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A foodborne outbreak caused by staphylococcal enterotoxins in cheese sandwiches in northern Italy

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SHORT COMMUNICATION

Abstract

Staphylococcus aureus can produce numerous toxins, including staphylococcal enterotoxins, namely, SEs, SEA to SEE, SEG to SEI, and SER to SET. They have demonstrated emetic activity and can cause food poisoning outbreaks (FBOs). Here, we report a multidisciplinary investigation conducted by authorities competent in food safety in collaboration with veterinary and health and hygiene services. The issue was foodborne intoxication which involved eight members (aged 12–74 years) of the same family. Notification reported that time to symptom onset was about 3 hours after lunch, which suggests that the food contained preformed toxins. Ham and cheese used for the preparation of sandwiches consumed by all symptomatic relatives were suspected to be the source of FBO. The day after symptoms appeared, samples were collected at the restaurant and delivered to the food safety laboratory. All analyses performed on official food samples are validated according to ISO 17025:2017 and accredited. The cheese subsamples were all positive for coagulase-positive staphylococci CPS (range: $1.1*10^3$ CFU/g to $8.1*10^3$ CFU/g). The pooled sample tested positive for staphylococcal enterotoxin D (SED) at 0.649 ng/g. This concentration can cause symptoms of FBO. Following the notification of suspected food poisoning, a rapid response by the district food safety agency is necessary. In the case described here, the multidisciplinary collaboration facilitated the collection of samples at the food business plant aimed at identifying a suspected source of intoxication and to withdraw unsafe food from the market.

Keywords: foodborne illness; staphylococcal enterotoxins; dairy products; food safety

Introduction

Staphylococcus aureus is a gram-positive, facultative, anaerobic, nonmotile, nonsporing, catalase, and coagulase-positive bacteria (Decastelli *et al.,* 2023). This commensal and opportunistic pathogen can cause a wide range of infections, from superficial skin infections to severe and potentially fatal invasive disease (Kadariya *et al.,* 2014). It produces numerous toxins, including staphylococcal enterotoxins (SEs), specifically named SEA to SEE, SEG to SEI, and SER to SET, with demonstrated emetic activity. The enterotoxins are among the most common foodborne causative agents of food poisoning worldwide (Balaban and Rasooly, 2000; Hennekinne, 2012). SEs are a major cause of food poisoning, which typically occurs after ingestion of foods, particularly processed meat and dairy products, contaminated with *S. aureus* due to improper handling and

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inadequate storage at elevated temperature (Argudín *et al.*, 2010). Staphylococcal food poisoning is usually associated with the consumption of protein-rich processed foods, with neutral pH and without high background microflora (e.g. meat preparations, creams, and dairy products) (De Buyser *et al.,* 2001). These food items are vehicles of amino acids and low-molecular-weight peptides that support the survival and growth of *S. aureus* (Peles *et al*., 2007).

In general, bacterial toxins are among the most frequent causes of foodborne outbreaks (FBOs) in western countries. The European Food Safety Authority (EFSA) (The European Union, 2022) reported that in 2021 FBOs were caused by bacteria (28.5%), bacterial toxins (17%), viruses (6.8%), parasites, and other causative agents (2%).

Toxigenic *S. aureus* strains can synthesize SEs during the logarithmic phase or during the transition from the exponential to the stationary phase.

The molecular weight of SEs ranges between 20 and 30 kDa. Their mechanism seems to disrupt intestinal activity and induce food poisoning, with nausea, vomiting, abdominal pain, and diarrhea, but usually without fever or hypotension (Castro *et al.,* 2018; Otto, 2014; Hu *et al.,* 2021). Based on their antigenic heterogeneity, more than 20 SEs (SEA–SElV) have been identified. Of the 24 SEs reported in literature, five (SEA, SEB, SEC, SED, SEE, the so-called classic enterotoxins) are well described and can be detected by commercially available assays or in-house methods (Nia *et al.,* 2016; Grispoldi *et al.,* 2021).

The effective dose of SEs causing symptoms has not been defined in humans: estimates range from 6.1 ng SEA (Guillier *et al.,* 2016) to 0.1 μg (Le Loir *et al.,* 2003*;* Asao *et al.*, 2003). In monkeys, all SEs can induce an emetic reaction at a dose of 100 μg/kg (10 μg/kg for SEA) (Omoe *et al*., 2013).

Regulation (EC) 2073/2005 regulates food microbiological safety in Europe (EU, 2005) based on microbiological criteria for foodstuff. The Regulation expounds the criteria for food safety and process hygiene. If test results for either type of criteria are unsatisfactory, food businesses must take specific action in compliance with the Regulation. For food categories, such as cheese, milk, and whey powder, SEs are listed under food safety criteria applicable to products placed on the market during their shelf life; SEs must not be detectable in 25 g.

Coagulase-positive staphylococci (CPS) are listed under the criterion of process hygiene for a variety of dairy products (made from raw milk, ripened cheese, unripened soft cheese, etc.), with limits ranging from 10 CFU/g to 10^5 CFU/g depending on the type of heat treatment that they have undergone. In any case, if the number of CPS is $>10^5$ CFU/g, the cheese batch is to be tested for SEs.

An FBO is suspected when two or more cases of a similar disease seek medical attention at a hospital or from their family physician after ingestion of a common food. The Foodborne Disease Outbreaks Guidelines for Investigation and Control (WHO, 2008) specify the actions to be undertaken in the event of an FBO, as well as delineate the roles, objectives, operative procedures, and management of investigations for communicating information that the competent agencies can and must release.

Here, we report a multidisciplinary investigation that food competent authorities conducted in collaboration with veterinary and health and hygiene service, after a notification of suspected symptoms in consumers of a common meal.

Materials and Methods

Case Presentation

A family (two grandparents, two daughters, four young grandchildren) were having a short vacation in the Alps in Piedmont, northern Italy, in the summer of 2022. On 9 August, four of them presented with gastrointestinal symptoms (vomiting and diarrhea) or headache at around 6.30 p.m. No fever was reported by any of the patients (Table 1). On this day, the family had breakfast at their place of stay and lunch at a restaurant during a mountain hike (Table 2). The family received medical attention for symptoms of suspected food poisoning. Food safety competent authority inspectors were notified for collecting food samples. Due to self-limiting illness, biological fluid samples were not collected.

Food Sample Collection

On 10 August, food safety agency inspectors were asked to make an inspection in a small restaurant on the Alps where the family (eight members) had lunch the day before and after which one adult and three children showed gastrointestinal symptoms. The food inspectors collected food samples at the cafeteria where the family consumed sandwiches. Five portions of ham and five portions of raw milk cheese, both used for preparing sandwiches, were collected with sterile devices (scissor, laboratory plastic bags, cutter, and scalpel) and transported refrigerated to the Food Safety and Quality Laboratory, Istituto Zooprofilattico Sperimentale del Piemonte Liguria and Valle d'Aosta, Turin. The food

Table 1. Members of the family involved in the notifications and symptoms they exhibit.

Family member				Symptoms				
		Date of birth	Gender	Diarrhea	Vomiting	Cramps	Nausea	Headache
1	Grandfather	30/12/1948	M	x	x	x	x	
2	Grandmother	n.r.	F					
3	Daughter A	n.r.	F					
4	Daughter B	n.r.	F					
5	Granddaughter	n.r.	F					
6	Grandson	29/10/2005	M	x	x	x	x	x
7	Grandson	17/02/2011	M				x	
8	Granddaughter	20/01/2012	F	x	x	x	x	

NR. data not reported in the epidemiological questionnaire.

Table 2. Food composition of meals consumed before the onset of symptoms.

Consumer	Breakfast				Lunch			
	Coffee	Sponge cake	Ham	Cheese	Ham and cheese sandwich	Tomato and cheese sandwich	Orange juice	Chocolate pudding
1	x	x	x	x	x			
2	x	x	x	x				
3	x	x	x	x				x
4	x	x	x	x			x	
5	x	x	x	x	x			
6	x	x	x	x	x	x		
7	x	x	x	x	x	x		
8	x	x	x	x	x			

business operators were informed that a microbiological investigation for suspected food poisoning was being conducted; they were summoned to appear in person or send their legal representative to the laboratory at 11 a.m. on 11 August. From the moment of collection to the moment of analysis, samples were always kept refrigerated at 4±2 °C for 24 h.

In the present FBO, the district food safety agency conducting the investigation requested the laboratory to test for microbial pathogens with longer incubation times and hygiene indicator microbes. This was done to exclude other pathogens or causative agents and to provide the health inspectors with information about the level of hygiene at the food business where the cheese sandwiches were prepared.

Food Sample Analysis

The Food Safety and Quality Laboratory is accredited according to ISO17025:2017; all analyses performed on official food samples are validated and accredited. The laboratory was requested by the food safety agency to conduct microbiological analysis to detect bacterial pathogens and bacterial toxins. Table 3 presents the potential causative agents and the laboratory method applied.

Confirmatory quantitative analysis for quantification of SEs was performed at the Anses Maison-Alfort Laboratory for Food Safety European Union Reference Laboratory for CPS, including *S. aureus* (EURL-CPS).

Results and Conclusion

Laboratory analyses were performed on two food matrices (ham and cheese) collected at the restaurant and used for the preparation of lunch sandwiches. Each sample was divided into five subsamples. Laboratory analysis was negative for *Listeria monocytogenes*, *Salmonella* spp, and *Campylobacter* spp. Table 4 presents the results of testing for hygienic criteria and enumeration of food contaminant bacteria if >10 CFU/g. The cheese subsamples were all positive for CPS (range, $1.1*10^3$ CFU/g to

Table 3. Laboratory methods applied to food samples.

Causative Agent	Purpose	Method	Reference
Listeria monocytogenes	Detection	Real-time PCR	AFNOR BRD 07/10 – 04/05
L. monocytogenes	Enumeration	Colony count	ISO 11290-2:2017 ISO 11290-2 (2017)
Salmonella spp.	Detection	Real-time PCR	ISO 6579-1:2020 UNI EN ISO 6579-1 (2017)
Anaerobe enumeration	Enumeration	Colony count	ISO 15213:2003 ISO, 15213 (2003)
Campylobacter spp.	Enumeration	Colony count	ISO 10272-2:2017 ISO 10272-2 (2017)
Coagulase-positive *staphylococci*	Enumeration	Colony count	ISO 6888-2:2021 ISO 6888-2 (2021)
Yersinia enterocolitica	Detection	Colony isolation	ISO 10273:2017 ISO 10273 (2017)
Bacillus cereus	Enumeration	Colony count	ISO 7932:2004 ISO 7932 (2004)
Enterobacteriaceae	Enumeration	Colony count	ISO 21528-2:2017 ISO 21528-2 (2017)
Clostridium perfringens	Enumeration	Colony count	UNI-EN ISO 7937:2005 UNI-EN ISO 7937 (2005)
Eschericha coli	Enumeration	Colony count	UNI ISO 16649-2:2010 UNI ISO 16649-2 (2010)
Cereulid encoding gene (*ces*)	Detection	Real-time PCR	Horwood *et al*. (2004)
Staphylococcal enterotoxins A,B,C,D, and E	Detection	Enzyme-linked fluorescence	ISO 19020:2017 ISO 19020 (2017)

Table 4. Microbiological analyses' results.

Food sample	*E. coli* (CFU/g)	*Enterobactericeae* (CFU/g)	CPS (CFU/g)
Ham			
Subsample 1		40	40
Subsample 2		100	
Subsample 3			
Subsample 4		150	
Subsample 5		40	
Cheese			
Subsample 1	40	50	1.300
Subsample 2	40	40	1.100
Subsample 3	40	40	1.600
Subsample 4	40	50	3.300
Subsample 5	60	100	8.100

*When 40 CFU/g is reported, readers should interpret the results as low bacterial concentration (range: 10–40 CFU/g). CPS is coagulase-positive staphylococci.

$8.1*10^3$ CFU/g). Enzyme-linked fluorescence detected SEs A to E in all five cheese subsamples. A portion of the five subsamples was sent to EURL-CPS; the pooled sample tested positive for staphylococcal enterotoxin D (SED) (estimated concentration, 0.649 ng/g).

In this FBO, the time to symptom onset was about 3 hours after lunch, which suggests that the food contained preformed toxins. Laboratory analysis detected SEs (about 0.649 ng/g) in the cheese sandwiches. Even if diarrhea is not the most representative symptom for SEs intoxication, it is often mentioned among symptoms of SEs FBO (Argudín *et al.*, 2010). The other presenting symptoms were indicative of the causal agent and the quantity of toxins in the food. The sandwiches were approximately made with a standard portion of cheese (about 30 g); the likely amount of SED each case consumed was approximately 20 ng.

From an analytical perspective, a CPS dose between $1.3*10^3$ and $8.1*10^3$ CFU/g may seem to contradict the published data (Wong and Bergdoll, 2002) and the European food safety criterion (EU, 2005). It is widely recognized that the production and the release of emetic toxins by *S. aureus* strains occur at a concentration of about 10^5 CFU/g. This concentration is cited by food safety norms. Indeed, health agencies require that official laboratories detect preformed toxins as a safety criterion and as a hygiene criterion whenever the concentration is above the limit. The laboratory tests revealed low concentrations of *Escherichia coli* and *Enterobacteriaceae*, indicating a fecal contamination, being nevertheless compliant with food safety legislation (EU, 2005). Although the hazard analysis and critical control point (HACCP) manual indicated that good manufacturing protocols were followed and well described, the food safety agency inspectors asked the food business operator to strictly respect the food safety own-check program, as well as the procedures for good hygiene practices, namely, cleaning and disinfection. In addition, the operator was asked to conduct staff training on food hygiene and to meet safety standards.

On further investigation by the district food safety competent authority, it was noted that the food business operator was unable to provide accurate information of the supplier of the cheese, and no invoices of the cheese were available. This violates general food safety regulation (EU, 2002) that requires food business operators to

maintain records that can allow for traceability and backward traceability of the products they sell. Furthermore, the lack of traceability of the product did not permit the competent authority to perform additional inspections at the farm/facility where the cheese was made and to know the characteristics of the milk used. Due to the preformed toxins detected in the cheese sample, a primary CPS contamination at the production step is suspected. A secondary contamination deriving from human handling during the preparation of the sandwiches at the cafeteria wouldn't have allowed SEs synthesis, due to short time.

During an official investigation into a suspected case of food poisoning, the microbiological examination of collected samples is a one-time process, primarily due to the perishable nature of the product under investigation. In addition, the food business operator or their legal representative could ask to be present at the laboratory. Finally, to ensure a representative sample for the laboratory, acknowledging that the causative agents may not be uniformly distributed throughout the sample, the official samples are divided into multiple smaller subsamples. Also in the reported case, the subsampling was crucial for the detection of toxins and pathogenic bacteria in single subsamples, which would have permitted to declare the entire original sample as noncompliant with food safety standards.

Here, we report an instance of FBO in which doctors, food safety agency staff, and official laboratory staff collaborated. Following notification of suspected food poisoning, rapid response by the district food safety agency facilitated the collection of samples of the same lot of cheese used for preparing the sandwiches at the food business facility. There was no other report or notification of suspected FBO in the same period and in the same area, probably due to small-scale cheese production in the mountain area in the summer or because of underreporting of self-limited illness.

According to this report, the current limit of 10^5 CFU/g CPS for the search for SE must also be questioned. In cases of FBO with symptoms compatible with the presence of enterotoxins, it may be necessary to consider lower *S. aureus* loads. This paper showed that this parameter for detecting staphylococcal toxins may be suboptimal for identifying microbial contamination potentially harmful for consumers.

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