Staphylococcal food poisoning outbreaks occurred in Sicily (Italy) from 2009 to 2016

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Abstract

Notification of foodborne outbreaks has been mandatory in Europe since 2005, and surveillance is carried out along the entire food chain. Here we report the results obtained from laboratory investigations about four cases of foodborne outbreaks that occurred in Sicily between 2009 and 2016, deemed to be related to staphylococcal enterotoxins (SEs) and coagulase-positive *Staphylococci* (CPS) by the Local Public Health Authority. *Primosale* cheese samples were processed by culture methods for enumeration of CPS and immunoenzymatic assays for detection and differentiation of the SEs possibly contained in food samples. In all cases, the mistrusted foods were found to be contaminated by CPS at bacterial loads between 5 and 8 log CFU/g and contained SE type C (SEC). The reported data confirm the risk of staphylococcal food poisoning associated with the consumption of raw milk cheese. SEC is the most commonly occurring SE in goat milk and dairy products and the most represented enterotoxin in Sicilian dairy products. Our results highlighted the need for improving the current monitoring efficiency and implementing the available laboratory methods to collect more faithful epidemiological data on the current prevalence of staphylococcal toxins in the food chain, including SEs currently not detectable by validated analytical methods.

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Introduction

Staphylococcal enterotoxins (SEs) represent an important cause of foodborne disease outbreaks (FBO) in Europe, where notification has been mandatory since 2005. In 2019, 74 outbreaks caused by SE poisoning were reported by 13 member states, including Italy, and involved a total of 1324 cases. The most serious outbreak was described in Italy, where 44 out of 70 cases (62%) required hospitalization (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021).

Staphylococcus aureus is commonly harbored by 30-60% of healthy people in the skin and respiratory mucosa (Argudín *et al.*, 2010); it is frequently isolated from raw milk, and its presence in this product represents a source for the introduction of *S. aureus* into the dairy product supply chain (D'Amico and Donnelly, 2011; Johler *et al.*, 2018), with the consequent risk of bacterial growth and release of enterotoxins in the final product. SE production, however, will not occur in all foods; in fact, environmental factors and conspicuous combinations of food parameters may influence and contribute to the formation of SEs, such as water activity, pH, redox potential, and temperature. Besides, bacterial antagonism is known to play an important role (Genigeorgis, 1989; Hennekinne *et al.*, 2012; Bianchi *et al.*, 2014; Schelin *et al.*, 2011). SEs are particularly stable and highly resistant to heat, proteolytic enzymes, and low pH, all qualities contributing to their full functionality in the gastrointestinal tract (Chen and Xie, 2019).

A wide variety of SEs responsible for staphylococcal food poisoning outbreaks (SFPOs) in humans have been reported (Nunes and Caldas, 2017). The main symptoms of SE poisoning are nausea, emesis, abdominal cramps, and diarrhea, which occur within 2-8 hours after the ingestion of contaminated food (Normanno *et al.*, 2007). More than 20 SEs and SE-like toxins have been described in the literature over more than 50 years (Etter *et al.*,

2020), but only 5 enterotoxins [types A (SEA), B (SEB), C (SEC), D (SED), and E (SEE)] can be detected using commercial immune-assays or in-house developed methods, while no validated method is available for the detection/quantification of other enterotoxin types such as SEG, SEH, and SEI, which are yet known to be a risk for consumers (Hennekinne *et al.*, 2010). However, with the emergence of novel SEs, there is a growing need for the characterization of suspected strains, including the identification of novel SE genes, to improve SFPO diagnosis. Several SFPOs in Europe have shown a typical SE symptomatology, but SEA-SEE enterotoxins could not be detected. However, genes such as *seg, seh, sei, sem, sen,* and *ser* were detected by polymerase chain reaction assay, suggesting the presence of non-SEA-SEE toxin types (Argudín *et al.*, 2010).

Here, we describe the epidemiological and laboratory investigations concerning four cases of FBOs due to pecorino *Primosale* cheese, which occurred in Sicily (southern Italy) (Figure 1) from December 2009 to January 2016 and involved several people.

Materials and Methods

Description of outbreak cases

Below is the background for each of the four outbreak cases investigated.

Case 1

In Acireale (Catania, eastern Sicily), on December 12, 2009, a husband and wife, aged 52 and 46, respectively, experienced gastrointestinal symptoms such as vomiting and severe abdominal cramps 1 hour after eating pork sausage and Sicilian *Primosale* cheese. After 12 hours, there was a remission of symptoms. Four days later, the same subjects consumed the same cheese again, reporting the same symptoms.

Case 2

In October 2011, three people went to the emergency department of the hospital in Mazara del Vallo (Trapani, northwestern Sicily) with gastrointestinal symptoms such as nausea, vomiting, severe abdominal cramps, and diarrhea. The patients were part of the same family: the mother (30 years old) and two children aged 5 and 7 who had eaten a *piadina* with bresaola and Sicilian *Primosale* cheese. The symptoms appeared 1-2 hours after the meal. The father had eaten *piadina* without cheese and developed no symptoms. The two children were hospitalized for 2 days.

Case 3

In April 2013, six people arrived at the emergency department of Marsala Hospital (Trapani, north-western Sicily) with gastrointestinal symptoms such as vomiting, severe abdominal cramps, and diarrhea. The patients belonged to a first family group (group A), including husband and wife aged 64 and 63, respectively; a second family group, including parent and son aged 68 and 35 (group B), respectively; and two unrelated men aged 44 and 52. Three of the people involved required hospitalization. Group A reported having eaten Sicilian *Primosale* cheese with black pepper, roasted sausage, and beef. The symptoms occurred 1 hour after the meal. One day later, the same individuals consumed the cheese again, reporting the same symptoms. Group B reported the development of symptoms half an hour after eating pasta and Sicilian *Primosale* cheese with black pepper.

Case 4

In January 2016, in Enna (Sicilian inland), three people from the same family, aged respectively 74 years old (grandmother), 53 years old (mother), and 13 years old (daughter), showed symptoms such as abdominal pain, vomiting, and diarrhea 2 hours after eating Sicilian *Primosale* cheese with black pepper. They needed medical care, but nobody required hospitalization.

Epidemiological investigation

From December 2009 to January 2016, four cases of FBOs occurred in Sicily (Southern Italy). In all FBOs, the investigations were carried out by the Local Public Health Authority as follows: interviewing of the involved people, sampling, investigations to trace the incriminated production batches and identify the source cheese-making factories, *in loco* inspection of the involved food-producing plant, and implementation of control measures. Based on the history and clinical symptoms reported by patients, the Local Health Authority collected Sicilian *Primosale* cheese samples and oriented laboratory investigations toward the search for SEs and coagulase-positive *Staphylococci* (CPS), including *S. aureus*. In the fourth FBO, they also oriented laboratory investigations toward *Salmonella* spp. and *Listeria monocytogenes* assessment. Furthermore, to investigate the hygienic level of the cheeses, the following analyses were performed: enumeration of β-glucuronidase *Escherichia coli* and coliform bacteria. Suspicious cheese residuals and cheese samples collected at supermarkets or cheese-making factories were kept under refrigeration and sent to the official laboratory for further analysis.

Sample collection

A total of 12 samples of *Primosale* cheese were brought to the laboratory of Food Microbiology (Experimental Zooprophylactic Institute of Sicily, Palermo) following four SFPOs recorded in Sicily, Italy. Referring to Case 1, the sample consisted of a leftover meal of suspected cheese. The sample could not be traced because it was purchased from a street vendor and lacked packaging and labeling. Referring to Case 2, sample collection included a residual of incriminated cheese and a cheese sample that was bought at the supermarket. Referring to Case 3, no leftovers were available for the suspected cheese sample. The inspection performed by the Local Health Authority led to the seizure of the incriminating batch at both the supermarket and the artisan dairy manufacturing plant. Also, five cheeses found at the supermarket without a batch number were seized. Referring to Case 4, no leftovers for the suspected

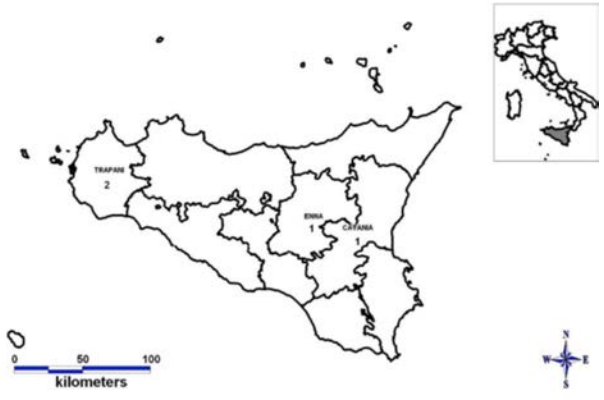

Figure 1. Map of outbreaks in Sicily.

cheese sample were available. The investigation and the official activities performed by the Local Health Authority led to the sampling and seizure of one unlabeled suspect cheese sample at the supermarket and the sampling and seizure of the presumptive incriminated batch taken at the artisan dairy manufacturing plant.

Microbiological analysis

CPS were counted in samples according to the standard method of UNI EN ISO 6888-2:2004 (ISO, 2004). Briefly, 30 g of the sampled cheese were diluted 1/10 (w/v) in buffered peptone water and mixed with a stomacher. Serial dilutions of each sample were plated on Baird-Parker agar + rabbit plasma fibrinogen and incubated at 37±1°C for 48 hours. At least five colonies per sample were tested with commercial biochemical identification kits API® ID32 STAPH (bioMerieux, Marcy L'Etoile, France) for *S. aureus* strain identification. Food specimens were also tested for SEs, according to ISO 19020:2017 (ISO, 2017a), to simultaneously detect SEA, SEB, SEC, SED, and SEE in food matrices without differentiating the five SEs. The European Union Reference Laboratory (EURL) for CPS performed the confirmation of positive samples and identified the specific SE contained in each of them by means of an in-house enzyme-linked immunosorbent assay (ELISA) method (Hennekinne *et al.*, 2007). In addition, some cheese samples (Case 3) were analyzed for the enumeration of β-glucuronidase-positive *E.* coli (ISO, 2001) and Coliforms (ISO, 2006), as well as the detection of *Salmonella* spp. (ISO, 2002) and *L. monocytogenes* (ISO, 2017b).

Staphylococcus aureus strain characterization

Five CPS strains isolated from each food sample underwent multiplex PCR (mPCR) to detect genes encoding SEs according to EURL CPS methods. The protocols included the detection of the genes from *sea* to *see* and *ser* for the first mPCR and from *seg* to *sej* and *sep* for the second mPCR (De Buyser *et al.*, 2009a, 2009b).

Results

The investigation, performed by the Local Health Authority, suggested *Primosale* cheese as a likely source of contamination for all the FBOs considered. The microbiological analyses performed on cheese samples detected CPS at high bacterial loads for all four FBOs (Table 1). CPS specimens isolated from each cheese sample were further analyzed for species identification, and all of them were identified as *S. aureus.*

The qualitative immunoassay detected SEs (type SEA to SEE) in the food matrices sampled per case. Further analysis by ELISA assay for specific SE identification confirmed the sample contamination and the presence of SEs at different levels of concentration.

Referring to the sample from Case 1 and to the two samples from Case 2, CPS bacterial loads were 5.1×10^6 CFU/g, 1.2×10^8 CFU/g, and 2.6×10^8 CFU/g respectively, while SEC concentrations were 4.94 ng/g and over 19 ng/g respectively (Table 2). Referring to Case 3, CPS bacterial loads were 1.1×10^8 CFU/g and 2.2×10^6 CFU/g for the samples attributable to the event, seized at the supermarket and the dairy manufacturing plant respectively, while referring to the five without-batch units collected at the supermarket, the bacterial load detected was above 10^5 CFU/g. Quantitative ELISA analysis detected SEC with a range of concentrations between 120.31 ng/g and 399.96 ng/g (Table 2). Also, referring to Case 3, the enumeration of *E. coli* and coliforms returned high bacterial loads, about 10^6 and between 10^6 and 10^7

Table 1. Staphylococcal count in cheese samples.

Case ID	Date	Location	Sampling place	CPS (CFU/g)
1	December 2009	Acireale (Catania)	House	5.1×10^6
2	October 2011	Mazara del Vallo (Trapani)	House	1.2×10^8
			Supermarket	2.6×10^8
3	April 2013	Marsala (Trapani)	Dairy plant	2.2×10^6
			Supermarket	1.1×10^8
			Supermarket (1)	4.0×10^7
				7.4×10^5
				4.3×10^7
				1.2×10^7
				4.8×10^8
4	January 2016	Gagliano Castelferrato (Enna)	Supermarket	1.5×10^5
			Dairy plant	<10

(1), sampling of five cheese units without batch; CPS, coagulase-positive *Staphylococci.*

Table 2. Staphylococcal enterotoxins detection and identification in cheese samples.

Case ID	Staphylococcal enterotoxin (detection)	Staphylococcal enterotoxins (specific identification)
1	Detected	SEC (4.94 ng/g)
2	Detected	SEC (>19 ng/g)
3	Detected	SEC (387.80 ng/g)
	Detected	SEC (399.96 ng/g)
	Detected	SEC (224.22 ng/g)
	Detected	SEC (120.31 ng/g)
	Detected	SEC (213.62 ng/g)
4	Detected	SEC (28.38 ng/g)

SEC, staphylococcal enterotoxins type C.

CFU/g respectively (Table 3). *Salmonella* spp. and *L. monocytogenes* were not detected in any of the samples tested. Referring to Case 4, CPS contamination was detected in the suspected samples, and the bacterial loads were 1.5×10^5 CFU/g for the cheese related to the toxigenic event collected at the supermarket and below the method's detection limit (10 CFU/g) for the cheese sample taken at the dairy plant. Specific quantitative ELISA analysis detected SEC at a concentration of 28.39 ng/g (Table 2). Referring to all cases, the characterization of *S. aureus* strains showed that at least 1 of the 5 strains isolated for each food sample carried the *sec* gene.

Discussion

This report describes four cases of SFP due to the consumption of contaminated fresh cheese, Sicilian pecorino *Primosale.*

Pecorino *Primosale* is a fresh, typical soft cheese made from raw sheep milk and produced in Central and Southern Italy. It is characterized by a white color and no evident rind. The name itself means "first salt" and is used to describe a cheese variety with a short ripening time (maximum 15 to 20 days) that is consumed immediately after the first salting and differs from other varieties of pecorino cheese, which usually have a longer period of ripening (more than 60 days) (Hennekinne *et al.*, 2010).

Primosale is an appropriate substrate for the preservation of vitality and multiplication of several bacteria due to its high moisture, high pH, and low concentration of NaCl. Reports regarding FBOs involving dairy products and food surveillance point out fresh cheese as a potential vehicle for food pathogens such as *Salmonella* spp., *L. monocytogenes,* and enterotoxigenic *S. aureus* (Giammanco *et al.*, 2011). The study conducted by Johler *et al.* (2018) shows that *S. aureus* is commonly present in artisanal dairy products and raw milk cheeses produced in Italy, where 80% of the artisanal cheeses sampled were positive for *S. aureus*.

The diagnosis of staphylococcal food poisoning (SFP) is generally confirmed by at least one of the following points: recovery of *S. aureus* at a bacterial load above 10^5 CFU/g from food remnants, detection of SEs in food remnants, or isolation of *S. aureus* of the same phage type from both patients and food remnants (Kérouanton *et al.*, 2007). A conclusive diagnosis of SFP is mainly based on the demonstration of SEs in the suspected food.

Clinical symptoms reported by the people involved and laboratory test results (loads of CPS above 10^5 CFU/g, staphylococcal enterotoxin detection in cheese samples, and *sec* gene detection in the isolated strains) confirmed the etiology of the FBOs in all cases. However, the absence of stool and emesis samples from the patients did not allow a relationship between isolated food and human strains.

The SE type detected in cheese samples from all four SFP cases was SEC; it was quantified, and its amount was between 4.94 and 399.96 ng/g. Scientific literature reports SEA as the most common cause of SFP worldwide, followed by SED and SEB (Kadariya *et al.*, 2014; Abdel-Hameid Ahmed *et al.*, 2019). However, SEC and SEE are also implicated in SFP. SEC was linked to the SFPO that occurred in Canada in 1980, and it was also involved in the SFPOs recorded in Taiwan between 2001 and 2003 and in Japan in 2009 (Kadariya *et al.*, 2014). Weiler *et al.* (2011) reported an epidemic outbreak by SEC associated with the consumption of ultrapasteurized milk in the Republic of Paraguay. Also, SEC and SEA are the most commonly occurring SEs in milk and dairy products (Etter *et al.*, 2020).

Enterotoxigenic CPS contamination in dairy products is related to different factors, such as dairy animals, the environment, and humans. *S. aureus* is the most common cause of mastitis in dairy animals (Kadariya *et al.*, 2014). Animals affected by clinical or subclinical mastitis are a source of milk contamination. In sheep, goats, and cattle, SEC was the predominant toxin type detected among the *S. aureus* specimens isolated from mastitis milk. Other studies have documented SEC producers as the most prevalent enterotoxin-producing *S. aureus* isolated from goat's milk and goat's skin of the udder, teats, and milk (Kadariya *et al.*, 2014). In addition, Basanisi *et al.* (2016) analyzed 37 *S. aureus* isolates from Italian sheep and goat cheeses. The *sec* gene was the most frequently recovered (42.8%), followed by *seh* (28.6%), *sea* (14.3%), and *see* (14.3%). Vitale *et al.* (2015) found *sec* to be the most represented enterotoxin gene in Sicilian dairy products.

However, *S. aureus* contamination in milk and dairy products can also occur during cheesemaking, through contact with contaminated equipment, or *via* handling by infected food workers.

Finally, during the epidemiological investigation of SFPs, no environmental or nasopharyngeal swabs from food handlers were collected, so it was not possible to trace the contamination source, although it is likely that dairy animals were the origin of the SFP strains. In addition, in the third SFPO case, high levels of *E. coli* and coliforms exceeding 10^6 CFU/g were found in cheese samples; this result was possibly related to the use of raw milk, bad hygiene of equipment, or poor hygiene practices during milk collection or cheese processing. Referring to the fourth SFPO case, other interesting results were the high bacterial loads recorded for CPS together with the detection of enterotoxin in the suspected cheese taken at the supermarket, and the absence of CPS counts in the cheese sample taken at the dairy plant. This result may be attributed to the purchase of illegal products or bad storage. In fact, in the traditional markets of Palermo, it was quite common to find *Primosale* cheese stored at room temperature (Giammanco *et al.*, 2011). The discussion is further fueled by the fact that in many outbreaks, strains exhibiting both classical and newly described enterotoxin genes can be detected but only classical enterotoxins can be identified in food by commercially available immunological-based methods.

Table 3. Results from the microbiological analyses performed on the cheese samples involved in the third outbreak

Outbreak	Outbreak date	Sampling place	*Escherichia coli* (CFU/g)	Coliforms (CFU/g)
3	April 2013	Artisan dairy plant	7.0×10^6	1.3×10^7
		Supermarket	4.6×10^6	4.5×10^7
		Supermarket (1)	3.5×10^6	5.0×10^6
			3.5×10^6	4.2×10^6
			4.2×10^6	5.2×10^7
			9.2×10^6	1.5×10^7
			1.3×10^6	4.4×10^6

(1), sampling of five cheese units without batch.

Conclusions

The combination of microbiological and immunological analyses plays a fundamental role in elucidating the FBOs, and once the microbial agent has been identified and isolated, the use of different characterization methods represents an effective approach to understanding the origin and the dynamics of contamination.

Complying with good hygienic practices and maintaining cold meals at refrigerated temperatures proved to be essential.

Moreover, due to the identification of several SE genes other than the ones encoding for SEA-E, currently detected by commercially available immunological-based methods, it would be desirable to implement validated methods targeting other SEs.

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