-Maurice Cedex
Weekly, print and online. In French.
<http://www.invs.sante.fr/beh/default.htm>

GERMANY

Epidemiologisches Bulletin
Robert Koch-Institut, Berlin
Weekly, print and online. In German.
http://www.rki.de/DE/Content/Infekt/EpidBull/epid__bull__node.html

GREECE

HCDCP Newsletter
Hellenic Centre for Disease Control and Prevention (HCDCP/KEELPNO), Athens
Monthly, online. In English and Greek.
<http://www2.keelpno.gr/blog/?lang=en>

HUNGARY

Epinfo (az Országos Epidemiológiai Központ epidemiológiai információs hetilapja)
National Center For Epidemiology, Budapest
Weekly, online. In Hungarian.
<http://www.oek.hu/oek.web?to=839&nid=41&pid=7&lang=hun>

ICELAND

EPI-ICE
Landlæknisembættið
Directorate Of Health, Seltjarnarnes
Monthly, online. In Icelandic and English.
<http://www.landlaeknir.is>

IRELAND

EPI-INSIGHT
Health Protection Surveillance Centre, Dublin
Monthly, print and online. In English.
<http://www.hpsc.ie/hpsc/EPI-Insight>

ITALY

Notiziario dell'Istituto Superiore di Sanità
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
<http://www.iss.it/publ/noti/index.php?lang=1&tipo=4>

Bolletino Epidemiologico Nazionale (BEN)
Istituto Superiore di Sanità, Reparto di Malattie Infettive, Rome
Monthly, online. In Italian.
<http://www.epicentro.iss.it/ben>

LATVIA

Epidemiologijas Biļeteni
Sabiedrības veselības agentūra
Public Health Agency, Riga
Online. In Latvian.
<http://www.sva.lv/epidemiologija/bileteni>

LITHUANIA

Epidemiologijos žinios
Užkrečiamųjų ligų profilaktikos ir kontrolės centras
Center for Communicable Disease Prevention and Control, Vilnius
Online. In Lithuanian.
<http://www.ulac.lt/index.php?pl=26>

NETHERLANDS

Infectieziekten Bulletin
Rijksinstituut voor Volksgezondheid en Milieu
National Institute of Public Health and the Environment, Bilthoven
Monthly, print and online. In Dutch.
<http://www.rivm.nl/infectieziektenbulletin>

NORWAY

MSIS-rapport
Folkehelseinstituttet, Oslo
Weekly, print and online. In Norwegian.
<http://www.folkehelse.no/nyhetsbrev/msis>

POLAND

Meldunki o zachorowaniach na choroby zakaźne i zatruciach w Polsce
Panstwowy Zakład Higieny,
National Institute of Hygiene, Warsaw
Fortnightly, online. In Polish and English.
<http://www.pzh.gov.pl>

PORTUGAL

Saúde em Números
Ministério da Saúde,
Direcção-Geral da Saúde, Lisbon
Sporadic, print only. In Portuguese.
<http://www.dgs.pt>

ROMANIA

Info Epidemiologia
Centrul pentru Prevenirea și Controlul Bolilor Transmisibile, National Centre
of Communicable Diseases Prevention and Control, Institute of Public Health,
Bucharest
Sporadic, print only. In Romanian.
Sporadic, print only. In Romanian.
http://www.insp.gov.ro/cnscbt/index.php?option=com_docman&Itemid=12

SLOVENIA

CNB Novice
Inštitut za varovanje zdravja, Center za nalezljive bolezni, Institute of Public
Health, Center for Infectious Diseases, Ljubljana
Monthly, online. In Slovene.
<http://www.ivz.si>

SPAIN

Boletín Epidemiológico Semanal
Centro Nacional de Epidemiología, Instituto de Salud Carlos III, Madrid
Fortnightly, print and online. In Spanish.
<http://revista.isciii.es>

SWEDEN

Folkhälsomyndighetens nyhetsbrev
Folkhälsomyndigheten, Stockholm
Weekly, online. In Swedish.
<http://www.folkhalsomyndigheten.se/>

UNITED KINGDOM

ENGLAND AND WALES

Health Protection Report
Public Health England, London
Weekly, online only. In English.
<https://www.gov.uk/government/collections/health-protection-report-latest-infection-reports>

NORTHERN IRELAND

Communicable Diseases Monthly Report
Communicable Disease Surveillance Centre, Northern Ireland, Belfast
Monthly, print and online. In English.
<http://www.cdscni.org.uk/publications>

SCOTLAND

Health Protection Scotland Weekly Report
Health Protection Scotland, Glasgow
Weekly, print and online. In English.
<http://www.hps.scot.nhs.uk/ewr/>

EUROPEAN UNION

“Europa” is the official portal of the European Union. It provides up-to-date
coverage of main events and information on activities and institutions of the
European Union.
<http://europa.eu>

EUROPEAN COMMISSION - PUBLIC HEALTH

The website of European Commission Directorate General for Health and
Consumer Protection (DG SANCO).
<http://ec.europa.eu/health/>

HEALTH-EU PORTAL

The Health-EU Portal (the official public health portal of the European Union)
includes a wide range of information and data on health-related issues and
activities at both European and international level.
<http://ec.europa.eu/health-eu/>

EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

European Centre for Disease Prevention and Control (ECDC)
The European Centre for Disease Prevention and Control (ECDC) was
established in 2005. It is an EU agency with aim to strengthen Europe’s
defences against infectious diseases. It is seated in Stockholm, Sweden.
<http://www.ecdc.europa.eu>

All material in *Eurosurveillance* is in the public domain and may be used and reprinted without special permission. However, the source should be cited properly and we suggest adding a link to the exact page on the *Eurosurveillance* website.

Articles published in *Eurosurveillance* are indexed in PubMed/Medline.

The *Eurosurveillance* print edition is a selection of short and long articles previously published on the *Eurosurveillance* website. The full listing of all *Eurosurveillance* articles can be found in the Archives section of the website.

The opinions expressed by authors contributing to *Eurosurveillance* do not necessarily reflect the opinions of the European Centre for Disease Prevention and Control (ECDC) or the Editorial team or the institutions with which the authors are affiliated. Neither the ECDC nor any person acting on behalf of the ECDC is responsible for the use which might be made of the information in this journal.



Visit our website at www.eurosurveillance.org

The *Eurosurveillance* print edition is a compilation of short and long articles that have previously been published on our website.

All the articles in this issue are available online: you can print each page separately or download the whole quarterly in pdf format.

The website archives all articles since 1995, and offers a search facility.

To receive *Eurosurveillance's* free **electronic releases** and e-alerts by email, please subscribe on our website.

Papers published in the former monthly release are indexed for MEDLINE since January 2001, and papers published in the weekly release from January 2005 (with the exception of short, non-scientific notices) are also indexed for MEDLINE.

The Index Medicus abbreviation for *Eurosurveillance* is Euro Surveill.

Follow us on Twitter : #eurosurveillanc

Contributions to Eurosurveillance are welcomed.
Full instructions to authors are available on our
website www.eurosurveillance.org

ISSN 1025 496X

Graphic design © ECDC, Stockholm



Publications Office