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Tracing sources of *Listeria* contamination in traditional Italian cheese associated with a US outbreak: investigations in Italy

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SUMMARY

In 2012 a US multistate outbreak of listeriosis was linked to ricotta salata imported from Italy, made from pasteurized sheep's milk. Sampling activities were conducted in Italy to trace the source of *Listeria monocytogenes* contamination. The cheese that caused the outbreak was produced in a plant in Apulia that processed semi-finished cheeses supplied by five plants in Sardinia. During an 'emergency sampling', 179 (23·6%) out of 758 end-products tested positive for *L. monocytogenes*, with concentrations from <10 c.f.u./g to $1{\cdot}1 \times 10^6$ c.f.u./g. Positive processing environment samples were found in two out of four processing plants. A 'follow-up sampling' was conducted 8 months later, when environmental samples from three out of six plants tested positive for *L. monocytogenes* and for *Listeria* spp. PFGE subtyping showed 100% similarity between US clinical strains and isolates from ricotta salata, confirming the origin of the outbreak. The persistence of strains in environmental niches of processing plants was demonstrated, and is probably the cause of product contamination. Two PFGE profiles from clinical cases of listeriosis in Italy in 2011, stored in the MSS-TESSy database, were found to have 100% similarity to one PFGE profile from a US clinical case associated with the consumption of ricotta salata, according to the US epidemiological investigation (sample C, pulsotype 17). However, they had 87% similarity to the only PFGE profile found both in the US clinical case and in 14 ricotta cheese samples collected during the emergency sampling (sample B, pulsotype 1). Sharing of molecular data and availability of common characterization protocols were key elements that connected the detection of the US outbreak to the investigation of the food source in Italy. Simultaneous surveillance systems at both food and human levels are a necessity for the efficient rapid discovery of the source of an outbreak of *L. monocytogenes*.

Key words: Food safety, *Listeria*, molecular epidemiology, outbreaks, surveillance system.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that causes severe illness in humans. In the European Union (EU), invasive listeriosis is an infection of great concern to public health due to its clinical severity (hospitalization rate >90%) and high fatality rate

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(20–30%), despite its low incidence [1]. This micro-organism is responsible for severe outbreaks and substantial economic losses due to food recalls [2]. It is ubiquitous in the environment and frequently shed by food-producing animals [3]. Nevertheless, it is more frequently transferred to foodstuffs via processing environments [4]. Semi-soft cheeses, with a production process including curing and seasoning, are considered products at high risk of *L. monocytogenes* contamination [5].

Surveillance of human listeriosis in the EU is carried out within the Foodborne and Waterborne programme (FWD) coordinated by the European Centre for Disease Prevention and Control (ECDC). Data on both sporadic and outbreak-associated cases of infection are collected and disseminated through the European Surveillance System (TESSy) and the European Epidemic Intelligence Information System for FWD (EPIS-FWD), a web-based communication platform bringing together experts from EU and non-EU countries including the United States, whose main objective is to allow the rapid detection of multi-country outbreaks and thereafter facilitate their investigation [6, 7]. Since November 2012, ECDC has implemented TESSy with MSS (Molecular Surveillance System) in order to routinely collect pulsed-field gel electrophoresis (PFGE) molecular-typing data of *L. monocytogenes* and other foodborne pathogen strains isolated from humans [8, 9]. The Statens Serum Institut (Denmark) in collaboration with ECDC organizes an annual External Quality Assessment in order to verify the competence of laboratories to perform PFGE on clinical isolates of *L. monocytogenes*.

Routine monitoring of *L. monocytogenes* in food and animals is carried out in the EU according to directive 99/2003 EC which provides for compulsory data collection. Monitoring data from Member States of the EU are annually collected and published by the European Food Safety Authority (EFSA) together with data on human cases of listeriosis provided by ECDC. The European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm), within the framework of a pilot study with the voluntary participation of some national reference laboratories for *L. monocytogenes*, has implemented a database of PFGE profiles from food and animal *L. monocytogenes* isolates.

In order to investigate the molecular epidemiology of listeriosis in humans and *L. monocytogenes* in ready-to-eat food through application of comparable molecular-typing methodology, the European Commission (EC) in 2009 requested collaboration between EFSA, ECDC and EURL Lm in terms of simultaneous and representative collection of food and human isolates at the EU level to be further investigated through molecular-typing methods [10]. This transectorial collaboration at the European level (joint EFSA-ECDC database) is in the process of being implemented [11] but in 2012 it was not yet in place and therefore was not part of the activities described hereafter.

On 8 August 2012, an urgent inquiry on the EPIS-FWD was posted to alert the countries participating in the EPIS-FWD network (38 countries mainly from the EU) of an ongoing multistate outbreak of listeriosis in the United States caused by a *L. monocytogenes* serotype 1/2a strain with an uncommon PFGE profile, extremely rare in the United States and possibly associated with imported cheese.

From March to October 2012 the US outbreak of listeriosis involved 22 patients and caused four deaths [12]. The US investigation team identified a semi-soft seasoned cheese (ricotta salata) made from pasteurized milk and imported from Italy, as the source of the outbreak.

The aim of this work is to describe the results of the investigation activities performed in Italy, to trace back the source of *L. monocytogenes* contamination at the production level, and assess the possible persistence of strains in processing environments several months after the US outbreak.

BACKGROUND

On 12 September 2012, the Italian Ministry of Health was officially informed of the *Listeria* outbreak through the International Food Safety Authority Network (INFOSAN) and on the same day the Italian Contact Point for the Rapid Alert System for Food and Feed (RASFF) disseminated the information to the local health authorities throughout the country. An international recall of the suspect cheese was promptly launched.

A national, multidisciplinary outbreak investigation team (OIT) was set up with the aim of identifying domestic human cases of listeriosis possibly linked to the outbreak, tracing back the production chain of the suspect cheese and carrying out the microbiological investigation to further support evidence implicating the ricotta salata as the likely source of the outbreak. The OIT included the Ministry of Health, National Public Health Institute, National Reference Laboratory for *L. monocytogenes*, police forces for health and environmental protection

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(Carabinieri and Corpo Forestale dello Stato), veterinary services of the local health authority and the official food laboratory (Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata). Active case-finding was conducted by sending an alert to the official health authorities of all the Italian regions requesting information on any laboratory-confirmed cases of listeriosis with similar PFGE profiles (100% similarity or one-band difference), but no cases could be detected. An intense sampling activity was then conducted.

MATERIALS AND METHODS

Cheese processing flow

Ricotta salata is a salt-cured and seasoned ricotta cheese made from pasteurized sheep's milk. Processing of cheese intended to be exported to the United States is always carried out through two different establishments: one plant produced semi-finished cheese and then supplied it to another plant where cheese manufacturing was completed and the final product was packaged and delivered to the United States. There were five different supplier plants, all of which were located in Sardinia region (plants B, C, D, E, F). These plants performed the initial stages of the production process, starting from pasteurized sheep's milk, up to the early days of the seasoning period. Semi-finished cheeses intended for export were all delivered to the same plant (plant A) located in the Apulia region, where the remaining seasoning period (up to 30 days) and the washing, cleaning, oiling, cutting and packaging operations were usually performed. The flow of the supply chain and processing activities throughout the different plants is shown in Figure 1. In addition to supplying semi-finished ricotta salata to plant A, supplier plants also produced end-products including ricotta salata and other types of seasoned cheese for domestic and European markets.

Sampling

Food and environmental samples were taken from all the manufacturing plants identified in Apulia and Sardinia. Two sessions of sampling were carried out: the first ('emergency sampling') was conducted soon after the epidemiological, laboratory and trace-back investigations conducted in the United States implicated ricotta salata as the likely source of the listeriosis outbreak in September 2012. The second ('follow-up sampling') was performed 8 months later, in May 2013, when both the sheep milking season and the cheese manufacturing activities in the plants were at their peak.

During the emergency sampling (Table 1), a total of 758 cheese and 183 environmental samples were taken. A first group of 557 cheese samples (ricotta salata and similar seasoned cheeses from pasteurized sheep's milk), including cheese batches exported to the United States, were taken from plant A and supplier plants B, C and D. In the Apulia region, the remaining 201 samples of cheese made by these plants were collected, including 108 samples of cheese from wholesale markets, and 93 samples of cheese from retail stores. Environmental samples from plants were distributed as follows: 79 samples in plant A, 18 in plant B, 49 in plant C, and 37 in plant D. Thirty-six samples of ingredients used for cheese production (lactic cultures, rennet, salt) were sampled at plants B, C and D.

During the follow-up sampling, 179 environmental and 35 cheese samples (ricotta salata and similar seasoned cheeses from pasteurized sheep's milk) were collected from all the six plants potentially linked to the outbreak. Environmental samples (27 samples in plant A, 29 in plant B, 33 in plant C, 30 in plant D, 29 in plant E, 31 in plant F) were evenly distributed throughout the different processing areas in each plant.

In both sampling sessions, environmental samples, collected from food contact surfaces (FCS) and non-food contact surfaces (NFCS), were taken using sponge-bags and swabs, according to ISO 18593:2004 [13] and guidelines from the EURL Lm [14]. Five sample units were collected for each batch, according to European Union Regulation 2073/2005 [15]. When <5 samples per batch were available, all available samples were collected.

Laboratory analysis

Environmental samples were examined by ISO 11290-1 for detection of *L. monocytogenes*; food samples were examined by ISO 11290-1 and 11290-2 for detection and enumeration of *L. monocytogenes* [16, 17]. The ISO 11290-1 method was also used for the detection of other *Listeria* spp. in processing environments and foodstuffs. Isolates were serotyped [18], including sera for somatic antigens O and flagellar antigens H (Denka Seiken Co. Ltd, Japan). When *L. monocytogenes* was detected, one isolate per sample unit underwent molecular characterization. PFGE was performed according to the PulseNet protocol [19]

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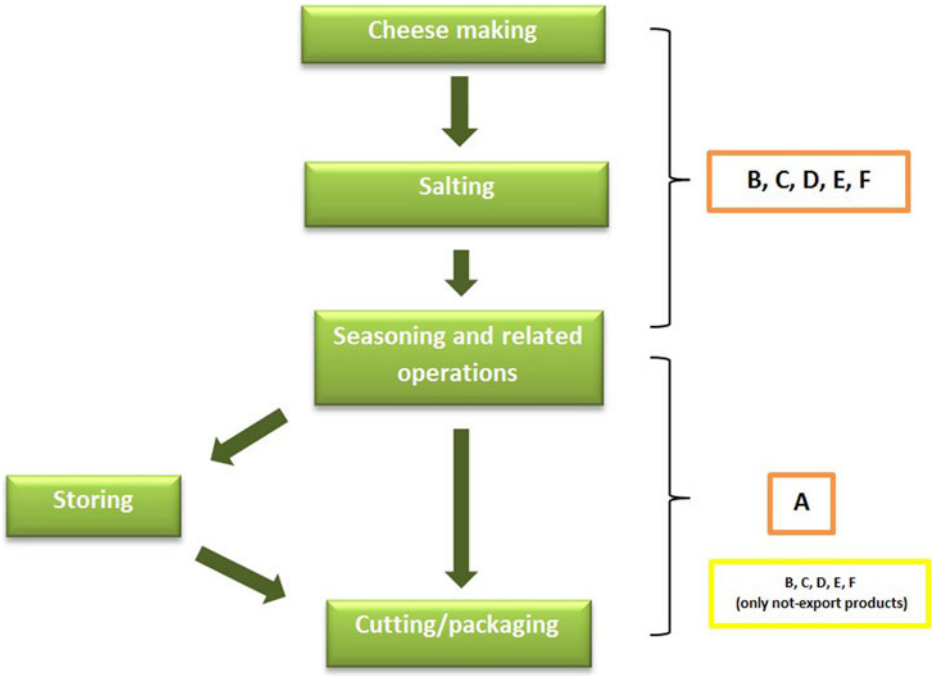

Fig. 1. Flow of processing activities for the production of ricotta salata and other similar seasoned cheeses throughout the different plants.

Table 1. *Summary of samples taken during the 'emergency' sampling*

Site	End products	Lm positive	Ingredients	Lm positive	Environment	Lm positive
Plant	557	128 (23·0%)	36	0	183	2 (1·1%)
Wholesale	108	32 (29·6%)				
Retail	93	19 (20·4%)				
Total	758	179 (23·6%)	36	0	183	2 (1·1%)

Lm, *Listeria monocytogenes*.

using restriction enzymes *Asc*I and *Apa*I. PFGE macrorestriction profiles were analysed with BioNumerics v. 6.6 software (Applied Maths, Belgium). Similarities between the profiles were derived using the Dice correlation coefficient [20] with a 1·0% band position tolerance and 1·0% optimization according to Martin *et al*. [21]. The software performed the clustering and construction of dendrograms by unweighted pair-group method analysis. Four PFGE profiles from the United States clinical strains could be shared between the US and Italian authorities thanks to a confidentiality agreement signed between the FDA and the Italian Ministry of Health. The profiles could be compared with PFGE profiles yielded from our research (food isolates) because they had been produced with the same PulseNet protocol.

RESULTS

During the emergency sampling, *L. monocytogenes* was found in two (1·1%) out of 183 environmental samples, taken in plants A, B, C, and D. Only environmental samples from plants B and D were positive, including one FCS sample (brush in washing machine) from the seasoning area in plant B, and one NFCS sample (discharge water into manhole) from the packaging area in plant D. Another NFCS sample (steel sink) from the packaging area in plant D tested positive for *Listeria* spp.

Of 758 finished cheese products, 179 (23·6%) tested positive for *L. monocytogenes*, with contamination levels ranging from <10 c.f.u./g to $1{\cdot}1 \times 10^6$ c.f.u./g. Even though other types of similar cheeses were sampled, *L. monocytogenes* was only detected in

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Table 2. *Detail of the results of all environmental sampling carried out during the 'emergency' and 'follow-up' sampling*

Surface	Stage of sampling	Surface type	Processing area	Plant	Lm serotype	Lm pulsotype	Identification
Brush	Emergency	FCS	Seasoning (washing)	B	4b	14	*L. monocytogenes*
Manhole	Emergency	NFCS	Cutting/packaging	D	4b	11	*L. monocytogenes*
Steel sink	Emergency	NFCS	Cutting/packaging	D			*L. innocua*
Operator shoe sole	Follow-up	NFCS	Seasoning (plastification)	A			*L. innocua*
Knife	Follow-up	NFCS	Cheese making	D	1/2a	3	*L. monocytogenes*
Cutting machine	Follow-up	FCS	Cutting/packaging	D	4b	11	*L. monocytogenes*
Table	Follow-up	FCS	Cheese making	E	1/2a	2	*L. monocytogenes*
Floor	Follow-up	NFCS	Cheese making	E	1/2a	7	*L. monocytogenes* *L. innocua*
Manhole	Follow-up	NFCS	Cheese making	E	1/2a	8	*L. monocytogenes* *L. innocua*
Trolley shelf (bottom)	Follow-up	NFCS	Salting	E	1/2a	7	*L. monocytogenes*
Salting machine	Follow-up	FCS	Salting	F			*L. innocua*
Manhole	Follow-up	NFCS	Salting	F			*L. innocua*
Shelf (bottom)	Follow-up	NFCS	Seasoning	F			*L. innocua*

Lm, *Listeria monocytogenes*; NFCS, Not food contact surface; FCS, food contact surface.

ricotta salata. The highest concentration was found in a ricotta salata that originated from plant B and was then further processed and packaged in plant A. All positive cheese samples had been processed in plant A and originated from semi-finished products from all supplier plants, including plant B (23·9%, 31/134 positive samples of known origin), plant C (18·6%, 22/134), plant D (44·8%, 60/134), plant E (5·2%, 7/134) and plant F (7·5%, 10/134). For 45 positive cheese samples, all taken at retail or at wholesale market, the supplier plant was not known because it was not specified on the label. All 36 samples of ingredients tested negative.

All finished cheese products sampled during the follow-up stage tested negative. However, the presence of *L. monocytogenes* and *Listeria* spp. in processing environments was confirmed. Samples were performed throughout the whole processing chain, both FCS and NFCS were positive for *L. monocytogenes* in plants D (2/30, 6·7%) and E (4/29, 13·8%). Surfaces in cheese-making (NFCS) and cutting/packaging (FCS) areas tested positive for *L. monocytogenes* in plant D, while surfaces in cheese-making (NFCS) and salting (NFCS) areas tested positive in plant E. Environmental samples from plants A (NFCS) and F (FCS and NFCS) tested positive for *Listeria* spp., with a ratio of 1/27 (3·7%) and 3/31 (9·7%), respectively, in salting and seasoning areas. During this follow-up sampling, no positive samples were found from plants B and C. The details of all environmental samples collected during both emergency and follow-up sampling, including the type of surface the sample was collected from, the serotype and the pulsotype for each sample, are reported in Table 2.

Of 187 food and environmental samples positive to *L. monocytogenes* (181 from the emergency sampling and six from the follow-up sampling), 182 isolates were subtyped. Two serotypes were identified from the isolates, including 1/2a (78% of cases) and 4b (22% of cases). PFGE analysis yielded 11 macrorestriction profiles for *Asc*I (designated *Asc*1–*Asc*11) and 18 for *Apa*I (designated *Apa*1–*Apa*18), including *Asc*I and *Apa*I profiles from four clinical strains identified in the US outbreak and all associated with the consumption of ricotta salata. The profiles of these clinical strains were provided to the competent Italian authorities by the FDA and identified as samples A, B, C, D ('clinical' A, B, C, D in Fig. 2). Only the serotype of two out of four clinical strains was communicated by the FDA, i.e. samples B and C, both belonging to serotype 1/2a. Combining *Asc*I and *Apa*I results, 18 different pulsotypes (designated 1–18) were identified.

The dendrogram showing the results of comparison between all strains from our study, including the four clinical strains, is reported in Figure 2. The *Asc*I/*Apa*I profile of one clinical strain (sample B, pulsotype 1) had 100% similarity to the *Asc*I/*Apa*I profile of 14 isolates from ricotta salata samples taken during the emergency sampling. All these 14 isolates were from ricotta salata samples produced in plant A using semi-finished cheese supplied from plants B, C or F. Another two

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clinical strains were also highly similar to the pulsotype 1, i.e. 87·9% (sample C, pulsotype 17) and 86·5% (sample A, pulsotype 16). On the other hand, the fourth clinical strain (sample D, pulsotype 15) only had 51·4% similarity to pulsotype 1.

Two PFGE profiles from clinical cases of listeriosis in Italy in 2011, stored in the MSS-TESSy database as A367 and A345 (both assigned to MSS-TESSy pulsotype '*Asc*I 0087–*Apa*I 02249'), were found to have 100% similarity to the profile of one of the four US clinical strains, i.e. sample C (pulsotype 17) (Fig. 3). They also had 87% similarity to the only PFGE profile found in both the US clinical case and in cheese samples collected during the emergency sampling (sample B, pulsotype 1). The Italian strains were isolated from elderly people during May and December 2011, of whom one died. No information on food exposure of these two cases was available.

Regarding environmental isolates, none was found to be similar to pulsotype 1. Isolates from plant D were the ones that more frequently showed 100% similarity to other ricotta salata isolates, in contrast to pulsotype 1. Moreover, isolates from plant E had 100% similarity to isolates from ricotta salata.

DISCUSSION

Widespread *L. monocytogenes* contamination was found in products and processing environments during both sampling periods. The first sampling stage was mostly focused on cheese sampling, showing the presence of contamination in >25% of sampled cheeses. PFGE demonstrated 100% genetic similarity in strains from US clinical cases and some samples of ricotta salata collected in Italy, providing support for the epidemiological investigation conducted in the United States to confirm the origin of the outbreak [22]. During the second sampling stage, *L. monocytogenes* was isolated from different processing areas of plants D and E, including cheese-making (plants D and E), cutting/packaging (plant D), and salting (plant E) areas, which suggests widespread contamination throughout the whole establishments, even many months after the first sampling. Both FCS and NFCS were contaminated. The presence of *Listeria* spp. in plant A could also be indicative of environmental conditions that favour harbouring of *L. monocytogenes*.

Persistence of contamination is a particular characteristic of *L. monocytogenes* in food processing environments, including dairy plants [23]. *L. monocytogenes* readily produces biofilms, allowing the organism to attach and survive on contact surfaces while resisting sanitization techniques employed in food-processing environments [24]. In the establishments subjected to our study, the PFGE results suggest the strains' persistence in environmental niches within plants and possible spread across plants over time, in particular in plant D. One isolate from plant D had 100% similarity to another isolate obtained from the same plant 8 months previously (pulsotype 11, Fig. 2). Moreover, isolates from the environment of plant D had 100% similarity (pulsotypes 3 and 11) or at least 85% similarity (pulsotypes 12 and 13) to other isolates found in cheese end-products processed in plant A, even if they were produced from semi-finished products of other suppliers. Further, isolates from the environment of plant E showed 100% similarity to isolates from cheeses sampled during the emergency sampling, but only if the cheeses had been produced from semi-finished products of plant E (pulsotype 8, Fig. 2).

On the basis of these PFGE results, the processing environment of plant D seems to be the most probable origin of contamination that could have spread to a variety of end-products through the environment of plant A. The environmental origin of the contamination is corroborated also by the fact that all sampled cheeses were made from pasteurized milk.

According to the outcomes from the OIT, the veterinary services of the local health authorities immediately took measures against food business operators, requiring implementation of thorough plant cleaning and monitoring. The competent Italian authority suspended production activities and export licenses until the veterinary services had verified the effectiveness of these corrective measures. The period of suspension of activities in plant A lasted about 3 months, up to the end of the emergency sampling and after the implementation of hygiene measures that were judged to be satisfactory by the competent Italian authority (December 2012). After that, production of cheese only for the domestic and European markets was allowed. The follow-up sampling (May 2013) confirmed compliance with EU legislation as no more positive end-products were found. However, considering the persistence of *L. monocytogenes* in the processing environment of two supplier plants (D and E), the export licence to the United States for plant A has not been renewed.

To the best of our knowledge, no further circulation of the *L. monocytogenes* strain associated with the US outbreak has been reported in the EU so far. A

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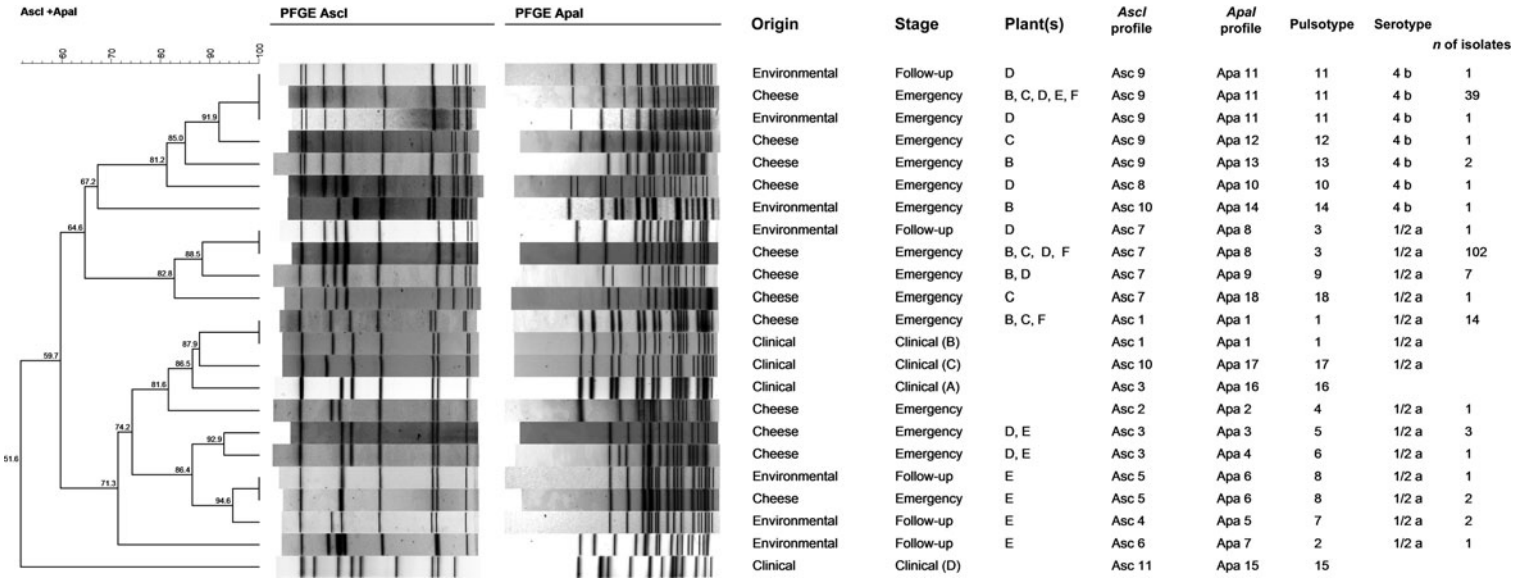

Origin	Stage	Plant(s)	*AscI* profile	*ApaI* profile	Pulsotype	Serotype	*n* of isolates
Environmental	Follow-up	D	Asc 9	Apa 11	11	4 b	1
Cheese	Emergency	B, C, D, E, F	Asc 9	Apa 11	11	4 b	39
Environmental	Emergency	D	Asc 9	Apa 11	11	4 b	1
Cheese	Emergency	C	Asc 9	Apa 12	12	4 b	1
Cheese	Emergency	B	Asc 9	Apa 13	13	4 b	2
Cheese	Emergency	D	Asc 8	Apa 10	10	4 b	1
Environmental	Emergency	B	Asc 10	Apa 14	14	4 b	1
Environmental	Follow-up	D	Asc 7	Apa 8	3	1/2 a	1
Cheese	Emergency	B, C, D, F	Asc 7	Apa 8	3	1/2 a	102
Cheese	Emergency	B, D	Asc 7	Apa 9	9	1/2 a	7
Cheese	Emergency	C	Asc 7	Apa 18	18	1/2 a	1
Cheese	Emergency	B, C, F	Asc 1	Apa 1	1	1/2 a	14
Clinical	Clinical (B)		Asc 1	Apa 1	1	1/2 a	
Clinical	Clinical (C)		Asc 10	Apa 17	17	1/2 a	
Clinical	Clinical (A)		Asc 3	Apa 16	16		
Cheese	Emergency		Asc 2	Apa 2	4	1/2 a	1
Cheese	Emergency	D, E	Asc 3	Apa 3	5	1/2 a	3
Cheese	Emergency	D, E	Asc 3	Apa 4	6	1/2 a	1
Environmental	Follow-up	E	Asc 5	Apa 6	8	1/2 a	1
Cheese	Emergency	E	Asc 5	Apa 6	8	1/2 a	2
Environmental	Follow-up	E	Asc 4	Apa 5	7	1/2 a	2
Environmental	Follow-up	E	Asc 6	Apa 7	2	1/2 a	1
Clinical	Clinical (D)		Asc 11	Apa 15	15		

Fig. 2. Dendrogram of PFGE profiles of all isolates the study. For each pair of *Asc*I/*Apa*I profiles (pulsotype) the following are detailed: origin (cheese, environmental or clinical strain), stage of sampling ('emergency' or 'follow-up'), plant where environmental pulsotypes have been found or plant of origin of the semi-finished cheese before processing in plant A, serotype and the number of isolates assigned to the pulsotype.

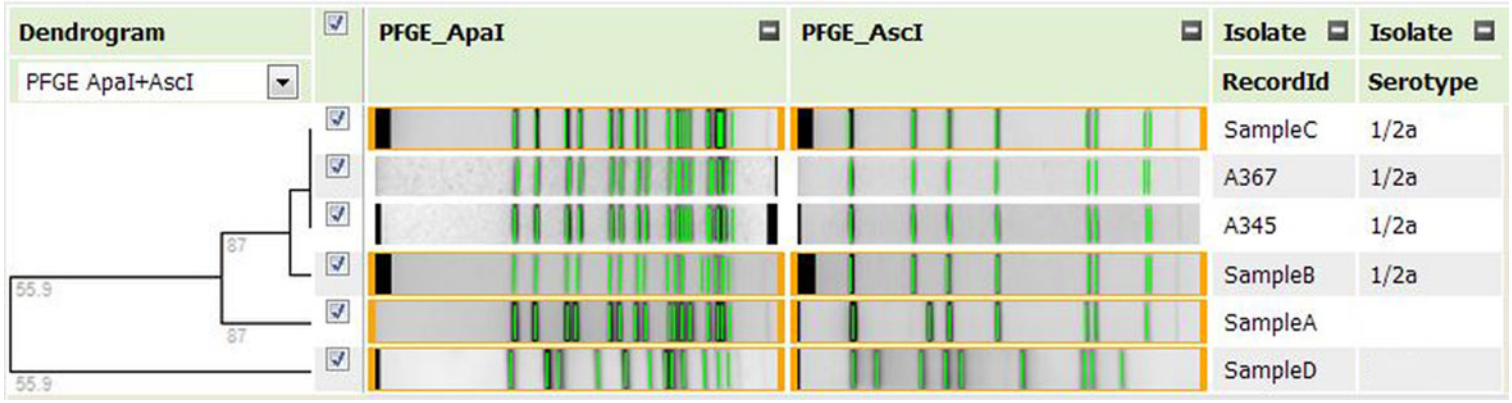

Fig. 3. Cluster analysis with TESSy-MSS database of the four US clinical strains isolated during the outbreak (samples A, B, C, D).

retrospective cluster analysis of *L. monocytogenes* strains isolated from humans in the EU was recently performed based on the MSS-TESSy database. Only two isolates matching the same *Asc*I/*Apa*I pattern profiles of one of the US outbreak strains could be found; both were cases that occurred in 2011. This result suggests that the circulation of this pulsotype (pulsotype 17 according to our nomenclature, in MSS-TESSy database designated *Asc*I 0087–*Apa*I 02249) is extremely rare also in the EU.

CONCLUSION

National and international collaborations successfully identified the contamination origins, which caused a severe outbreak in the United States. The ubiquitous and persistent presence of *L.monocytogenes* and structural failures may have resulted in the contamination of food-processing environments, and ultimately food products. However, overall this outbreak was a good opportunity to verify that the available systems for a rapid exchange of information between the EU and the United States, in cases of cross-border foodborne outbreaks, actually allow a prompt response in case of emergency. Despite an extremely long food-production chain, trace back of the suspected cheese could be performed promptly, revealing the effectiveness of the tracing tools implemented in Italy according to mandatory traceability foreseen by regulation EC 178/2002 [25]. The availability of common protocols for molecular characterization of *L. monocytogenes* in different sectors and geographical areas was the key element that connected the detection of an outbreak in the United States to investigation of the food source in Italy. This highlights the utility of promoting harmonized molecular subtyping of *L. monocytogenes* isolates of human and non-human origin for the integrated analyses necessary to support outbreak investigation and inform risk-mitigation options in the food production chain [11, 26, 27].

The prompt intervention of the Italian investigation team prevented more *L. monocytogenes* contaminated food from entering the distribution chain at the international level.

In conclusion, all actions taken for the management of this outbreak could be seen as a successful integration of food and human surveillance and control systems in Italy. More efforts are needed to ensure the integrated collection and rapid comparison of epidemiological and molecular data from different origins, and to allow for a prompt response to listeriosis outbreaks.

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DECLARATION OF INTEREST

None.

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