

Investigation of a staphylococcal food poisoning outbreak combining case–control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014

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In June 2014, a staphylococcal food poisoning outbreak occurred at an international equine sports event in Luxembourg requiring the hospitalisation of 31 persons. We conducted a microbiological investigation of patients and buffet items, a case–control study and a carriage study of catering staff. Isolates of *Staphylococcus aureus* from patients, food and catering staff were characterised and compared using traditional typing methods and whole genome sequencing. Genotypically identical strains (sequence type ST8, *spa*-type to24, MLVA-type 4698, enterotoxin A FRI100) were isolated in 10 patients, shiitake mushrooms, cured ham, and in three members of staff. The case–control study strongly suggested pasta salad with pesto as the vehicle of infection ($p < 0.001$), but this food item could not be tested, because there were no leftovers. Additional enterotoxigenic strains genetically unrelated to the outbreak strain were found in four members of staff. Non-enterotoxigenic strains with livestock-associated sequence type ST398 were isolated from three food items and two members of staff. The main cause of the outbreak is likely to have been not maintaining the cold chain after food preparation. Whole genome sequencing resulted in phylogenetic clustering which concurred with traditional typing while simultaneously characterising virulence and resistance traits.

Introduction

Food poisoning caused by enterotoxigenic *Staphylococcus aureus* is one of the most common foodborne diseases [1]. In France, which has a long-established foodborne disease surveillance system able to detect fairly rare events [2], staphylococcal food poisoning (SFP) has ranked in recent years as the first cause of foodborne outbreaks: of 1,288 reported foodborne outbreaks in

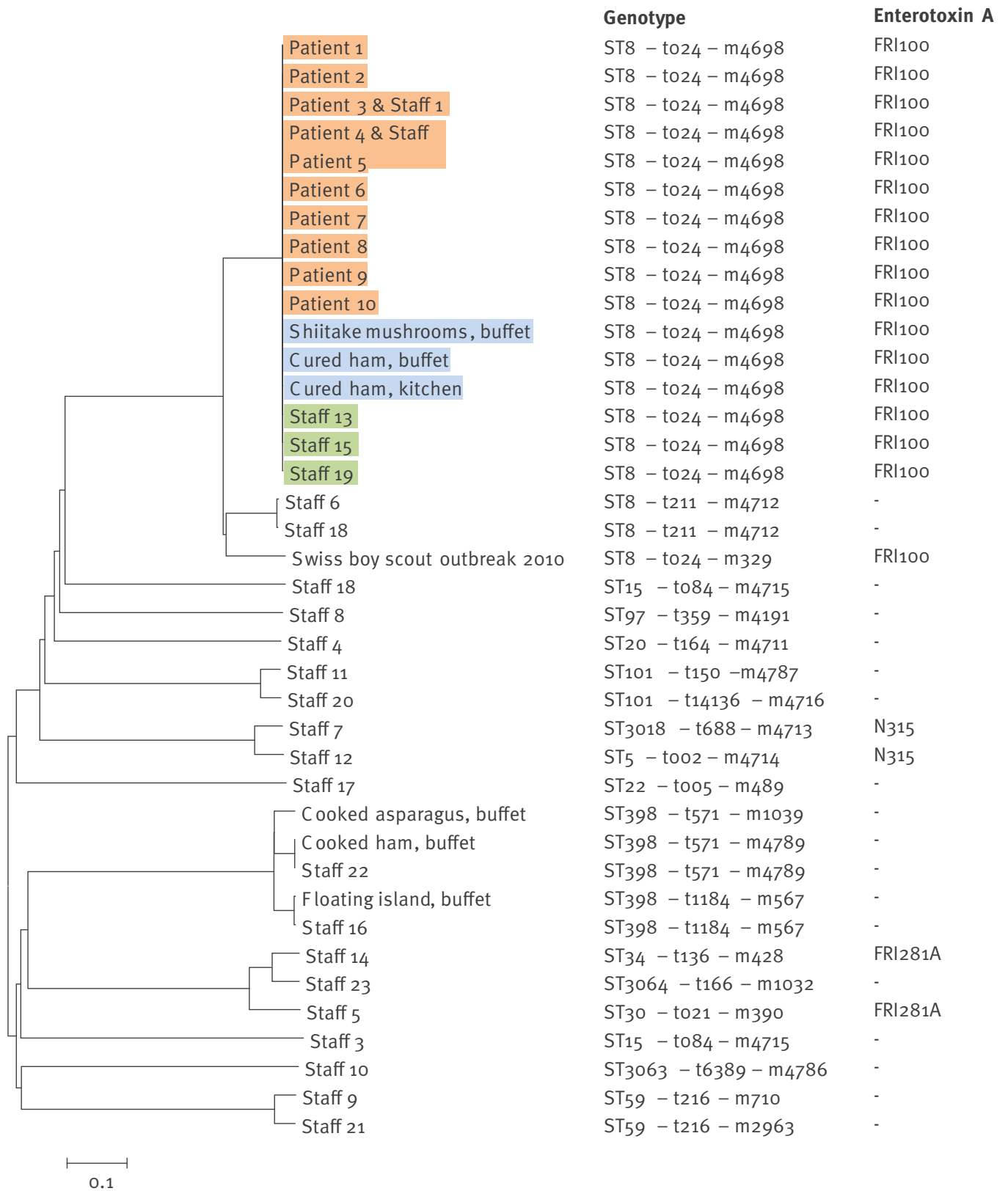
2012, 300 (23%) were due to SFP [3]. SFPs are thought to be under-reported for several reasons. First, because of the short duration of symptoms, only an estimated 10% of SFP patients visit a hospital [4]. Even if patients seek medical care, the physician often does not deem a stool analysis necessary. If a stool analysis is performed, the microbiological routine procedures often do not include testing for the presence of enterotoxigenic *S. aureus* unless specifically requested by the physician [5]. In addition, staphylococcal enterotoxin (SE) is highly stable and heat-resistant. Although the bacteria may have been inactivated by heating the food prior to consumption and can therefore be isolated neither from food nor the stool of the patient, the highly stable enterotoxins performed by *S. aureus* in the food may still be emetically active [6].

In contrast to most other gastrointestinal infections, the onset of SFP symptoms is very rapid, usually within a few hours after ingestion of the contaminated food. The median incubation period of aetiologically confirmed SFP outbreaks occurring in the United States between 1998 and 2008 was estimated to be four hours (5–95 percentile: two to seven hours) [7]. Symptoms in cases in these outbreaks typically included abdominal cramps (72%), vomiting (87%), and diarrhoea (89%). Fever (9%) was infrequently reported. The median duration of illness was 15 hours (5–95 percentile: 4–60 hours) [7].

Here, we report a SFP outbreak that occurred in a buffet restaurant at an international show-jumping event in Luxembourg in June 2014. A total of 31 persons had to be transferred by ambulance from the event site to emergency departments of three local hospitals. We describe findings of the ensuing epidemiological case–control study, the microbiological contamination

FIGURE

Clonal relationship between patient, food, and catering staff isolates, staphylococcal food poisoning outbreak, Luxembourg, 12–13 June 2014



A phylogenetic dendrogram (neighbour joining tree) was generated for 39 *Staphylococcus aureus* isolates based on the allelic profiles of 1,625 available of 1,878 queried MLST+ target genes. The scale bars indicate the number of differing alleles comprising the calculated distance. The colours represent the origin of outbreak-related strains (orange: stool samples from hospitalised patients; blue: food samples; green: throat or nose samples from colonised staff members). The genotype column shows the combined data of multilocus sequence typing (prefix ST), *spa*-typing (prefix t), and MLVA typing (prefix m).

TABLE

Results from analytical case-control study of food exposures, staphylococcal food poisoning outbreak, Luxembourg, June 2014

Food item at VIP buffet	Cases N (%)	Controls N (%)	Odds ratio	p value
Cooked ham	10 (45%)	6 (27%)	2.08 (0.50–9.04)	0.25
Cured ham	13 (59%)	10 (45%)	1.59 (0.41–6.28)	0.45
Grilled shrimps	12 (55%)	8 (36%)	1.95 (0.49–7.84)	0.28
Lamb	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Melon	13 (59%)	12 (55%)	1.08 (0.27–4.30)	0.90
Panna cotta	13 (59%)	9 (41%)	1.93 (0.49–7.68)	0.29
Pesto pasta salad	18 (82%)	3 (14%)	27 (4.35–195.43)	<0.0001
Potato gratin	15 (68%)	13 (59%)	1.32 (0.31–5.59)	0.67
Potato salad	10 (45%)	11 (50%)	0.76 (0.19–2.94)	0.65
Prawns	3 (14%)	1 (5%)	3.16 (0.22–173.90)	0.32
Raw vegetables	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Risotto	12 (55%)	9 (41%)	1.6 (0.41–6.31)	0.44
Ruccola salad	10 (45%)	10 (45%)	0.92 (0.24–3.57)	0.89
Salmon tartare	3 (14%)	2 (9%)	1.5 (0.15–19.7)	0.67

of food samples, and colonisation by *S. aureus* of catering employees at the event. In particular, we characterised the *S. aureus* isolates from patients, food items obtained from the buffet, and food handlers using traditional typing methods (PCR, *spa*-typing, and multilocus variable-number tandem repeat analysis (MLVA)), as well as whole genome sequencing.

The event

From 12 to 15 June 2014, an equestrian show-jumping event with approximately 140 participating international athletes and 300 horses took place in Luxembourg. Approximately one to three hours after eating a buffet lunch in the tented VIP restaurant on 12 June, 11 persons with symptoms of vomiting, diarrhoea, and prostration were taken by ambulance to the emergency departments of two hospitals where they received parenteral fluids. The official health inspection service was informed immediately of the incident and microbiological analysis of stool samples from hospitalised patients was ordered. Official food safety inspectors proceeded immediately to take samples from remaining buffet items for microbiological analysis. An inspection of the professional caterer's onsite restaurant and offsite kitchen did not reveal any major food safety deficiencies as specified in regulation (EC) 852/2004. The next morning, on 13 June, local newspapers announced salmon tartare as a potential culprit. A few hours after having the buffet lunch in the VIP restaurant on 13 June, a further 20 persons fell ill with the same symptoms and were transferred by ambulance to hospital emergency departments. The event organiser stopped serving any prepared meals for the remainder of the event. On 14 and 15 June, there were no further reports of gastrointestinal illness related to the event. While approximately 150–200 persons were estimated to have consumed the buffet lunch in the VIP

restaurant on both days and a total of 31 persons were admitted to hospital emergency departments over the two days, the exact number of affected persons is unknown. There were no reports of illness among those people who ate at the other food-serving premises at the event: a non-VIP lunch buffet operated by the same caterer but with different menus, and a barbecue stall hosted by non-professional club members.

Methods

Microbiological examination of stool samples

Culture of stool samples for bacterial pathogens (including *Salmonella*, *Campylobacter* and verotoxinogenic *Escherichia coli*) conducted in three hospital laboratories revealed the presence of *S. aureus* in ten patients and *Enterococcus* in one patient. Isolates of *S. aureus* were immediately referred to the National Health Laboratory for further molecular characterisation.

Case-control study

Following their recovery from illness and after the food samples had been analysed, eight cases who had been admitted to emergency care were contacted by telephone to get initial information on potential food exposures. All food items and symptoms reported by cases were included in a final questionnaire administered by telephone to 22 cases and 21 controls. Cases were defined as persons with sudden gastrointestinal illness (at least one symptom: vomiting, diarrhoea, abdominal cramps or nausea) who had eaten buffet lunch at the VIP restaurant on 12 or 13 June. Controls were defined as persons who had eaten buffet lunch at the VIP restaurant on 12 or 13 June, without any gastrointestinal symptoms. Non-hospitalised cases and controls were contacted using information provided by the event organiser.

Testing of food samples

Food samples were tested by accredited methods for aerobic plate count, *E. coli*, coagulase-positive staphylococci (ISO 6888-2:1999), *Salmonella*, and *Bacillus cereus*. The salmon tartare and floating island dessert samples were additionally tested for *Listeria monocytogenes*.

Staphylococcal carriage study among caterer's employees

Following the detection of *S. aureus* in patients, a staphylococcal carriage study was conducted on 19 and 20 June among the caterer's employees who worked in the onsite restaurant or in the offsite kitchen where buffet items were prepared, including the slicing of ham. Catering employees screened included waiters, cooks, and other kitchen staff. Throat and nose swabs were taken by doctors and sent the same day to the laboratory where they were streaked onto selective Chapman media (reference 51053, BioMérieux, Marcy l'Etoile, France).

Characterisation of *S. aureus* isolates and whole genome sequencing

Isolates of *S. aureus* obtained from patients, food, and catering employees were confirmed by MALDI-TOF mass spectrometry (Bruker, Brussels, Belgium). Confirmed isolates of *S. aureus* were further characterised for the presence of *nuc*, *mecA*, toxic shock syndrome toxin 1 (TSST-1), and Pantone-Valentine leukocidine (PVL) [8] as well as genes coding for staphylococcal enterotoxins A (*sea*), B, C, D E, H, I, and J [9]. Isolates exhibiting *sea* were further characterised by sequencing the PCR products and compared to strains containing allelic *sea* variants FRI100, FRI287A, and N315. In addition, isolates were subjected to *spa*-typing [8] and MLVA typing [10]. Whole genome sequencing of isolates was performed on a Miseq Desktop Sequencer using the Nextera DNA sample preparation kit (Illumina, Eindhoven, The Netherlands) with an average coverage of 59 fold (range 27–140 fold). Antimicrobial resistance genes, virulence factors and multi-locus sequence types (MLST) were determined by submitting the raw read files to public webserver tools hosted by the Center for Genomic Epidemiology in Denmark [11–13]. After sequencing, whole genome MLST+ was conducted using the Seqsphere+ v2.3 pipeline (Ridom, Münster, Germany). Briefly, after trimming reads until the average quality was 30 in a window of 30 bases, the trimmed reads were mapped to the reference genome NC_002951.2 and the allelic profiles of 1,878 target genes were determined based on the MLST+ scheme developed previously [14]. Final phylogenetic analysis was based on 10 patient isolates, 6 food isolates, 22 carrier isolates of members of staff, and an isolate with *spa*-type to24 from an unrelated Swiss outbreak in 2010. The whole genome reads were deposited at the European Nucleotide Archive (study accession no. PRJEB7847). For the purpose of this study, we use the terminology 'genotype' to denote the combined typing results of MLST, *spa*- and MLVA typing.

Results

Fifteen (48%) of the 31 patients admitted by ambulance to emergency care were women. The median age was 35 years (range 14–58 years). Three patients were catering staff at the event. Six patients were non-residents of Luxembourg. Information on clinical symptoms was available for 22 of the patients and included diarrhoea (20 patients), nausea (19 patients), cramps (19 patients) and vomiting (17 patients). All admitted patients were discharged within 48 hours. All 10 isolates obtained from patients' stool samples represented the same genotype (MLST sequence type (ST)-8, *spa*-type to24, MLVA-type 4698), possessed genes encoding *sea* allele FRI100 and conferring penicillin resistance mediated by *blaZ*.

Results from the analytical epidemiological case–control study (Table) implicated consumption of pasta salad with pesto as the most likely vehicle of SFP. Eighteen of 22 cases reported eating this food item compared to 3 of 21 controls ($p < 0.0001$). All 14 interviewed cases who had been hospitalised reported eating the pasta salad with pesto. Unfortunately, there were no leftovers of the pasta salad with pesto when sampling was taking place and so this dish was not available for microbiological testing. Eating cured ham or salmon tartare were not statistically significant risk factors ($p = 0.45$). One interviewed patient reported not having eaten ham at the buffet for religious reasons.

Food samples

Isolates of *S. aureus* with a genotype identical to patient isolates (MLST ST-8, *spa*-type to24, MLVA-type 4698) were detected in cured ham samples (range < 40 – $5,200$ colony-forming units (CFU)/g) and shiitake mushrooms (< 40 CFU/g) sampled at the event site and in cured ham samples (enumeration range < 40 – 120 CFU/g) obtained at the offsite catering kitchen where the ham was sliced and stored (Figure). Non-enterotoxigenic isolates of *S. aureus* with a different genotype to patient isolates were found in cooked asparagus (< 40 CFU/g, MLST ST-398, *spa*-type t571, MLVA type 1039), the floating island dessert (< 40 CFU/g, MLST ST-398, *spa*-type t1184, MLVA-type 567) and several samples of cooked ham (range 50–320 CFU/g, MLST ST-398, *spa*-type t571, MLVA-type 4789). Unsliced complete legs of cured and cooked hams obtained from the supplying butcher were negative for *S. aureus*. All 18 food items sampled from the event buffet were negative for *Salmonella* and *E. coli*. One food item (cooked asparagus) was positive for presumptive *Bacillus cereus* (840 CFU/g).

The pasta salad with pesto could not be sampled during food inspection, as there were no leftovers from this dish. The primary ingredients used to make the pesto sauce for the pasta salad (fresh basil, hard cheese, and pine nuts) were all negative for *S. aureus*.

Staphylococcal carriage study

Thirty-eight of the 49 catering employees at the event were screened for nasal/throat carriage of *S. aureus*.

Median age of the screened employees was 32.5 years (range 17–50 years), and 11 were women. Twenty-two employees were found to be colonised by *S. aureus*: three staff members were colonised by strains identical to those found in patients (Figure). Another four employees were colonised by *S. aureus* isolates exhibiting *sea*, but a different genotype than the outbreak strain. None of the seven employees colonised by isolates exhibiting *sea* reported wounds or gastrointestinal disease prior to the event. Overall, 17 different genotypes were observed among the 22 colonised employees. None of the isolates in food, patients, or catering employees were meticillin-resistant or exhibited *pvl*.

Whole genome sequencing

The whole genome phylogeny (Figure), as determined by 1,625 of 1,878 MLST and MLST+ target genes that were present in all 39 isolates, clearly delineated the outbreak isolates. *S. aureus* isolates found in 10 patients were identical to those isolated from cured ham, shiitake mushrooms and from three catering employees. Interestingly, the Luxembourg outbreak strain had 347 allele differences with a strain that led to the intoxication of 27 boy scouts in Switzerland in 2010, although both strains share a common *spa*-type t024 [15]. Two of the three food isolates which differed from the outbreak strain were also observed among catering employees. These belonged to livestock-associated sequence type ST398 with *spa*-types t571 or t1184.

Discussion

Studies of foodborne outbreaks, in which enterotoxigenic isolates were detected in patients, food, and food handlers, are rare [16–18]. Our report shows that, even in the era of whole genome sequencing, public health investigations of foodborne outbreaks remain very dependent on classical case–control investigations for interpretation of events. Whereas initial microbiological typing results suggested cured ham as the main vehicle for the intoxication, the case–control study clearly identified the pasta salad with pesto as the most likely source, which was no longer available for microbiological testing.

In our outbreak, there was good evidence that the pathogen responsible for the outbreak was *S. aureus*, because identical enterotoxigenic strains of *S. aureus* with a common *spa*-type but rare MLVA type were recovered from the stools of 10 hospitalised cases. Because three catering employees were colonised by a strain with the same genotype, it is likely that at least one of them may represent the source of food contamination, either via manual contact or through respiratory secretions [19]. However, because catering employees were screened a week after the outbreak, it cannot also be ruled out that some staff members became colonised only during or after the event [20].

One of the probable factors contributing to the outbreak may have been the unusually hot weather for the season, with maximum temperatures ranging between 25 °C and 32 °C during the week preceding the event, compared with a historical average of 21 °C. The food safety inspection at the catering facility revealed that a fridge had stopped working properly a few days prior to the event, although the catering staff denied using this fridge to store any of the dishes. The pasta salad with pesto was reported to have been pre-cooked and sealed into plastic bags in 2 kg portions, and then cooled down in a fast refrigeration unit. Nevertheless, the fact that *S. aureus* was detected in several dishes including cured and cooked ham, at concentrations up to 5,200 CFU/g, suggests that the cold chain before or during the event was interrupted to allow sufficient microbial growth during or following food manipulation.

A major limitation of our study is that the food item identified by the case–control study, pasta salad with pesto, was no longer available for testing and thus there is no microbiological evidence that the pasta salad with pesto was contaminated with the outbreak strain. However, matrices with similar biochemical properties like potato salad have been confirmed before as vehicles of SFP in France [21] and Switzerland [15]. In the latter case, a strain with identical *spa* type t024 and enterotoxin A FRI100 allele led to the intoxication of 27 boy scouts. The *sea* gene found in our outbreak strain is the dominant *sea* allele described in *S. aureus* isolates that are associated with food poisoning outbreaks worldwide [19,21–23] and in enterotoxigenic isolates recovered from food handlers [24].

The epidemiological results from our carriage study are consistent with previous findings in similar studies. Our finding of 58% carriers among food handlers concurs with longitudinal studies showing that approximately 20% of persons are persistent nasal carriers and an additional 30% are intermittent carriers of *S. aureus* [25]. The high genetic diversity among asymptomatic carriers was also observed in similar studies in Germany [26], Switzerland [27], and Bosnia [28]. Interestingly, we found meticillin-susceptible livestock-associated strains with ST398 *spa* type t571 and variants thereof in both catering employees and in food. Similar clones have recently emerged causing severe infections in neighbouring France and Belgium [29,30], while remaining rare in Germany [31].

Although WGS has been applied to meticillin-resistant *S. aureus* in hospital and long-term care settings [32–34] and to other foodborne pathogens [35,36], to our knowledge our study is the first to report WGS as a tool in a staphylococcal food poisoning outbreak. While WGS showed virtually identical groupings to MLVA, one major advantage of WGS is that it is a universal method applicable to any bacterial species and that it provides further data on the presence of genes encoding virulence and resistance factors.

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Conflict of interest

None declared.

Authors' contributions

JM coordinated the various investigations, collated strains from different sources, constructed phylogenies, conducted the statistical analysis for the case-control study, and wrote the manuscript; FD conducted the classical genotyping including MLVA, spa typing and virulence factor detection by PCR; GM was responsible for the laboratory analysis of food items; CR and CO conducted the whole genome sequencing; CO assisted with bioinformatics and with preparing the figure; SJ provided reference material and assisted with interpretation; MP was responsible for the microbiological analysis of human strains; PH led the food inspection; PW was responsible for the public health response and the case-control data collection.

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