International Dairy Journal 42 (2015) 12–15

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Short communication

Enterotoxin genes, enterotoxin production, and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy

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ARTICLE INFO

Article history:
Received 27 August 2014
Received in revised form
14 October 2014
Accepted 18 October 2014
Available online 10 November 2014

ABSTRACT

A total of 227 *Staphylococcus aureus* colonies, isolated from 54 samples of raw milk and dairy products of bovine, ovine, caprine and bubaline origin were tested for the presence of genes coding for staphylococcal enterotoxins (SEs/SE*ls*) and for methicillin resistance. Ninety-three colonies, from 31 of the 54 samples (57.4%) and from 18 (69.2%) of the 26 farms of origin tested positive for SEs/SE*ls* genes. Most isolates harboured more than one toxin gene and 15 different toxinotypes were recorded. The most frequent were "*sec*" gene alone (28.6%), "*sea*, *sed*, *ser*, *selj*" (20%), "*seg*, *sei*" and "*seh*" (8.6%). The 77 colonies harbouring "classical enterotoxins" genes (*sea-sed*) were further tested for enterotoxin production, which was assessed for 59.2% of the colonies. Three methicillin-resistant *S. aureus* (MRSA) isolates were detected in three different ovine milk/dairy product samples (1.3%). All isolates belonged to *spa* type t127, sequence type 1, clonal complex 1, SCC*mec* type IVa.

1. Introduction

Staphylococcus aureus is involved in a wide variety of diseases in humans and animals and its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance (Argudin, Mendoza, & Rodicio, 2010). In 2009, the European Food Safety Authority underlined the increasing concern for Public Health represented by the presence of methicillin-resistant *S. aureus* (MRSA) in food producing animals, and recommended that further work should be performed on sampling, detection and quantification of MRSA carriage in both humans and animals, as well as on the contamination of food and the environment (EFSA, 2009). *S. aureus* is also considered a major foodborne pathogen (Hennekinne et al., 2010). Some strains are able to produce enterotoxins within foodstuff, causing staphylococcal food-poisoning (SFP), (Argudin et al., 2010). *S. aureus* enterotoxins (SEs) have been divided into 5 serological "classical types" (SEA, SEB, SEC, SED, and SEE), and among them SEA is considered as the main cause of SFP outbreaks in the United States, Japan, France, and UK (Argudin et al., 2010). In the last few years, new types of SEs (recently discovered SEs) and staphylococcal-like (SE*l*) proteins have been described (Hennekinne et al., 2010).

The aim of this work was to study *S. aureus* isolated from a variety of milk and dairy products produced in Central Italy from different animal species. The isolates were studied in terms of: (i) presence of genes coding SEs and SE*ls* by using Multiplex-PCR (M-PCR); (ii) expression in vitro of SEs by using a Reversed Passive Latex Agglutination (RPLA) assay; (iii) cefoxitin susceptibility for MRSA screening. Detected MRSA isolates were further phenotypically and genomically characterised.

2. Materials and methods

2.1. Staphylococcus isolation and identification

Between 2011 and 2013, 565 milk and dairy products samples were collected from bovine, ovine, caprine and bubaline farms (78) located in Central Italy. Samples included raw milk (428), thermised milk (9), curd (8), "Ricotta" cheese (7), yoghurt (8) and

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http://dx.doi.org/10.1016/j.idairyj.2014.10.009

cheese (105), also including typical/traditional unripened, soft ("stracchinato" cheese), "pasta filata" ("mozzarella" cheese), semi-hard ("caciotta" cheese) and hard cheese ("pecorino romano" cheese). All the samples were analysed for the enumeration of coagulase-positive staphylococci (CPS) according to ISO 6888-2/Amd1 (ISO, 1999/2003). Coagulase positive colonies were identified as *Staphylococcus spp*. by microscopic observation, Gram-staining, and catalase determination.

From each positive sample, suspected colonies (up to 5) were further analysed. *S. aureus* identification was performed by a modified species-specific PCR, using primers targeting *femA* gene (132 *bp*) (Mehrotra, Wang, & Johnson, 2000).

2.2. SEs/SEls gene detection and enterotoxins production

All *S. aureus* colonies were investigated for the presence of genes coding for 9 selected SEs (*sea, seb, sec, sed, see, seg, seh, sei, ser)* and 2 SE*l*s (*selj, selp*), according to what recommended by the European Union Reference Laboratory for coagulase-positive staphylococci (EU-RL CPS) by using two multiplex PCR protocols (M-PCR) (ANSES EU-RL CPS, 2009a, 2009b), as described by Bianchi et al. (2014). Every *S. aureus* colony positive for at least one of the "classical SEs" coding genes by PCR was tested for staphylococcal enterotoxins production (SEA-SED), performed by the RPLA method, using the kit SET-RPLA (TD 9000, Oxoid, Basingstoke, UK), according to the manufacturer's instructions.

2.3. Antimicrobial susceptibility testing

All *S. aureus* colonies were screened for methicillin resistance by cefoxitin disk diffusion method according to the criteria of Clinical Laboratory Standards Institute (CLSI). Results were interpreted following the performance standards for antimicrobial susceptibility testing (23rd informational supplement; CLSI., 2013). Methicillin resistant isolates were further tested for phenotypic susceptibility to β-lactams and other antimicrobials representative of the most relevant classes active against *Staphylococcus spp*. by the broth micro-dilution method in 96-well microtitre plates (Trek Diagnostic Systems, Westlake, OH, USA). Results (minimum inhibitory concentrations, MICs) were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST., 2014) epidemiological cut-offs.

2.4. Molecular characterisation

Phenotypically suspected MRSA isolates were further confirmed by molecular methods. The detection of *mec*A and *bla*Z genes by PCR assay was performed using primers and protocols described by Martineau et al. (2000) and Strommenger, Kettlitz, Werner, and Witte (2003).

MRSA isolates were genotyped by *spa* typing, multilocus sequence typing (MLST) and by typing/subtyping of the staphylococcal cassette chromosome *mec* (SCC*mec*) using multiplex PCR methods as previously described (Battisti et al., 2010).

3. Results and discussion

3.1. S. aureus isolates, SEs/SEls gene detection and enterotoxin production

From 54 out of the 565 samples tested (9.6%), a total of 227 *Staphylococcus spp*. colonies were obtained. Positive *Staphylococcus spp*. samples were from eleven ovine, nine bovine, three caprine, one bubaline, and two mixed bovine-ovine farms, for a total of 26 positive farms. Samples included raw milk (32), thermised milk (1) and cheese (21). All the 227 colonies were identified by PCR as *S. aureus* and further tested for the presence of SEs/SE*l*s genes by using M-PCR. Ninety-three colonies tested positive for the presence of one or more SEs/SE*l*s genes. These colonies derived from 31 of the 54 *S. aureus* positive samples (57.4%), collected from 18 of the 26 (69.2%) positive farms. In particular, SEs/SE*l*s genes were detected in isolates from 21 raw milk, 1 thermised milk, and 9 cheese samples. These results confirm that enterotoxigenic *S. aureus* can be commonly found in milk and dairy products, as reported in other studies conducted in Northern Italy (Bianchi et al., 2014) and Switzerland (Hummerjohann, Naskova, Baumgartner, & Graber, 2014), adopting a similar approach.

Many of our samples (11/31; 35.5%) yielded both SEs/SE*l*s-positive and SEs/SE*l*s-negative colonies at the same time, as previously described (Hummerjohann et al., 2014; Normanno et al., 2007), underlining the importance of testing more than one colony per sample. Using the PCR approach, the possibility of testing more colonies or even colony pools to increase the chances of detecting positive samples could be taken into account.

Of the 93 positive colonies originally isolated, the analysis of the gene profiles was carried out only on those obtained from the same sample and showing the same SEs/SE*l*s PCR profile, which were considered as single isolates. The enterotoxin gene profiles (toxinotypes) of the resulting 35 SEs/SE*l*s positive selected isolates, in relation with the species, the farm of origin and the type of sample, are shown in Table 1.

The SEs/SE*l*s genes most frequently detected were *sed*, present in 40% of the isolates, followed by *sec* (34.3%), *sea* and *selj* (both present in 31.4% of the isolates) and *ser* (28.6%). None of the isolates harboured *see* and *selp* genes. Toxinotypes composed by a single gene were observed in 16 of the 35 isolates (45.7%), while 19 (54.3%) harboured more than one toxin gene, displaying a remarkable heterogeneity. A total of 15 different toxinotypes were identified among the 35 isolates. The most frequent were "*sec*" gene alone (10/35; 28.6%), mainly present in isolates from small ruminants samples, "*sea, sed, ser, selj*" (7/35; 20%), of bovine and bubaline origin, followed by "*seg, sei*" and "*seh*" (8.6%).

Overall, one or more of the recently discovered SEs/SE*l*s genes were detected in 22 of the 35 isolates (62.9%), in most cases (16/22; 72.7%) associated with "classical SEs". Recent studies have shown that some of these toxins can be responsible for staphylococcal gastroenteritis outbreaks. In particular, SEH producing strains have been involved in SFP outbreaks (Ikeda, Tamate, Yamaguchi, & Makino, 2005; Jørgensen et al., 2005), while SER has been recognised to possess emetic properties at concentrations over 100 ng mL^{-1} (Lis et al., 2012).

However, also considering that the screening of the recently discovered SEs and SE*l*s in food is at present not routinely performed, it is still difficult to assess the threat that they could pose to public health and further investigations are needed to evaluate their contribution to the foodborne disease burden (Argudin et al., 2010; Bianchi et al., 2014).

Overall, 77 colonies out of 93 were found positive for the "classical SEs" coding genes (*sea, seb, sec,* or *sed*) by PCR and were all tested for enterotoxin production by the SET-RPLA kit. Some colonies presented more than one of the above mentioned genes simultaneously, for a total of 103 PCR positive results. The expression in vitro of SEA, SEB, SEC, SED in relation with the presence of the corresponding genes, is reported in Table 2. The frequent detection (61/103; 59.2%) of enterotoxin producing colonies indicates that a potential food safety risk associated with dairy products does exist, in particular when proper strategies to avoid *S. aureus* growth and SEs/SE*l*s formation in foods are not implemented (Normanno et al., 2005).

Table 1
SEs/SE*l*s gene profiles (toxinotypes) of the 35 selected *S. aureus* isolates in relation with the farm, the species of origin and the type of sample.[a]

SEs/SE*l*s gene profiles (toxinotypes)	Number of positive isolates per species of origin					Total number of positive isolates	Farm ID/species of origin (type of sample)
	Bovine	Ovine	Caprine	Bubaline	Bovine/ovine (cheese)		
sea	0	1	0	0	0	1	Farm: S/Ov (raw milk)
sec	1	4	3	0	2	10	Farms: B/Bov (raw milk); L/Ov (raw milk); O/Ov (raw milk); P/Ov (cheese); Q/Ov (raw milk); V/Cap (raw milk and cheese); Y/Mixed (ovine cheese); Z/Mixed (bovine/ovine cheese)
sed	1	0	0	0	0	1	Farm: B/Bov (raw milk)
seg	0	0	1	0	0	1	Farm: W/Cap (cheese)
seh	0	3	0	0	0	3	Farms: L/Ov (cheese); S/Ov (raw milk)
sea, sei	1	0	0	0	0	1	Farm: D/Bov (raw milk)
seb, sec	0	0	1	0	0	1	Farm: V/Cap (cheese)
sed, selj	1	0	0	0	0	1	Farm: A/Bov (raw milk)
seg, sei	2	1	0	0	0	3	Farms: C/Bov (raw milk); G/Bov (raw milk); M/Ov (cheese)
sea, sed, selj	1	0	0	0	0	1	Farm H/Bov (raw milk)
sea, sed, ser	1	0	0	0	0	1	Farms H/Bov (raw milk)
sec, seg, sei	0	1	0	0	0	1	Farm N/Ov (thermised milk)
sed, seg, sei	1	0	0	0	0	1	Farm G/Bov (raw milk)
sed, ser, selj	2	0	0	0	0	2	Farm H/Bov (raw milk)
sea, sed, ser, selj	4	0	0	3	0	7	Farms: H/Bov (raw milk); X/Bub (raw milk and cheese)

[a] Farms were identified with letters from A to Z. No positive SEs/SE*l*s isolates detected from samples of farms E, F, I, J, K, R, T, or U; abbreviations are: Bov, bovine; Bub, bubaline; Ov, ovine; Cap, caprine; Mixed, mixed bovine+ovine.

Table 2
Expression in vitro of "classical enterotoxins" (SEA, SEB, SEC, SED), as detected by the SET-RPLA, in relation to the presence of the corresponding genes as detected by M-PCR.

Enterotoxin	Number of colonies positive for the corresponding gene	Number (%) of enterotoxin producing colonies
SEA	31	16 (51.6%)
SEB	2	2 (100%)
SEC	32	20 (62.5%)
SED	38	23 (60.5%)

3.2. Phenotypic and genomic characterisation of methicillin resistant isolates

All the 227 *S. aureus* colonies were screened for cefoxitin susceptibility and three of them were found to be resistant (1.3%). All the three MRSA isolates, positive to both *mec*A and *bla*Z genes, were isolated from ovine samples and were all identified as MRSA belonging to *spa* type t127, ST1, clonal complex (CC) 1, SCC*mec* type IVa.

Information on host specific-MRSA of small ruminants is scant: MRSA strains *spa* type t843 ST130 and *spa* type t034 ST398 of ovine origin have been described by Eriksson, Espinosa-Gongora, Stamphøj, Larsen, and Guardabassi (2013), while Gharsa et al. (2012) isolated five MRSA *spa* type t044, ST153 CC80 from healthy sheep. The MRSA lineage identified in this work is usually considered as a human community-associated MRSA, but it has also been identified in cattle (Huber, Koller, Giezendanner, Stephan, & Zweifel, 2010; Hummerjohann et al., 2014; Juhász-Kasanyitzky et al., 2007; Pilla et al., 2012) and pigs (Franco et al., 2011; Hasman et al., 2010). The three isolates harbored a *SCCmec* element, previously described in both human and animal *spa* type t127 ST1 isolates (Franco et al., 2011). All our MRSA isolates harboured the *seh* gene, which is a common finding in isolates of human and animal origin (Franco et al., 2011; Hummerjohann et al., 2014). Antimicrobial susceptibility testing showed that the three MRSA displayed the same resistance pattern, being resistant to cefoxitin, penicillin, erythromycin, streptomycin, kanamycin and tetracycline. This co-resistance pattern (tetracycline, macrolide and aminoglycoside) has been frequently detected in both ST1 MRSA of human and animal origin in Italy (Battisti et al., 2010; Franco et al., 2011).

Two of our MRSA isolates were from two different "pasta filata" cheese samples produced by the same ovine dairy farm, and one from an ovine bulk tank milk sample from another farm. The two farms, both located in the province of Rome, were apparently not epidemiologically related.

The presence of MRSA in food is not routinely investigated (EFSA, 2009). In Italy, MRSA have been sporadically identified in bovine milk (Antoci, Pinzone, Nunnari, Stefani, & Cacopardo, 2013; Normanno et al., 2007; Pilla et al., 2012), while they have never been detected in surveys conducted on ovine, caprine or bubaline milk (Cremonesi et al., 2013; Morandi, Brasca, Andrighetto, Lombardi, & Lodi, 2010; Perillo et al., 2012; Spanu et al., 2013; Virdis et al., 2010). At a global level, only a few data are available on the presence of MRSA in dairy products. To our knowledge, they have been isolated from ovine pecorino cheese and bovine mozzarella in Italy (Normanno et al. 2007), from traditional cheese samples produced from goat or sheep's fatty milk in Iran (Shanehbandi, Baradaran, Sadigh-Eteghad, & Zarredar, 2014) and from ice cream in Turkey (Gucukoglu, Cadirci, Terzi, Kevenk, & Alisarli, 2013). In all the above mentioned studies, the genotypic characterisation of the strains was not performed. Recently, Hummerjohann et al. (2014) identified one MRSA out of 623 isolates from Swiss bovine raw milk cheese. The strain belonged to *spa* type t127, the same *spa* type identified in our study.

4. Conclusion

In conclusion, the occurrence of SEs/SE*l*s positive isolates in a high proportion of milk and dairy product samples, as well as the detection of recently discovered SEs/SE*l*s genes in more than half of our isolates, also from "ready to eat" products, is of concern and underline the need of standardised diagnostic methods to verify and quantify the presence of the "new" enterotoxins directly in food. In fact, the screening of the recently discovered SEs and SE*l*s in food is at present not considered by the European Union even from "ready to eat" products. This issue, as well as the lack of specific guidelines recommending the number of colonies to be tested for SEs/SE*l*s genes, either during routine testing or investigations of SFP outbreaks, should be taken into account by both microbiologists and risk assessors.

Finally, the identification and genetic characterization of three MRSA isolates from two ovine farms represents the first Italian report on the occurrence of MRSA in ovine milk, and to the Authors knowledge, the first genotyping of MRSA strains from ovine dairy products at international level. Although the prevalence was low, the isolation of MRSA from "ready to eat" food is of concern.

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