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Staphylococcus aureus harbouring egc cluster coding for non-classical enterotoxins, involved in a food poisoning outbreak, Romania, 2012

Staphylococcus aureus purtător de gene codante pentru enterotoxine non-clasice (cluster egc), implicat într-un focar de toxiinfecţie alimentară, România, 2012

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Abstract

In March 2012, a food poisoning outbreak was reported in a Romanian county, with a total number of 30 children affected. The symptoms (vomiting, diarrhea and abdominal pain), with onset within 1-2 hours after the ingestion of a particular food (milk), suggested a possible staphylococcal aetiology. An outbreak investigation was carried out, in accordance with the national surveillance methodology and 25 samples: stool (n=9), vomit (n=5), nasal swabs (n=9), and milk (n=2) were collected from the affected children, food handlers and suspected food. All isolated strains were sent to the Reference Centre for Staphylococci within the "Cantacuzino" National Institute of Research-Development for Microbiology and Immunology, Bucharest, Romania, for confirmation and further analysis. The aim of this study was to increase the reference laboratory capacity to confirm staphylococcal food poisoning (SFP) outbreak by defining the molecular basis of toxicity of Staphylococcus aureus (S. aureus) isolates and assessing their genetic relatedness. PCR methods have been used to detect 14 enterotoxin genes and the expression of some of these genes was proved by using a reverse transcription real-time PCR. Pulsed-field gel electrophoresis (PFGE) and Staphylococcus protein A coding gene sequence typing (spa typing) have been used to track the origin of the S. aureus contamination and to confirm the food poisoning outbreak.

Two enterotoxigenic S. aureus strains isolated from milk, twelve isolated from patients and two from food handlers were of the same spa- type (t902) and revealed an indistinguishable SmaI macrorestriction pattern after a PFGE analysis. All these strains harboured the same toxin genes profile, namely the enterotoxin gene cluster (egc), which strongly supports the evidence that the milk was the incriminated food vehicle of the outbreak and a food-handler was the most likely source of the staphylococcal food poisoning (SFP) incident.

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Rezumat

În martie 2012, într-un judeţ din România, a fost raportat un focar de toxiinfecţie alimentară ce a afectat un număr de 30 de preşcolari. Debutul simptomelor (vărsături, diaree şi dureri abdominale), la 1-2 ore de la ingestia unui anumit produs alimentar (lapte), a sugerat o posibilă etiologie stafilococică a acestui focar.

Ancheta epidemiologică a fost efectuată în conformitate cu metodologia naţională de supraveghere a focarelor de toxiinfecţii alimentare (TIA), fiind recoltate 25 de probe de la copiii afectaţi, de la personalul ce a manipulat hrana şi din alimentul incriminat: materii fecale (n=9), lichide de vărsătură (n=5), exsudate nazale (n=9), lapte (n=2). Tulpinile izolate au fost trimise la INCDMI "Cantacuzino", Bucureşti, România, pentru confirmare şi analize suplimentare. Scopul studiului a fost sporirea capacitatii laboratorului de referinta pentru confirmarea focarelor de toxiinfectii alimentare stafilococice prin determinarea profilului toxic al tulpinilor de Staphylococcus aureus şi stabilirea înrudirii lor genetice. Pentru evidenţierea genelor codante a 14 enterotoxine stafilococice au fost folosite metode bazate pe studiul acizilor nucleici (PCR), iar expresia câtorva dintre aceste gene, a fost evidenţiată cu ajutorul reacţiei de revers- transcriere în timp real (RT- real- time PCR).

Pentru a determina sursa contaminării cu S. aureus şi pentru a confirma acest focar de toxiinfecţie alimentară au fost folosite electroforeza în câmp pulsator (PFGE) şi tipizarea pe baza secvenţierii genei codante a proteinei A stafilococice (spa typing). Două tulpini enterotoxigene de S. aureus izolate din lapte, douăsprezece izolate de la pacienţi şi două de la personalul din sectorul alimentar au prezentat acelaşi tip spa (t902) şi un profil de macrorestricţie identic. Aceste tulpini au prezentat acelaşi profil enterotoxigenic, clusterul egc (enterotoxin gene cluster), informaţii ce susţin ipoteza că laptele a fost alimentul vehicul iar una dintre persoanele care au manipulat alimentele a reprezentat probabil, sursa acestui incident.

***Cuvinte cheie**: Staphylococcus aureus, toxiinfecţie alimentară stafilococică, tipizarea spa, egc-enterotoxin gene cluster, PFGE*

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Introduction

Staphylococcal food poisoning (SFP) is widespread and relatively frequent, being one of the main worldwide acute food intoxications; milk and dairy products representing 1-9% of all incriminated foods in Europe (1).

SFP occurs following ingestion of staphylococcal enterotoxins (SEs) preformed in food by enterotoxigenic *Staphylococcus* strains, usually by *S. aureus,* introduced in food through improper handling (2), cooking and/or storage conditions (3). SFP can be suspected when the interval between eating contaminated food and onset of symptoms (such as nausea, vomiting, diarrhea and abdominal cramps) is between 30 minutes and 8 hours, usually 2 to 4 hours (2). After consumption, enterotoxins from the contaminated food are absorbed in the gastro-intestinal tract and further into the blood, thus triggering the emetic reflex (4). The onset of illness occurs when *S. aureus* is found in a quantity of at least 10^5 CFU/g in food; generally, the specific symptoms are registered after the ingestion of 100-200 ng of enterotoxin (2). From clinical point of view, the severity of symptoms is directly linked to the quantity of enterotoxins ingested and the patient's sensitivity (5).

The illness is usually self-limiting, commonly lasting only a day or two, but can take longer in severe cases; in rare cases, the intensity of symptoms may require hospitalization (6). The main source of contamination is represented by food handlers that can be carriers of enterotox-

in-producing *Staphylococcus* in their nose or on their hands (3). Ingestion of a food product containing staphylococcal enterotoxin, particularly those that come into contact with the food handlers' hands, either without subsequent cooking or with inadequate heating or refrigeration, represents the transmission pathway.

To date, more than 20 SEs have been characterized: the "classical" types: SEA, SEB, SEC, SED, and SEE (7) and more recently described the "non-classical" SEs: SEG, SEH, SEI, SER, SES and SET, with emetic activity. Staphylococcal enterotoxin-*like* (SE*l*) toxins: SE*l*J, SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, SE*l*Q, SE*l*U, SE*l*V, SE*l*X (8, 9, 10, 11) are the staphylococcal enterotoxins of which the emetic activity is unknown or has not been yet tested (12).

A recent study confirmed the emetic potential of SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, and SE*l*Q in monkeys (*cynomolgus monkeys*) and proposed renaming them as SEK, SEL, SEM, SEN, SEO, SEP, and SEQ, respectively (13).

The classical SEA to SEE enterotoxins (SEs) generated by *Staphylococcus aureus* were considered to be the main reason for staphylococcal food-poisoning (SFP) worldwide (7). Only SEG, SEH and SEI-producing strains of the new enterotoxins, have clearly been involved in SFP outbreaks (14, 15). Results from different researchers have shown the high incidence of genes encoding new SEs and SE*l*s among food borne diseases caused by *S. aureus* (16, 17).

All SEs and SE*l*s are single-chain proteins with a molecular weight of 19-29 kDa (18, 19) heat-stable and resistant to proteolytic enzymes such as pepsin and trypsin, allowing their activity to continue in the digestive tract after ingestion (20). These proteins showed a potential superantigenic activity that stimulates nonspecific-T cell proliferation (7).

SEs genes are located on mobile genetic elements: plasmids, phages or pathogenicity islands- SaPIs (3). In 2001, Jarraud et al. (21) reported the existence of an *egc* (*e*nterotoxin *g*ene *c*luster) operon regarded as a reservoir of *se*/*sel* genes in continuing evolution. The *egc* operon comprising *seg*, *sei*, *sem*, *sen* and *seo* genes also contains two pseudogenes: ϕent1 and ϕent2 that are frequently found among clinical and food *S. aureus* isolates (3). To date, four different *egc* subtypes (22) were suggested: *egc*1 (*selo*, *selm*, *seli*, Ψ*ent1*, Ψ*ent2*, *seln*, *seg*); *egc*2 (*selo*, *selm*, *seli*, *selu*, *seln*, *seg*); *egc*3 (*selo*$_v$, *selm*$_v$, *seli*$_v$, *selu*$_v$, *seln*$_v$, *seg*$_v$), and *egc*4 (*selo, selv*, *selu2*, *seln, seg*).

egc SE genes are arranged in tandem orientation in the *egc* cluster and are co-expressed (23). The *egc* locus may also be present in an incomplete form lacking one or more genes (10).

A complete characterization of SFP outbreaks is provided by an integrated gene-to-protein approach (24), and to improve SFP characterization, various authors (25) have demonstrated a possible transcription of mRNA from *se* genes. PCR based-methods are specific and quick methods that inform about the presence or absence of genes encoding SEs, but not about the expression of these genes.

Taking into consideration the ubiquity of *S. aureus* strains, detection of this microorganism in food, food handlers and patients is not sufficient to support *S. aureus* involvement in an outbreak. Supporting evidence is needed based on molecular typing and toxic profile detection. Non A-E SE producing *S. aureus* strains ought to be evaluated equally by molecular methods in order to prove their ability to cause staphylococcal food poisoning.

In our study, the absence of classical SEA to SEE enterotoxin producing isolates prompted us to further investigate the molecular basis of *S. aureus* isolates toxicity and to assess their genetic relatedness in order to confirm the SFP suspected outbreak.

In order to improve the reference laboratory capacity based on the translation of research re-

sults into public health microbiology practice, the authors considered that characteristics of the 2012 outbreak isolates as a paradigm to be reported.

Materials and methods

Bacterial strains

Clinical and food samples were collected and processed by the county public health authorities after having obtained an informed consent and following an epidemiological investigation. A total of 25 *S. aureus* isolates from milk (*n*=2), stools (*n*=9) and vomit (*n*=5) from patients, and nasal swabs from food handlers (*n*=9) were submitted to the Reference Centre for Staphylococci within the "Cantacuzino" NIRDMI, Bucharest, Romania, for classical enterotoxins detection.

Other epidemiological evidence concerning this outbreak (number of cases, patient ages, symptoms, treatment and evolution) were not available.

All the isolates were identified by coagulase production to species level using rabbit plasma (bioMérieux, France) and by an "*in house*" Lachica method, in order to detect thermal stable nuclease production (26); biochemical characterization was performed automatically using the Vitek2 system (bioMérieux, France). To confirm methicillin-resistance, a PCR for *mec*A gene detection was performed (27).

Molecular typing – PFGE and spa typing

All isolates were characterized by pulsed-field gel electrophoresis (PFGE) and *spa* typing. PFGE was performed with a CHEF mapping system (Bio-Rad), as previously described (28). The recommended standard strain NCTC 8325 was used as a size marker to allow later normalization of electrophoretic patterns across the gel. The digital image was analyzed with a BioNumerics 6.6 (Applied Maths) using the Dice coefficient and generated by the Unweighted-Pair Group Method using Average linkages (UPGMA) with 1.5% tolerance and 1% optimization settings. A similarity cut-off of 80% and the criterion of a difference of no more than 6 bands, as described by Tenover (29), were used to define a pulsotype. Isolates that exhibited identical or related PFGE patterns were considered to belong to the same clone. A single strain was nontypable using *SmaI* digestion.

Spa typing was performed according to a standard protocol, as previously described (30), using an Applied BioSystems 3130 Genetic Analyzer. For relevant genetic information on *spa* repeating code and assignment of a *spa* type, the sequence information was uploaded and synchronized with the central Ridom SpaServer (www.spaserver.ridom.de), curated at the Institute of Hygiene, University Hospital Münster, Germany, by SeqNet.Org. The clonal relation between isolates can be investigated by clustering related *spa* types using the Based Upon Repeat Patterns (BURP) algorithm. This algorithm is used to define spa clonal complexes (spa-CC) (31).

Identification of SE genes by PCR

Genomic DNA was extracted by thermal and/or enzymatic lysis (lysostaphin, proteinase K). The amplifications were performed using an Applied Biosystems Thermocycler with primers recommended by other researchers for 14 staphylococcal enterotoxin genes: *sea, seb, sec, sed, see* (32), *seg, seh, sei* (33), *sej* (34), *sel* (35), *sem, sen, seo* (16), *ser* (35), according to the technique reported in our previous study (36).

RNA Extraction and cDNA Synthesis

One (1) ml of a culture solution (stationary phase state) in tryptic soy broth was centrifuged at 10,000 x g at 4°C for 5 min. RNA was extracted using QuickExtract™ RNAExtraction Kit (Epicentre) according to the manufacturer's instructions. The residual DNA was digested using an optional DNase treatment included in the kit.

10 µl of total RNA were then used in a 20 µl reverse transcription reaction for cDNA synthesis using Reverta 50 L RT reagent kit (Sacace, Italy), containing random sequence hexamers.

Real-Time PCR

The real-time PCR analysis was performed using a Stratagene Mx3005P™ system, with the same primers used in the PCR reactions for *seg*, *seh*, *sei* and *sem* genes and 2 µl of cDNA using KAPA SYBER® FAST qPCR Kit Master Mix Universal. The cycling program was as follows: enzyme activation: 95°C for 5 min., followed by 40 cycles of denaturation for 3 sec. at 95°C, and annealing/extension step for 30 sec. at 60°C (25). Fluorescence readings were recorded after each extension step, followed by a melting curve analysis at 65-99°C (temperature transition rate of 0.1°C/sec) based on continuous fluorescence readings.

Results and discussions

The PFGE analysis revealed 7 different PFGE banding patterns (patterns A to G) and 7 clusters (1- 7) (Fig. 1, Table I).

In this study, one predominant *S. aureus* lineage, defining the outbreak pulsotype was identified, containing a number of 18 (72%) from all isolates, belonging to the PFGE pattern D and to the same cluster (cluster 4, with 88.7% similarity). These isolates showed the same enterotoxin gene profile defining the *egc* operon: *seg, sei, sem, sen, seo* (Table I).

16 isolates of this cluster exhibited the same *spa* type (t902) and the other two strains exhibited t449 and t6346 *spa* types. Using BURP clustering, we demonstrated that all these types belonged to the same *spa* clonal complex (*spa* CC005). This cluster included isolates from nasal swabs (*n*=4), milk (*n*=2), stool (*n*=9), vomit (*n*=3), samples from all parts involved in the

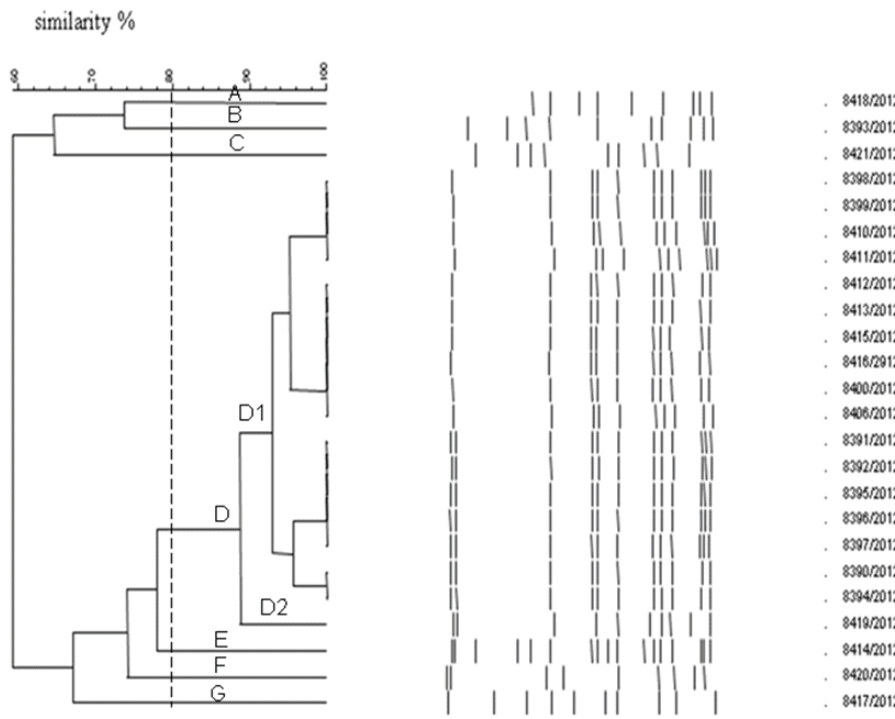


Figure 1. Dendrogram of PFGE clusters and relationships of *S. aureus* isolates

Table I. Analysis of *S. aureus* strains from the reported food poisoning outbreak

Isolates ID number	PFGE- pattern	PFGE cluster	Source	spa type	se genes *
8418/2012	A	1	nasal swab	t3216	*sec, sel, egc*
8393/2012	B	2	vomit	t127	*seh*
8421/2012	C	3	nasal swab	t056	*neg***
8398/2012	D1	4	stool sample	t902	*egc*
8399/2012	D1	4	stool sample	t902	*egc*
8410/2012	D1	4	stool sample	t902	*egc*
8411/2012	D1	4	stool sample	t902	*egc*
8412/2012	D1	4	stool sample	t902	*egc*
8413/2012	D1	4	nasal swab	t902	*egc*
8415/2012	D1	4	nasal swab	t449	*egc*
8416/2012	D1	4	nasal swab	t902	*egc*
8400/2012	D1	4	stool sample	t902	*egc*
8406/2012	D1	4	stool sample	t902	*egc*
8391/2012	D1	4	food (milk)	t902	*egc*
8392/2012	D1	4	vomit	t902	*egc*
8395/2012	D1	4	vomit	t902	*egc*
8396/2012	D1	4	stool sample	t902	*egc*
8397/2012	D1	4	stool sample	t902	*egc*
8390/2012	D1	4	food (milk)	t902	*egc*
8394/2012	D1	4	vomit	t902	*egc*
8419/2012	D2	4	nasal swab	t6346	*egc*
8414/2012	E	5	nasal swab	t056	*neg***
8420/2012	F	6	nasal swab	t318	*seg*
8417/2012	G	7	nasal swab	t059	*seg, sei, sen*
8405/2012	NT	-	vomit	t902	*egc*

*Tested for genes: *sea, seb, sec, sed, see, seg, seh, sei, sej, sel, sem, sen, seo, ser; egc*: *seg-sei-sem-sen-seo*
**neg: negative for toxin genes

epidemiological chain of this SFP outbreak: the source (food handlers), the suspected food (milk) and the patients.

The PFGE patterns were strongly correlated with SE gene profiles and *spa* types. One *S. aureus* vomit specimen harboring the same *spa* - type t902 with the majority of SFP strains and *egc* positive was nontypable using *SmaI* digestion.

For the strains that showed different *spa* types from the SFP clone, the following findings were available:

- two S. *aureus* isolates from nasal swabs were non-enterotoxigenic and exhibited the same *spa* type (t056), but had different PFGE patterns (PFGE types C and E < 60% similarity),
- one *S. aureus* isolate from vomit was methicillin-resistant, belonged to t127 *spa*- type, exhibited the *seh* gene and was unrelated with outbreak strains by PFGE analysis,
- in other *S. aureus* isolate from vomit that showed the *spa* type t3216 (PFGE pattern A < 60% similarity with pattern D), *sec* gene was found next to *sel* and *egc* genes,

- two isolates from nasal swabs (PFGE patterns F respectivly G < 60% similarity with pattern D) exhibited an incomplete form of *egc*: *seg*, and *seg*, *sei* and *sen* genes respectivly.

The most frequent SE gene profile was *egc*, containing: *seg, sei, sem, sen, seo* (20/25, 80%). This association is not unexpected because all these genes belong to the *egc* operon.

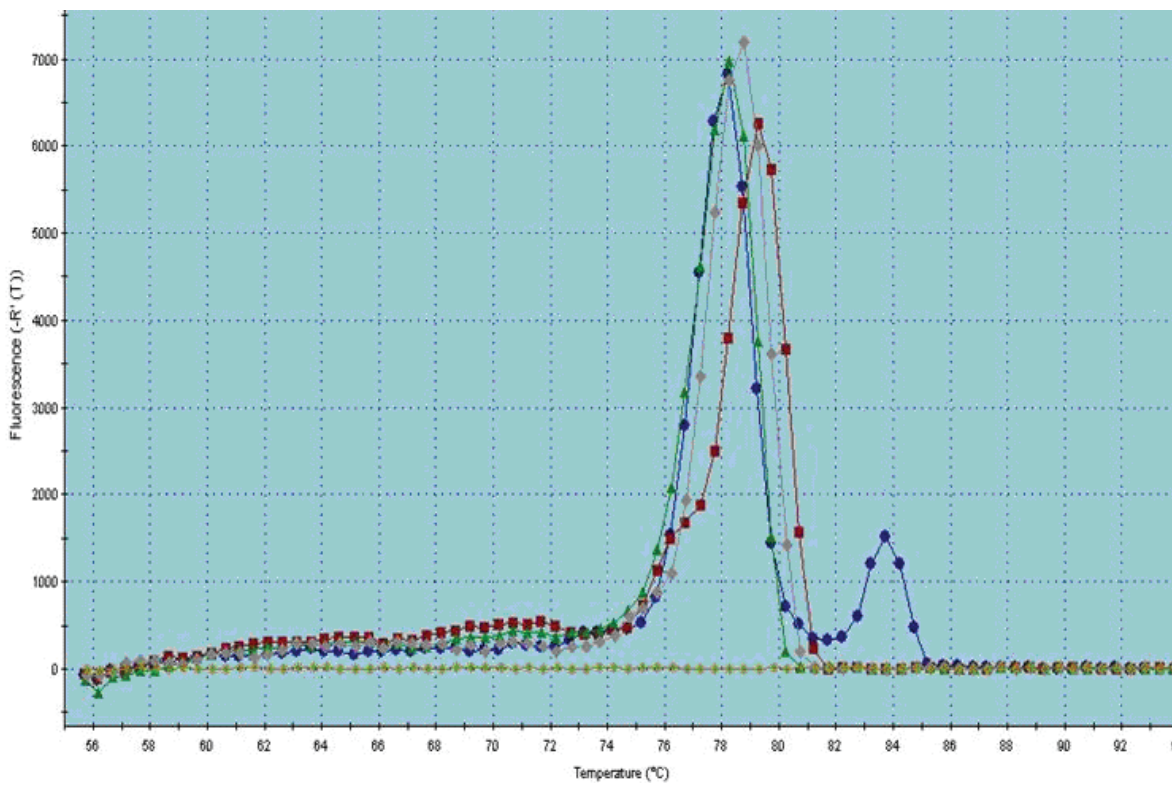
The *sea, seb, sed, see* encoding classical enterotoxins were not detected in any of the isolates, neither were the *sej* and *ser* genes.

The expression of *seg, seh, sei* and *sem* enterotoxin genes was confirmed by reverse transcription real-time PCR (Fig. 2). The results show a possible transcription of mRNA from these genes but do not indicate whether the strains will produce sufficient quantities of enterotoxins in food (2).

In our study, the frequency of the newly identified enterotoxin genes (80%) was higher than the one of classical genes. This indicates that the pathogenicity of the newly identified enterotoxins should be emphasized (4).

In this study, the presence of *egc* clusters in 20 out of 25 isolates is similar to studies from other countries like Poland (17), Spain (37) and Norway (38) that reported the presence of *egc* genes in food, associated to *S. aureus* contamination.

To our knowledge this is the first study published in Romania on the role of newly discovered staphylococcal enterotoxins in food borne outbreaks.



● *seg*,♦ *seh*, ■ *sei*, ▲ *sem*

Figure 2. Reverse real-time transcription PCR for amplification of *S. aureus seg, seh, sei* **and** *sem* **genes as determined by melting peak analysis on** *S. aureus* **8391 (***seg, sei, sem***) and** *S. aureus* **8393 (***seh***)**

For the first time, a report on the *egc* operon was performed by Blaiotta et al. in 2004 (16), in *S. aureus* strains isolated from meat and dairy products in Italy. A recent study from China reported that the occurrence of *egc* was higher than the classical SE genes in food (39). In 2007, Hwang *et al*. (40) revealed that the newly described SE or SE*l* genes, particularly those belonging to the *egc* cluster, were more frequently detected in *S. aureus* strains isolated from raw pork and chicken meat in Korea than genes encoding classical SEs.

Despite all this, *egc*-encoded SEs or SE*l*s have not been yet directly recognized in typical cases of SFP, although, SEG and SEI have been reported as the cause of chronic diarrhea in two malnourished neonates (41). In 2013, Omoe et al. confirmed in a primate model the emetic potential of SE*l*K, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P, and SE*l*Q (13).

In our study the SFP diagnostic was supported by: (i) isolation of *S. aureus* strains with the same enterotoxin genes profile from food and patients, (ii) isolation of *S. aureus* strains with the same enterotoxin genes profile from different patients, (iii) demonstration of *seg, seh, sei* and *sem* enterotoxin genes expression by RT- Real Time PCR and (iv) an identical or related *SmaI* macrorestriction pattern on PFGE analysis for *S. aureus* strains isolated from milk, patients and food handlers.

Conclusions

Routine microbiological investigations, in conjunction with clinical and epidemiological data, are useful when the staphylococcal etiology of a food poisoning outbreak is suspected.

As *Staphylococcus aureus* is ubiquitous, it may be frequently isolated from food, food handlers and patients, without being involved in a SFP. Molecular investigation made possible the confirmation of the staphylococcal foodborne outbreak and the tracking of the epidemiological chain by proving that 18/25 strains belonged to the same pulsotypes, *spa* type and toxigenic profile.

PFGE dendrogram, together with isolation of the same *spa*-type strains from food, food handlers and most of the patients support the etiological involvement of a t902, *egc* positive *Staphylococcus aureus* strain in this SFP.

To our knowledge, this is the first report of an *egc* positive, A, B, C, D, E enterotoxins negative *S. aureus* strain involvement in a SFP, in Romania.

The prevalence of these newly reported SE and SE-*like* toxin genes and their role in SFP outbreaks should be further investigated.

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Conflict of interest

We have no conflicts of interest to declare.

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