

A severe gastroenteritis outbreak of *Salmonella enterica* serovar Enteritidis PT8, with PFGE profile XbaI.0024 and MLVA profile 2-9-7-3-2 following a christening reception, Greece, 2016

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SUMMARY

In June 2016, a *Salmonella enterica* serovar Enteritidis outbreak ($n = 56$) occurred after a christening reception in Central Greece, mainly affecting previously healthy adults; one related death caused media attention. Patients suffered from profuse diarrhoea, fever and frequent vomiting episodes requiring prolonged hospitalisation and sick leave from work, with a 54% hospital admission rate. The majority of cases experienced serious illness within <12 h of attending the party. We investigated the outbreak to identify the source(s) of infection and contributing factors to the disease severity. From the retrospective cohort study, the cheesy penne pasta was the most likely vehicle of infection (relative risk 7·8; 95% confidence interval 3·6–16·8), explaining 79% of the cases. *S. enterica* ser. Enteritidis isolates were typed as phage-type PT8, pulsed-field gel electrophoresis type XbaI.0024, multiple locus variable-number tandem repeat analysis-type 2-9-7-3-2. The strain did not share the single-nucleotide polymorphism address of the concurrent European *S. enterica* ser. Enteritidis PT8 outbreak clusters. Following five consecutive years with no documented *S. enterica* ser. Enteritidis outbreaks in Greece, this outbreak, likely associated with a virulent strain, prompted actions towards the enhancement of the national *Salmonella* molecular surveillance and control programmes including the intensification of training of food handlers for preventing similar outbreaks in the future. Advanced molecular techniques were useful in distinguishing unrelated outbreak strains.

Key words: Epidemiology, outbreak, *Salmonella* molecular typing, *S. enterica* ser. Enteritidis.

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INTRODUCTION

Non-typhoidal salmonellosis usually causes mild, self-limiting gastroenteritis [1]. Symptoms usually appear 12–36 h after eating and last up to 7 days. The disease can be severe and life-threatening in vulnerable

populations like children, the elderly and people with underlying comorbidities [2]. Salmonellosis ranks second after campylobacteriosis as the most commonly reported food-borne disease across the European Union (EU) [3, 4]. Despite a significant downward trend in *Salmonella* infections since 2008, a 15.3% increase in the overall EU notification rate was noted in 2014 compared with 2013, followed by a further 1.9% increase in 2015 [3, 4]. Most European cases of non-typhoidal salmonellosis are currently caused by *Salmonella enterica* serovar Enteritidis [5]. *S. enterica* ser. Enteritidis is the predominant serovar associated with *Salmonella* outbreaks [4]. In the EU, in 2015, *S. enterica* ser. Enteritidis was implicated in 63% of 953 food-borne outbreaks caused by *Salmonella*. A Europe-wide large outbreak of *S. enterica* ser. Enteritidis PT8, with multiple locus variable-number tandem repeat analysis (MLVA) profiles 2-9-7-3-2 and 2-9-6-3-2, occurred from May 2016 to February 2017 [5].

In Greece, the mean annual salmonellosis notification rate for the period 2008–2015 was 4.1 cases per 100 000 population, with *S. enterica* ser. Enteritidis being the most common serotype, dropping yearly from 63% of total isolates in 2008 to 24% in 2015 [6]. The decreasing trend of *S. enterica* ser. Enteritidis cases was mainly attributed to the Greek National *Salmonella* Control Programme implemented in poultry in accordance with the EU legislation [7]. At the same period, only small, household, outbreaks of *S. enterica* ser. Enteritidis were identified in the country; the last documented community outbreak was in 2011 (Hellenic Centre for Disease Control and Prevention, data not published).

On 19 June 2016, hospital authorities in Central Greece informed public health authorities of the occurrence of several cases of gastroenteritis following a christening reception at a restaurant in a popular tourist village. A 57-year-old guest with no underlying condition died after being admitted to the hospital with severe gastroenteritis symptoms. Two otherwise healthy guests, aged 26 and 60, were admitted to the intensive care unit. As a consequence, the outbreak captured wide media and public attention. An outbreak control team (OCT) was dispatched to investigate the outbreak and identify the implicated food item, the causative pathogen and factors possibly contributing to disease severity. The OCT also investigated the possible association of the outbreak with the concurrent 2016/17 European outbreak.

METHODS

Epidemiological investigation

A retrospective cohort study was performed. Restaurant management provided a list of all food items served. Telephone interviews were conducted by the fellow of the European Public Health Microbiology Training Program of ECDC (EUPHEM) after special training, using a structured questionnaire. Data were obtained on demographics, the symptoms, the need for and the length of hospitalisation, and the time taken off from work. The presence of underlying conditions and the possible contact with ill persons unrelated to the christening party were recorded. To identify potential risk factors for illness, all guests were asked whether they had consumed any of the specified food items. Information on food portions and incubation period was also collected as they were considered to be proxy measures of infectious dose. Foods doses were categorised as ‘tasting portion’, ‘one portion’ or ‘more than one portion’. For food served on a common platter for four persons, serving size corresponded to about half of a standard portion size. Incubation periods were taken as the time (in hours) from serving the course until the onset of symptoms. After reviewing descriptive data, case was defined as a christening guest who had diarrhoea (≥ 3 loose stools in 24 h) within 72 h of the reception.

Statistical analysis

Categorical variables were compared using the χ^2 test. Continuous variables were expressed as median with the corresponding interquartile range, and were compared using the Mann–Whitney *U* test. *P*-values of <0.05 were considered statistically significant. The frequency and intensity of symptoms and association with outcomes were assessed. An epidemic curve was used to depict the length and case distribution of the outbreak. For each consumed food item, attack rate (AR), relative risk (RR) and 95% confidence interval (95% CI) were computed. All analyses were performed using Stata v.12.1 (StataCorp., College Station, Texas, USA).

Laboratory investigation

Stool samples were screened only for three bacterial pathogens, *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. The available identified *Salmonella* isolates were sent to the National Reference Centre for

Salmonella. Serotyping was performed according to the White–Kaufmann–Le Minor Scheme [8, 9]. Susceptibility testing was carried out using the disk diffusion method. Antimicrobial agents appropriate for monitoring of antibiotic resistance in human *Salmonella* isolates, according to the relevant EU protocol [10], were tested. The European Committee on Antimicrobial Susceptibility Testing breakpoints were applied [10]. Two outbreak isolates from patients who belonged to different families and were hospitalised at different hospitals, were phage-typed by the Ward–Colindale system [11]. Pulsed-field gel electrophoresis (PFGE) was performed after the digestion of genomic DNA with *XbaI* macrorestriction endonuclease, according to standard operating procedure [12]. Similarity and cluster analyses were performed using the Dice coefficient and the unweighted-pair group method with the use of average linkage, using BioNumerics software v.6.0 (Applied Maths, Belgium). PFGE profiles were uploaded to the European Molecular Surveillance System (TESSy-MSS), operated by the European Centre for Disease Prevention and Control, for matching with other characterised PFGE profiles at European level [13]. The isolates were submitted for MLVA analysis, following a laboratory standard operating procedure [14], based on the five-locus MLVA method proposed by Hopkins *et al.* [15]. Single-nucleotide polymorphism (SNP)-based whole genome sequencing (WGS) analysis was carried out by the PHE Genome Sequencing and Development Unit [16]. A hierarchical, ‘SNP address’, approach was used to assess the genetic distance between our isolates and those of the concurrent European outbreak [5, 16].

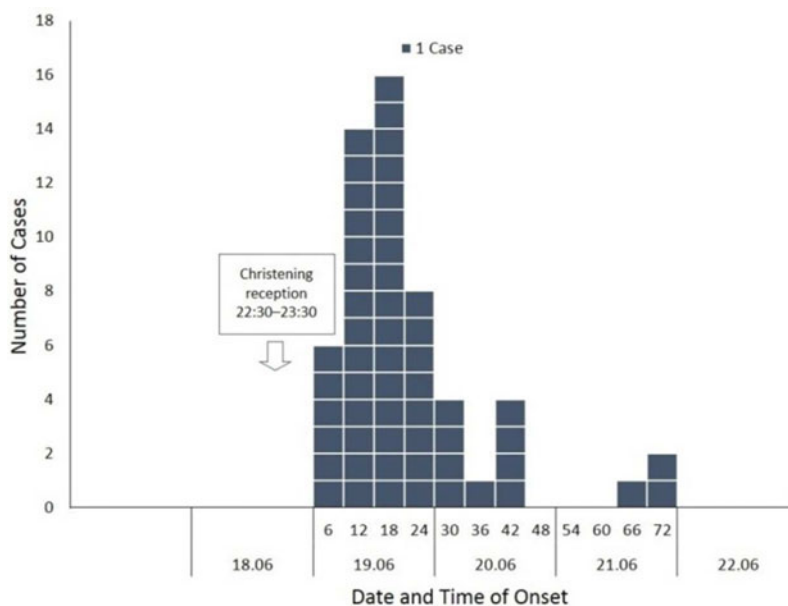
Environmental investigation

The OCT visited the restaurant where the christening event took place in order to collect information on preparation processes for the foods served. The order and delivery books of the restaurant were reviewed. Environmental swabs were taken from working benches. Food leftovers were no longer available; raw materials were collected for laboratory testing [17]. The OCT also attempted to trace raw materials back to the original suppliers. All the food handlers were interviewed regarding food handling practices and illness 1 week before and after the christening reception. They were also asked whether they had consumed foods served at the reception. Stool samples were obtained after verbal consent from all food handlers.

RESULTS

Epidemiological investigation

Of the 133 attendees, 122 (92%) individuals completed the questionnaire. Participants’ median age was 40·5 years (range 3–86); 70 (57%) participants were females. A total of 56 (46%) respondents met the case definition (median age, 43·5 years (range 3–86); 31 (55·4%) females). Of the 56 cases, 49 (87·5%) reported high body temperature (median, 39·2 °C; range 38·4–41), 37 (66%) abdominal pain and 22 (39·3%) vomiting, while 30 (54%) were hospitalised. Twenty-eight (50%) complained of intense abdominal pain. Eleven (50%) of those vomiting had at least four vomiting episodes per day (range 1–15). Half of those requiring hospitalisation stayed at the hospital for 5 days or more (range 1–10 days). Of the 42/56 (75%) working patients, 21 (50%) reported absenteeism from work, with one out of two being off work for ≥ 7 days (range 1–15 days). Age and sex did not significantly differ between cases and non-cases and were not associated with any of the symptoms and outcomes. Thirty-six cases were previously healthy adults. Eight cases had an underlying condition; cardiovascular disease ($n = 4$); diabetes ($n = 3$); hypothyroidism ($n = 1$). One patient reported a previous upper gastrointestinal tract surgery ($n = 1$). Their clinical presentation and outcome did not differ from those of other cases. None reported achlorhydria or prior use of antacids or acid reducers. Affected individuals had no contact with sick people unrelated to the reception. The shape of the epidemic curve is suggestive of a common point-source outbreak (Graph 1). The median incubation period was 15 h (range 6–71). Twenty of the 56 (35·7%) patients developed diarrhoeal illness in <12 h from serving the course (Table 1). This group of affected guests passed ≥ 7 loose stools in a day, had the highest proportion of fever and vomiting with more than four vomit episodes a day; and had the highest hospitalisation rate lasting >6 days. They also had symptoms of long duration (≥ 8 days; $n = 11$, 55%). Results of the univariate analysis are displayed in Table 2. A RR of 7·8; 95% CI 3·6–16·8 was associated with having eaten cheesy penne pasta at the christening reception. Of the 63 people who ate cheesy penne pasta, 50 fell ill (AR = 79·4%). As cheesy penne pasta was served on a common platter for four persons, half of a standard portion was allocated for each guest. Most (32/50; 64%) affected guests, who had eaten cheesy penne pasta, reported that they had eaten less than half-portion.



Graph 1. Interviewed guests ($n = 56$) who developed diarrhoea (≥ 3 loose stools in 24 hours) within 72 hours following a christening reception in Central Greece, June 2016, by date and six-hour time intervals of onset of illness.

No relationship between symptoms and outcomes with consumed portion of implicated food was shown. One case was reported to have exclusively consumed cheesy penne pasta served at the christening reception.

Laboratory investigation

Stool cultures from 17/56 (30.4%) cases were positive for *Salmonella* spp. Seven isolates were sent for further typing, and were identified as *S. enterica* ser. Enteritidis. The isolates were found to be fully susceptible to the tested antibiotics. Two isolates were typed as phage-type PT8, with PFGE profile XbaI.0024 and MLVA profile 2-9-7-3-2. The isolates shared the t5-level SNP address 1.2.3.323.323.24095628.% (with %-sign substituting any number standing for 0-SNP level) differing from each of the two European outbreak's WGS clusters by 175 (SNP address 1.2.3.175.175.175.%) and 360 (SNP address 1.2.3.18.359.360.%).

Other restaurant consumers with *S. enterica* ser. Enteritidis infections

The food handler that was tested positive for *S. enterica* ser. Enteritidis reported tasting food in the kitchen during the reception (penne pasta) and that he developed symptoms in the following days. It was also found that two persons with laboratory-confirmed *S.*

enterica ser. Enteritidis infection had consumed cheesy penne pasta served at the reception despite not attending the event themselves (family members of one of the food handlers). Finally, stool cultures from four clients who fell ill after they had eaten at the restaurant after the reception were positive for *S. enterica* ser. Enteritidis. Data regarding cases other than those among the reception guests are summarised in [Table 3](#).

Environmental investigation

The restaurant owners had voluntarily closed down their restaurant before the inspection took place. Prior to the arrival of investigators the café kitchen had been cleaned and most perishable goods had been discarded. All environmental and raw materials tested negative for *Salmonella*. Stool cultures from six out of seven food handlers were also negative for *Salmonella*. The remaining food handler, who reported diarrhoea after the event, was diagnosed with *Salmonella* infection. The food handler reported tasting food in the kitchen during the reception. The ingredients of the incriminated dish were penne pasta, yellow hard cheese, semi-hard cheese, cured meat, three packets of crème fraîche, six egg yolks, pepper and salt. The pasta was boiled and cheese was cut at noon. Afterwards, the ingredients were put into the refrigerator. Food was baked at some point in the afternoon. The dish was kept at room

Table 1. Range of symptoms and outcomes sorted by the length of incubation period among 56 guests falling ill within 72 h after attending a christening reception in Central Greece, June 2016

Symptom	Incubation period (hours)		
	≤12 Cases (n = 20/56; 35.7%) Number (%)	>12 to ≤18 Cases (n = 16/56; 28.6%) Number (%)	>18 Cases (n = 20/56; 35.7%) Number (%)
Diarrhoea	20 (35.7)	16 (28.6)	20 (35.7)
High body temperature (>38.3 °C)	18 (32.1)	15 (26.8)	16 (28.6)
Malaise/fatigue	18 (32.1)	12 (21.4)	14 (25.0)
Abdominal pain	13 (23.2)	12 (21.4)	12 (21.4)
Dehydration	15 (26.8)	10 (17.9)	10 (17.9)
Vomiting	16 (28.6)	5 (8.9)	1 (1.8)
Dizziness/loss of consciousness	7 (12.5)	1 (1.8)	4 (7.1)
Outcome	Number (%)	Number (%)	Number (%)
Hospitalisation	17 (30.4)	6 (10.7)	7 (12.5)
Taking time off from work ^a	8 (19.0)	10 (23.8)	3 (7.2)

^a The results refer to 42 cases of working age.

Table 2. Food-specific attack rate and relative risk of developing diarrhoea by food item among 122 persons who attended a christening reception in Central Greece, June 2016

Consumption of specified food item	Exposed			Unexposed			RR	95% CI
	Cases	Total	AR %	Cases	Total	AR %		
Savoury pie with cheese	47	95	49.5	7	24	29.2	1.69	0.88–3.27
Savoury pie with spinach	34	68	50.0	21	53	39.6	1.26	0.84–1.90
Aubergine with minced meat and béchamel	46	91	50.5	9	30	30.0	1.68	0.94–3.02
Vineyard leaves filled with rice	35	76	46.1	20	45	44.4	1.04	0.69–1.56
Burger	46	93	49.5	9	28	32.1	1.54	0.86–2.74
Stew meat	24	45	53.3	30	75	40.0	1.33	0.90–1.97
Cheesy penne pasta	50	63	79.4	6	59	10.2	7.80	3.62–16.8
Greek salad	17	37	45.9	38	84	45.2	1.02	0.67–1.55
Green salad	17	32	53.1	38	89	42.7	1.24	0.83–1.87
Feta cheese	20	35	57.1	34	84	40.5	1.41	0.96–2.08
Dip with cheese and pepper	22	40	55.0	33	81	40.7	1.35	0.92–1.98
Oven-roasted potatoes	17	30	56.7	35	88	39.8	1.42	0.95–2.14
Biscuit cake	24	60	40.0	32	60	53.3	0.75	0.51–1.11
Cheesecake	24	44	54.5	32	73	43.8	0.95	0.64–1.42
Chocolate pastry	24	43	55.8	35	81	43.2	1.24	0.86–1.81
Homemade cake (lemon pie)	5	13	38.5	46	98	46.9	0.82	0.40–1.68
Chocolate fondue fountain	4	11	36.4	49	105	46.7	0.78	0.35–1.75

Food specific attack rates (AR), relative risks (RR) and 95% confidence intervals (95% CI) were calculated.

temperature for more than 2 h before being served after the completion of the first course. It has to be mentioned that the outbreak occurred during a heat wave in Greece. The temperature at the day of the reception ranged between 25.7 and 39.5 °C. Inspectors recorded the lack of a Hazard Analysis and Critical Control Point system and of standard procedures inside the kitchen for avoiding cross-contamination.

Raw and cooked foods were not adequately separated. The oven's heating performance was not tested; no registry data were kept on refrigerator temperature. The origin of the eggs used to prepare the cheesy penne pasta was also investigated. An inspection of the implicated hatchery and packing station was initiated. Samples taken were negative for *S. enterica* spp.

Table 3. Other restaurant consumers with *Salmonella enterica* serovar Enteritidis in Central Greece, June 2016

Cases	#	Sex	Age (years)	Date of eating	Date of onset	Consumed food	Hospitalisation			Underlying condition
							Required	Duration (days)	Time off from work (days)	
							(days)	(days)	(days)	
Food handler	M	36	18-06	-	Cheesy penne pasta	N	-	-	N	
Non-attendees (consuming food served at the reception)	#1 M	8	19-06	20-06	Cheesy penne pasta	Y	3	3	N	
Regular consumers (falling ill after eating at the restaurant)	#1 M	50	19-06 ^a	19-06 ^b	Oven-roasted potatoes and tortellini à la crème	Y	7	10	Y ^c	
	#2 F	32	19-06	20-06	Silverside beef with rice (without cheese)	Y	8	12	N	
	#3 M	4	20-06 ^a	21-06	Pasta with minced meat and cheese/tortellini à la crème	Y	6	-	N	
	#4 M	1	20-06 ^a	21-06	Pasta with minced meat and cheese/tortellini à la crème	Y	3	-	N	

^a At 12:00 h.

^b At 23:00 h.

^c Previous lower gastrointestinal tract surgery.

M, male; F, female; Y, yes; N, no.

Measures taken

The local public health authorities suspended the license of the restaurant for 4 months. The suspension was lifted after a thorough review of the restaurant's food safety management system and the implementation of improvements to enhance processes associated with reduction in the potential for food contamination (focus at monitoring and recording of processes and temperatures, heat treatment and preservation of food). Furthermore, all employees were re-trained on the basic concepts of Hazard Analysis and Critical Control Point (HACCP) and their compliance was further assessed by the local public health authority. The enhancement of the national *Salmonella* molecular surveillance was also decided; MLVA typing will be introduced in the routine work of the National Reference for salmonellosis.

DISCUSSION

A serious *S. enterica* ser. Enteritidis PT8, MLVA profile 2-9-7-3-2 outbreak occurred in Greece in 2016, despite a recorded decreasing trend of cases in the previous years and the absence of recorded large outbreaks due to this serotype in the country since 2011. Remarkable morbidity was associated with this outbreak, even though non-typhoidal salmonellosis is generally mild. High rates of severe symptoms and hospital admission were reported. Patients suffered from severe gastrointestinal symptoms such as profuse diarrhoea, fever and frequent vomiting episodes demanding prolonged hospitalisation and sick leave from work. One death was associated with this outbreak. Another interesting feature was that the majority of affected guests experienced serious gastrointestinal illness within <12 h of attending the reception. There has been an inconclusive debate about the infectious dose and illness severity of non-typhoidal *Salmonella* [18–20]. In this outbreak, the small amount of consumed cheesy penne pasta did not allow us to assess the dose–response relationship. It is possible that bacteria may have survived inadequate heat processing and high-fat cheesy penne pasta may have protected *Salmonella* cells – encapsulated in fat – against stomach acidity [21]. Another possible explanation is that leaving this perishable food out of the refrigerator on a warm summer evening allowed bacterial multiplication in the likely vehicle of infection [22]. The short incubation period may be attributable to very high infectious dose and/

or co-infection with toxin-producing bacteria (e.g. *Staphylococcus aureus*), as described before [23]; their isolation though was not feasible in Greek local hospitals at that point of time. The severity of *Salmonella*-associated disease may additionally depend on host factors as well as the specific strain of *Salmonella* [24]. Regarding host status in the current outbreak, more than 60% of the cases were otherwise healthy adults. Over the last two decades, only a limited number of serious *S. enterica* ser. Enteritidis outbreaks affecting mainly non-high-risk adults have been published [19, 20, 25–28]. The fact that severe *S. enterica* ser. Enteritidis outbreaks are infrequent is perhaps in line with the serovar's weak human invasiveness in high-income countries [29]. Finally, the outbreak-associated isolates were found to belong to phage-type PT8.

Interestingly, despite lacking correlation between clonal lineage and virulence within the serovar, PT8 has been suggested to be one of the high virulent *S. enterica* ser. Enteritidis phage types [30, 31]. Our finding that the isolates of the present outbreak were identified as PT8 and produced the PFGE profile XbaI.0024 according to TESSy-MSS (corresponding to PFGE profile SENTXB.0002 according to the former PulseNet Europe network for food-borne infections in Europe) is consistent with studies showing a strong association between PT8 and SENTXB.0002 [32]. Phage-type PT8 has been one of the most frequently observed phage types in Eastern Europe since the 1990s [26, 33–37].

In 2011–2015, PT8 was the predominant phage type in human cases in 13 European countries [5]; no such data were available for Greek cases. In 2016, a gastroenteritis outbreak occurred amid a *S. enterica* ser. Enteritidis PT8 outbreak affecting different countries in Europe [5]. The MLVA profile of our PT8 isolates was indistinguishable from the MLVA profile 2-9-7-3-2 of the multi-country outbreak [5]. SNP-based WGS analysis, however, revealed that our outbreak did not belong to either of the two WGS clusters associated with the 2016/17 European outbreak despite their temporal relatedness.

Information on the factors that contributed to the occurrence of the outbreak involved the high ambient temperature at the time of the outbreak and the lack of standard procedures inside the kitchen for avoiding cross-contamination. The fact that four people fell ill after they had eaten at the restaurant the days following the reception is supportive of the hypothesis that probably cross-contamination occurred inside the

kitchen during the preparation for the reception. Even though cross-contamination after cooking is the most probable cause of the outbreak, the exact point that cross-contamination occurred remained unclear. The literature shows that this is a common limitation of the investigation of outbreaks at restaurant premises [38]. For this reason, environmental investigation of similar outbreaks should be more detailed, and inspection should be scheduled as soon as possible after the establishment being linked with an outbreak, ideally within a day. It is also recommended that the authorities conduct multiple establishment visits if needed to complete the environmental assessment and monitor poor handling practices. In this case due to the media attention, the restaurant owners had voluntarily closed down their restaurant before the inspection took place and investigators were restricted in their ability to hypothesise and test potential means of cross-contamination during the preparation of the incriminated meal. That most Greek local hospitals lacked the capacity to screen stools for other food-borne pathogens with very short incubation periods (at least shorter than that of *Salmonella*), such as *S. aureus*, constitutes one more limitation, since co-infection cannot be excluded. Finally, the current study is limited by the fact that no leftovers were available for microbiological testing and identification of the food vehicle.

CONCLUSION

In the aftermath of the herein described recent *S. enterica* ser. Enteritidis outbreak associated with high morbidity, after five consecutive years of no outbreak documentation, the occurrence of *S. enterica* ser. Enteritidis outbreaks remains a public health threat in Greece. Thus, despite the progress in *Salmonella* control, there can be no complacency; it is vitally critical to maintain and strengthen the continued implementation of the National *Salmonella* Control Programmes and to intensify efforts to (re) educate food handlers in the basic principles of food management and hygiene to prevent such outbreaks from occurring. A more detailed environmental investigation is needed in case of similar outbreaks so that the factors led to cross-contamination inside the kitchen be identified and the appropriate correction measures be taken. Advanced molecular data need to be incorporated into the current public health surveillance systems in Greece to help us distinguish unrelated outbreak strains and identify emerging clones.

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DECLARATION OF INTEREST

None.

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