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High prevalence of clonally diverse *spa* type t026 *Staphylococcus aureus* contaminating rural eggshells

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Abstract

Purpose. The presence of *Staphylococcus aureus* in poultry and poultry products, including eggs, increases its potential to enter the food chain, resulting in foodborne diseases. In this context, eggshell colonization by staphylococci may represent a risk factor. This study aimed to investigate the contamination of rural eggshell by *S. aureus* and to characterize the key features of the isolated strains.

Methodology. Antibiotic resistance was assessed by disc diffusion. Resistant isolates were analysed by PCR for the identification of associated genetic determinants of resistance. PCR was also used to screen for the presence of genes coding for toxins, namely, *sea*, *sec*, *sei*, *sem*, *seo* and *tst*. The genetic characterization was extended by means of *agr* locus typing and *spa* typing.

Results. 34 *S. aureus* were isolated. Macrolide- and tetracycline-resistant strains were prevalent. All strains were susceptible to oxacillin, cefoxitin and trimethoprim-sulfamethoxazole. PCR screening for genes encoding enterotoxins detected several virulence patterns, which, together with *spa*-typing and *agr*-locus typing, allowed cluster analysis and the description of novel clones.

Conclusion. Continuous monitoring of staphylococci is needed also in rural or natural settings. Increasing the number of samples and expanding the geographical region will be needed to further extend the significance of the study.

INTRODUCTION

In rural areas of Marche region in Italy, many farmers have the traditional tendency to collect fresh eggs for domestic consumption. External eggshell contamination caused by poor hygienic conditions, manipulation and lack of veterinary controls could be a potential risk for food safety, increasing the risk of illness in humans. It is known that the shell becomes infected after laying when it is exposed to a contaminated environment because contact with the important reservoir of micro-organisms represented by the animal faecal material is unavoidable [1–3]. The eggshell microflora is notably heterogeneous; nevertheless Gram-positive bacteria are prevalent, probably for their ability to tolerate dry conditions. Penetration of micro-organisms from eggshell to egg content (horizontal transmission) is the most frequent contamination route for several species, including *Staphylococcus aureus*, predominantly found on eggshell [4–6]. This pathogen colonizes a large number of animal species and is considered a significant cause of avian disease. The presence of *S. aureus* in poultry and poultry products, including eggs, increases its potential to enter the food chain, resulting in foodborne diseases [7]. Thus, staphylococcal eggshell colonization may present a greater risk factor for egg contamination. The first step in this study was to isolate *S. aureus* from eggshells collected in five farms of a rural area. A major objective was then the analysis of virulence and antibiotic resistance profiles of isolates and molecular typing to investigate the clonal diversity and genetic features of circulating strains. To our knowledge, this is the first work investigating staphylococcal contamination of rural eggshells in Italy.

METHODS

Isolation and identification of strains

During the period of September to December 2014, 50 eggs were collected at five distinct collection times from five farms randomly selected in a rural area of 15 km^2 located in the province of Macerata, Italy (n=10 eggs for each farm). At the time of sampling, each farm was breeding between 5

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Abbreviations: MSSA, methicillin-susceptible *Staphylococcus aureus*; SFP, staphylococcal food poisoning.

and 10 hens. All flocks considered in this study were housed in a non-cage system and no cracked eggs were collected. Poultry flocks did not receive any antibiotic treatment. Each egg was sampled within few hours after laying using clean gloves, placed in a sterile bag, refrigerated, transported to the laboratory and analysed within 24 h. Eggs were separately subjected to a washing treatment in a sterile plastic bag with 10 ml maximum recovery diluent to obtain bacteria from the eggshell [8]. An aliquot of the diluent (100 µl) was plated on Baird-Parker agar to determine the presence of *S. aureus*. The plates were incubated at 37 °C for 24 h [9]. Whenever multiple strains were likely to be present, as per the colony morphology, more than one isolate per sample was collected and further analysed. After genomic DNA extraction using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), presumptive positive isolates were confirmed as *S. aureus* by PCR detection of thermonuclease gene *nuc* [10].

Antimicrobial resistance profile

Antibiotic resistance profiles of isolates were analysed by disc diffusion test in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[11]. Antimicrobial agents tested were: penicillin (1 U), oxacillin (1 µg), cefoxitin (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), fusidic acid (10 µg), clindamycin (2 µg) and erythromycin (15 µg). In antibiotic resistant isolates, associated resistance genes were amplified by PCR. Erythromycin-resistant isolates were screened for the presence of *ermC* and *msrA*, whereas the *blaZ* gene was explored in penicillin-resistant isolates and *tetM* for tetracycline-resistant isolates [12].

Molecular typing

Amplification of selected toxin genes (*sea*, *sec*, *sei*, *sem*, *seo* and *tst*) and amplification of four accessory gene regulators (*agr*) were carried out by PCR as previously reported [13, 14] using the primer sets shown in Table 1. The PCR products were analysed by agarose gel electrophoresis. Toxin genes as well as *agr* groups were assigned to each strain by comparing the product with a positive control included in each run. All isolates were subjected to staphylococcal protein A typing (*spa* typing) as described by Shopsin and colleagues [15]. The PCR products were purified using the PCR Clean-up Kit (Sigma-Aldrich) and sequenced. Sequences were analysed using Ridom SpaServer website (http://www.spaserver.ridom.de) developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/).

Analysis of data and statistics

Partitions were defined and analysed following Severiano *et al.* [16] and the literature cited therein. Server-based software for the analysis was found at the Comparing Partitions

Table 1. Oligonucleotide primers used in this work and references

Gene	Primer	Sequence (5′–3′)	Annealing temp. (°C)	Reference
sea	SEA-1	GAAAAAAGTCTGAATTGCAGGGAACA	63	[13]
	SEA-2	CAAATAAATCGTAATTAACCGAAGGTTC		
sec	SEC-1	GTAAAGTTACAGGTGGCAAAACTTG	63	[13]
	SEC-2	CATATCATACCAAAAAGTATTGCCGT		
seo	SEO-1	AGTTTGTGTAAGAAGTCAAGTGTAGA	58	[13]
	SEO-2	ATCTTTAAATTCAGCAGATATTCCATCTAAC		
sem	SEM-1	CTATTAATCTTTGGGTTAATGGAGAAC	60	[13]
	SEM-2	TTCAGTTTCGACAGTTTTGTTGTCAT		
sei	SEI-1	CTCAAGGTGATATTGGTGTAGG	61	[13]
	SEI-2	AAAAAACTTACAGGCAGTCCATCTC		
tst	TSST-1	TTCACTATTTGTAAAAGTGTCAGACCCACT	61	[13]
	TSST-2	TACTAATGAATTTTTTTATCGTAAGCCCTT		
nucA	nuc-F	GCGATTGATGGTGATACGGTT	55	[10]
	nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
agr I	agrI-F	ATCGCAGCTTATAGTACTTGT	55	[14]
	agrI-R	CTTGATTACGTTTATATTTCATC		
agr II	agrII-F	AACGCTTGCAGCAGTTTATTT	55	[14]
	agrII-R	CGACATTATAAGTATTACAACA		
agr III	agrIII-F	TATATAAATTGTGATTTTTTATTG	55	[14]
	agrIII-R	TTCTTTAAGAGTAAATTGAGAA		
agr IV	agrIV-F	GTTGCTTCTTATAGTACATGTT	55	[14]
	agrIV-R	CTTAAAAATATAGTGATTCCAATA		
spa	spa1113	TAAAGACGATCCTTCGGTGAGC	50	[15]
	spa1514	CAGCAGTAGTGCCGTTTGCTT		

website (http://www.comparingpartitions.info/index.php?link=Tool). Clustering was performed on observations by the Nearest Neighbor (Single Linkage) method (standardized) using the Statgraphic Centurion ver. XVII software package. The distance metric used was the Squared Euclidean. Briefly, the computation was made as to create one cluster from the total number of observations (n=34). The clusters were groups of observations with similar characteristics. To form the cluster, the procedure began with each observation in a separate group. It then combined the two observations which were closest together to form a new group. After recomputing the distance between the groups, the two groups then closest together were combined. This process was repeated until only one group remained.

RESULTS AND DISCUSSION

The presence of *S. aureus* was recorded in 26 (52 %) out of 50 sampled eggshells. A total of 34 strains of *S. aureus* were isolated (farm I=12 strains, farm II=5, farm III=3, farm IV=5 and farm V=9). We mainly found one strain associated with a single eggshell, although nine (35 %) eggshells carried two different strains. The results of genotypic and phenotypic profiling are summarized in Table 2.

Toxin genes and antibiotic resistance were variably distributed among strains from different farms. Oxacillin-resistant strains were not detected and were thereby classified as methicillin-susceptible *S. aureus* (MSSA). All strains were also susceptible to cefoxitin and trimethoprim-

Table 2. Genotypic and phenotypic profiles of strains of *S. aureus* isolated from eggshells in this study

Strain*	Farm	Toxin gene	Antibiotic resistance†	*agr* group	*spa* type
$E1_a$	I	none	ERY-TE	3	t026
$E1_b$	I	*sea*	TE	3	t026
E2	I	*sea*	DA-ERY-TE	3	t026
$E3_a$	I	none	PEN-ERY	3	t026
$E3_b$	I	none	None	3	t026
$E4_a$	I	none	PEN-TE	3	t026
$E4_b$	I	*sea*	TE	3	t026
$E5_a$	I	none	PEN-TE	3	t026
$E5_b$	I	none	TE	3	t026
$E6_a$	I	none	TE	1	t026
$E6_b$	I	none	PEN-ERY	1	t026
E7	I	none	PEN-ERY-TE	1	t2493
E8	II	none	PEN-ERY-TE-DA	1	t026
E9	II	*tst-seo*	PEN-ERY-TE-FC	3	t012
E10	II	*sem*	PEN	1	t026
E11	II	*sem-seo*	PEN	1	t026
E12	II	*sem-seo*	PEN	1	t026
E13	III	*sem*	PEN	2	t002
E14	III	*sem-seo*	PEN	1	t091
E15	III	*sec-sem-seo-tst*	PEN-DA	3	t026
E16	IV	*sem-seo*	PEN	1	t5845
E17	IV	*sem-seo*	PEN	1	t026
$E18_a$	IV	*sem-seo*	PEN	2	t209
$E18_b$	IV	*sec-sem-seo-tst*	PEN	2	t091
E19	IV	*sem-seo*	PEN	1	t026
E20	V	*sea-sec-sem-tst*	PEN	3	t030
E21	V	*sea-sec-sem-tst*	PEN	3	t002
$E22_a$	V	*sem*	PEN-FC	1	t026
$E22_b$	V	*sem-seo*	PEN	3	t4558
$E23_a$	V	*sea-sem*	PEN	3	t030
$E23_b$	V	*sea-sec-sem-seo*	PEN	1	t026
$E24_a$	V	*sem-seo*	PEN	1	t026
$E24_b$	V	*sea-sec-tst*	PEN	1	t030
E25	V	*sem*	PEN	1	t030

*Subscript letters a and b indicate multiple strains isolated from the same eggshell.

†PEN, penicillin; ERY, erythromycin; FC, fusidic acid; DA, clindamycin; TE, tetracycline.

sulfamethoxazole. The highest resistance rate was towards penicillin (79 %), followed by tetracycline (32 %) and erythromycin (21 %) (Table 3). Importantly, only one strain was susceptible to all antimicrobial agents considered in this study whereas the remaining isolates showed 11 different resistance profiles. Most resistance profiles (64 %) included only one isolate, and four profiles were shared by three or more isolates.

Subsequent analysis of antibiotic determinant genes revealed that all penicillin-resistant strains carried *blaZ*, all tetracycline-resistant strains carried *tetM* and all erythromycin-resistant strains carried *msrA*. Our data confirmed the wide spread of penicillin resistance among *S. aureus* [17–19], and the resistance rate to erythromycin [20] and tetracycline [21] in poultry MSSA strains.

The most frequent virulence gene was *sem* (56 %), followed by *seo* (38 %) and *sea* (24 %), while *sei* was not detected. Of the 34 strains of *S. aureus* tested, 24 (71 %) exhibited at least one toxin gene, six isolates (18 %) carried three or more toxin genes, and overall, nine different genetic profiles were observed (Table 4).

The accessory gene regulator (*agr*) is considered an important regulatory component involved in the control of virulence factor expression [22]. For this reason, the classification of strains of *S. aureus* on *agr* groups has been considered. The distribution of *agr* groups was extremely variable among the strains collected from the same farm. The most frequent *agr* group was *agr* group I, accounting for 47 % of the isolates, followed by *agr* group III represented by 44 % of isolates. Strains belonging to the two most frequent *agr* groups showed greater combination of genes encoding three or more toxins (Table 4). As expected by the non-nosocomial origin of the isolates, no *agr* group IV-positive strain was detected in our study and there was a very low prevalence of *agr* group II-positives (9 % of all isolates).

The distribution of *spa* types is also shown in Table 2. A total of 29 (85 %) strains were grouped into nine different *spa* types, varying in length between two (t5845) and ten (t012; t002; t091) repeats, the most prevalent being t026 (21 strains – 62 %). This prevalence of *spa* type t026 strains is significantly higher than the global prevalence derived from data recorded into the SpaServer (0.51 %). This apparent discrepancy may be speculatively explained by the source of the strains, which was animal in our study and mainly human in the records within the SpaServer database [23–25]. Conversely, the absolute prevalence of MSSA t026 strains in our cohort correlated with the net prevalence of MSSA t026 strains reported globally. It is worth mentioning that this is the first description of t026 strains from Italy as per the SpaServer database (last date accessed: 08 May 2017) and a comprehensive search of the published literature databases. The analysis of *spa* types through the phylogenetic tree, largely reported in the literature [26, 27], revealed that although several t026 strains were from Farm I (n=11), they did not belong to the same clone (Fig. 1). As a matter of fact, the Simpson Index of Diversity for the resistance profile (0.855; Confidence Interval 95 % [CI95 %]: 0.719–0.990) counted six different partitions. Also the Wallace's coefficients with jack-knife pseudo-values CI95 % were determined to provide adirectional information about the relation between 'Farm' and '*spa* type' partitions. Coefficients were 0.293 (CI=0.000–0.663) for 'Farm' to '*spa* type' and 0.132 (CI=0.000–0.322) for '*spa* type' to 'Farm', meaning that if two strains are in the same cluster by 'Farm' they have about 30 % chance of having the same *spa* type, while conversely, this was about 13 %. Hence, it may be concluded that the high occurrence of t026 in the population was not due to sampling errors or to the spread of a single clone. Additionally, some strains belonging to a *spa* type different from t026 were sharing with t026 strains the same pattern related to the other traits considered. That is the case for the cluster largely represented by t026 strains comprising also E14-t091 and E16-t5845 and that of the cluster containing E10-t026 together with E25-t030. Four isolates were typed as t030, which is usually associated with methicillin resistance [28–30]. A single strain was detected for each one of the following types: t2493, t012, t5845, t209 and t4558. As reported by the SpaServer, the global presence of t2493, t5845 and t4558 types is very low (<0.01 %). Instead, the

Table 3. Antibiotic resistance rates and associated resistance genes

Antibiotic	No. of isolates (% of total)	Gene*
Penicillin	27 (79)	*blaZ*
Tetracycline	11 (32)	*tet*(M)
Erythromycin	7 (21)	*msr*A
Clindamycin	3 (9)	–
Fusidic acid	2 (6)	–

*Clindamycin and fusidic acid resistance genes were not investigated.

Table 4. Relationship between genotypic profile of toxin genes and *agr* groups

Toxin genes profile	No. of strains	*agr* group
No toxin genes	6	*agr* III
	4	*agr* I
sea	3	*agr* III
sem	3	*agr* I
	1	*agr* II
tst + *seo*	1	*agr* III
sea + *sem*	1	*agr* III
sem + *seo*	7	*agr* I
	1	*agr* II
	1	*agr* III
sea + *sec* + *tst*	1	*agr* I
sec + *sem* + *seo* + *tst*	1	*agr* II
	1	*agr* III
sea + *sec* + *sem* + *tst*	2	*agr* III
sea + *sec* + *sem* + *seo*	1	*agr* I

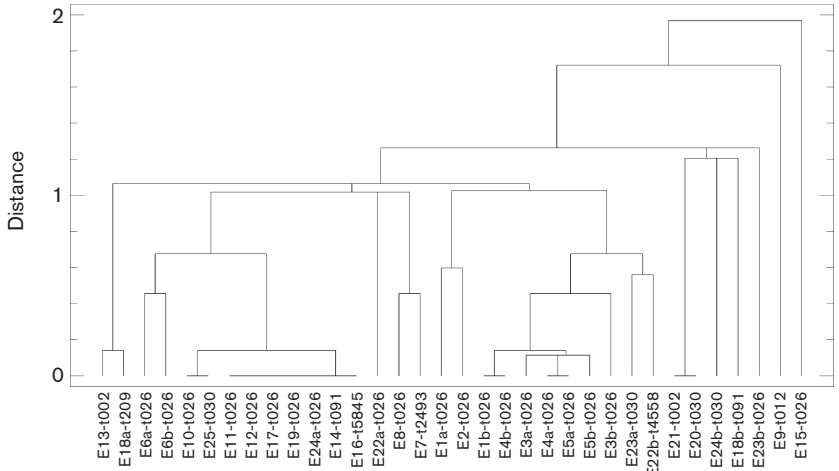

Fig. 1. Dendrogram showing the distances between strains belonging to different *spa* types based on *agr* group, toxin genes and antibiotic resistance profiles.

relative global frequency of the remaining *spa* types detected (t012, t209, t002 and t091) was 1.62, 0.20, 6.89 and 0.92 %, respectively. All of these showed a wide geographic distribution in Europe as reported by Grundmann *et al.* [31]. Specifically, t002 and t012 have been reported as the most frequent *spa* types isolated among European MSSA strains and they were associated to ST5-ST231 and ST30, respectively. Finally, t209 (ST109) was the second most frequent *spa* type isolated among MSSA strains in Belgium whereas t091 (ST7) was frequently recorded in Austria and in Hungary [31]. In addition, two of the types detected in our MSSA isolates (t002 and t091) were included into the world's 20 most frequent types recorded in the Ridom SpaServer. No information about multilocus sequence typing (MLST) was available for t2493, t5845 and t4558 according to the Ridom database. The association between different *spa* types to the same sequence type confirmed the high discriminatory power of the *spa* typing method [32].

Growing consideration has been given to *S. aureus* in poultry products including eggs. In recent years, the European Food Safety Authority reported an increasing number of food poisoning cases caused by consumption of eggs whose shells were contaminated by this pathogen. Our results confirmed the occurrence of *S. aureus* on eggshell, and the possibility of egg content contamination cannot be excluded. However, a comparison between farms is difficult, because the *S. aureus* occurrence varies according to the geographic areas and the hygiene conditions. There is evidence that animal foods contain several resistant bacteria; thus detection of eggshells carrying multidrug-resistant *S. aureus* strains poses risks for consumers in that they may be exposed via contact or egg consumption [7]. In our study, most strains were resistant to different antibiotics and carried several genes encoding enterotoxins whose presence is often associated to serious human staphylococcal food poisoning (SFP) [33]. Although *sea* is the enterotoxin gene most frequently found among SFPs [34–37], *sem* was the predominant gene in our study and was detected alone or in conjunction with other genes. *sem* belongs to the enterotoxin gene cluster (*egc*) that is frequently detected in strains isolated from food samples in various countries [38].

In conclusion, this study offers examples on how different the features of staphylococcal clones might be depending on the source of isolation, human or animal (see the high prevalence of *spa* type t026 and MSSA t030). Interestingly, the presence of strains with minor *spa* types (t2493, t5845, and t4558) focuses attention on the possible derivation of certain strains, only occasionally isolated from human samples, from animals or animal products. Notwithstanding the modest size of the study, the richness and variability of the genetic repertoire presented by strains of *S. aureus* isolated from eggshell represents a clear potential risk for consumer health once the egg content becomes contaminated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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