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Staphylococcus aureus isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes

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Abstract

Staphylococcus aureus (*S. aureus*) is recognized worldwide as one of the major agents of dairy cow intra-mammary infections. This microorganism can express a wide spectrum of pathogenic factors used to attach, colonize, invade and infect the host. The present study evaluated 120 isolates from eight different countries that were genotyped by RS-PCR and investigated for 26 different virulence factors to increase the knowledge on the circulating genetic lineages among the cow population with mastitis. New genotypes were observed for South African strains while for all the other countries new variants of existing genotypes were detected. For each country, a specific genotypic pattern was found. Among the virulence factors, *fmtB*, *cna*, *clfA* and leucocidins genes were the most frequent. The *sea* and *sei* genes were present in seven out of eight countries; *seh* showed high frequency in South American countries (Brazil, Colombia, Argentina), while *sel* was harboured especially in one Mediterranean country (Tunisia). The *etb*, *seb* and *see* genes were not detected in any of the isolates, while only two isolates were MRSA (Germany and Italy) confirming the low diffusion of methicillin resistance microorganism among bovine mastitis isolates. This work demonstrated the wide variety of *S. aureus* genotypes found in dairy cattle worldwide. This condition suggests that considering the region of interest might help to formulate strategies for reducing the infection spreading.

Keywords: mastitis; dairy cow; *S. aureus*; genotypes, virulence genes

Introduction

Staphylococcus aureus continues to be one of the most prevalent pathogens causing intramammary infections (IMI) in dairy cows. It's a worldwide pathogen recognized as a cause of subclinical

77 infections, resulting in increased somatic cell count (SCC), but may also cause clinical mastitis.
78 Staphylococcal mastitis is a major problem in dairy industry, affecting animal health and causing
79 economic losses of up to € 300 per cow per year, due to the reduced milk quality and production [1-
80 2]. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission usually
81 occurs from cow to cow during milking.

82 Successful infection depends on virulence factors produced by *S. aureus.* A wide spectrum of
83 secreted and cell surface-associated virulence factors can be expressed to promote adhesion to the
84 host extracellular matrix components, damage host cells, and fight the immune system [3]. At least
85 25 different toxins (such as enterotoxins SEA to SEQ, toxic shock syndrome toxin-1 TSST-1,
86 exfoliative toxins Eta, Etb), 15 microbial surface components recognizing adhesive matrix
87 molecules, which are important for adhesion to tissues (such as clumping factor A *clfA*, intercellular
88 adhesion genes *icaA* and *icaD*), 20 immune evasion molecules (such as protein A, coagulase,
89 haemolysins and leucocidins, factors associated with suppressing innate immunity) and several
90 other *S. aureus* virulence factors are known. Some virulence factors are expressed by genes that are
91 located on mobile genetic elements called pathogenicity islands (*i.e.*, TSST and some enterotoxins)
92 or lysogenic bacteriophages (*i.e.*, Panton-Valentine Leucocidin, PVL) and others such as the
93 staphylococcal complement inhibitor, *scn*, the chemotaxis inhibitory protein, *chp*, and
94 staphylokinase, *sak*, are integrated in the bacterial chromosome [4]. Furthermore, *S. aureus* can also
95 acquire the staphylococcal cassette chromosome SCC*mec*, giving rise to methicillin-resistant *S.*
96 *aureus* (MRSA) [5]. In fact, the expression of the *mecA* or *mecC* gene in *S. aureus* confers
97 resistance to most of β-lactams, drugs which are frequently used for treatment of mastitis [6].

98 The determination of the origin of the *S. aureus* isolates involved in the aetiology of bovine mastitis
99 is highly relevant from the epidemiological point of view. In such a context, the precise
100 characterization of this pathogen provides monitoring of the bacterial strains dissemination among
101 animal populations.

Over the past two decades, a wide range of phenotyping and genotyping methods have been used or developed for *S. aureus* including, but not limited to, ribotyping, RAPD-typing, PFGE, MLST, spa-typing, RS-PCR, coagulase gene RFLP, MLVA, micro-arrays and whole genome comparisons [7-8-9-10-11]. Many molecular epidemiological studies have been based on the use of selected targets in the genome, giving rise to banding patterns based on restriction- or primer binding sites, or to allelic profiles for housekeeping or virulence genes [12]. Such studies continue to be useful diagnostic tools when the aim is to understand pathogen sources and transmission mechanisms. Moreover, among the genotyping methods, the RS-PCR showed to be accurate, rapid and inexpensive with a discriminatory power like the other more-recognized genotyping methods [13].

The aim of this study was to genotype by RS-PCR and compare the molecular-epidemiologic profiles of a large world collection of *S. aureus* isolates to deepen the knowledge on the circulating genetic lineages among the cow population with mastitis.

Results

In this study, a total of 120 isolates collected from eight different countries were genotyped by RS-PCR and analyzed for 26 virulence factors related to *S. aureus* pathogenicity, such as genes related to host adhesion and invasion (*clfA*, *cna, fmtB*), genes that have the potential to interfere with host defense mechanisms (*tsst*, *scn*, *chp*, *sak*, enterotoxins from *sea* to *sel* and leukotoxins), and the gene encoding the acquisition of methicillin resistance (*mecA*).

RS-PCR Genotyping

New genotypes comprising GTAR, GTBZ, and GTCA were observed for South African strains (Table 1). For all the other countries, at maximum new variants of existing genotypes were detected. They included GTI^{V}, GTI^{VI} (Argentina), $GTAQ^{I}$, $GTBN^{I}$, $GTBN^{II}$, $GTBY^{I}$ (Brazil), $GTAO^{I}$, $GTAO^{II}$ (Colombia), GTR^{XIII} (Italy), GTC^{V} and GTI^{V} (New York State). For each country, a specific genotypic pattern was found. Major genotypes with their variants were combined into

genotypic clusters (CL) [14]. For Argentina (Table 1) it mainly consisted of CLI (56 % of GTI variants) and CLR (25% of GTR variants), whereas for Brazil CLBN (20% of GTBN plus variants) and CLBY (40% of GTBY plus a variants) were most prominent. The Colombian strains were mainly positive for GTAO and its variants (CLAO, 60%). In the case of Germany and Italy, the most prevalent genotypes were GTC^{I}, GTR plus variants, and GTB, combined into CLC (30%), CLR (64.7%) and CLB (29.4%), respectively. Finally, the main genotypes observed for the South African and Tunisian strains were GTR and its variants (CLR, 45%), whereas the American strains were mainly positive for GTC and variants of it (CLC, 70.6%). In conclusion, cluster C was observed mostly in Germany and New York State, while CLR was widely disseminated in seven countries; especially it was frequently detected in Argentina, Germany, Italy, South Africa and Tunisia but less in Colombia and New York State.

All the existing genotypes including their variants such as GTC and GTC^{I} had been previously isolated from bovine intramammary infection or bovine milk. Exceptions were GTBH (sandwich with Mozzarella) and GTAO (human nasal carriage).

Virulence genes

All the 120 isolates analyzed in this study were positive for coagulase (*coa*) and thermonuclease (*nuc*) genes, but negative for a gene involved in host cell invasion, the exfoliative toxin (*etb*), and for SEB and SEE enterotoxins. The distribution of the virulence genes for each country is described in detail below.

Argentina

As reported in Table 2, all the Argentinian isolates were positive for a leucocidin (*lukE-lukD*) and for an enterotoxin (*sei*), but negative for the gene encoding exfoliative toxin (*eta*), for *mecA*, *sel* and *sej*. All strains were also negative for two mobile genetic element genes (*chp*, *scn*), while 5 carried *sak*.

Out of 16 isolates, 15 (93.7%) had the genes encoding for *lukE* and *clfA*, 14 (87.5%) for a cell wall-associated protein (*fmtB*), 13 (81.2%) harboured the genes encoding for collagen-binding protein (*cna*), *lukM* and Panton-Valentine leucocidin *lukSF-PV*, whereas 5 (37.5%) were positive for *sak* and/or for *tsst*, respectively.

All the 16 isolates were enterotoxigenic, harbouring at least one of the genes coding for A, C, D, G and H enterotoxins genes. Three isolates from 3 different farms were positive for 5 different enterotoxins (combination of *sea*, *sec*, *seg*, *seh* and *sei* or *sea*, *sed*, *seg*, *seh* and *sei* or *sea*, *sed*, *seg*, *seh* and *sei*) while 8 isolates from 8 different farms were positive for 4 enterotoxins (combination of *sed*, *seg*, *seh* and *sei* or *sea*, *seg*, *seh*). Four isolates, collected in 4 different farms, were positive for 3 enterotoxins genes (combination of *sea*, *seg* and *sei* or *seg*, *seh* and *sei*) and 1 isolates for 2 different enterotoxins genes (*seh*, *sei*).

Brazil

Isolates collected from Brazil were all positive for *fmtB*, *cna*, *clfA* and for the genes encoding leucocidins (*lukE*, *lukE-lukD*, *lukM*, *lukSF-PV*) (Table 3). All the Brazilian isolates were negative for genes carried on mobile genetic elements and usually present in isolates involved in human infections, such as *chp*, *scn*, and *sak*. Moreover, they were negative for *tsst*, *eta*, *mecA*, and *sec*, *sed*, *sel*, *sej*. Out of 15 isolates, 5 (33.3%) were positive for *seh*, 8 (53.3%) for both *sea* and *seh*, while a single isolate (6.6%) harboured other 2 enterotoxin genes (*seg*, *sei*).

Colombia

As shown in Table 4, all the Colombian isolates were positive for *lukE-lukD* and *cna*, but negative for *chp*, *tsst*, *eta*, *mecA* and *sec*, *sel*, *sej*. Out of 15 isolates, 14 (93.4%) were positive for *clfA* and *fmtB* genes, 13 (86.7%) for *lukSF-PV*, 10 (66.7%) for *sak* and *lukM*, and 7 (46.7%) for *scn*. Fourteen (93.3%) isolates were enterotoxigenic harbouring at least one of the genes *sea*, *sed*, *seg*, *sei* or *seh*.

The most frequently detected genes were *seh* (93.3%) and *sea* (86.6%), followed by *sei* (26.6%) and *seg* (20%). One isolate harboured all the 5 enterotoxin genes (*sea*, *sed*, *seg*, *seh* and *sei*); 2 other isolates coming from 2 different farms harboured 4 enterotoxin genes (*sea*, *seg*, *seh* and *sei*) and 1 isolate 3 enterotoxin genes (*sea*, *seh* and *sei*). Finally, 9 isolates, from 6 different farms, had the combination of genes encoding for SEA and SEH.

Germany

All the German isolates were positive for *lukE* and *cna*, but negative for the mobile genetic element genes (*chp*, *scn*, *sak*), for *eta*, *lukSF-PV* and for enterotoxin genes *sed*, *seh*, *sel*, *sej* (Table 5). Out of 17 isolates, one (6%) harboured the *mecA* gene, 4 (23.5%) the *tsst*, 13 (76.5%) the *fmtB*, 15 (88.2%) the *lukM* and 16 (94.1%) both *clfA* and *lukE-lukD* genes.

Fifteen isolates out of 17 (88.2%), collected from 15 different farms, were enterotoxigenic, harbouring at least one of the genes coding for A, C, G and I enterotoxins. The most frequently detected genes were *sea* (88.2%) and *seg* (58.8%), followed by *sei* and *sec* (29.4%). Two isolates harboured all the 4 enterotoxin genes (*sea*, *sec*, *seg*, and *sei*); 3 and 8 other isolates harboured 3 (*sea*, *sec*, and *seg*) or 2 genes (combination of *sea* and *seg*, or *sea* and *sei*), respectively.

Italy

All the Italian isolates were positive for *lukE, lukE-lukD*, *cna* and *fmtB*, but negative for *chp*, *eta*, *lukSF-PV* and *seh*, *sel* enterotoxin genes (Table 6). Out of 17 isolates, 14 (82.3%) were positive for *clfA* and 9 (53%) had the gene encoding *lukM*. One isolate (6%) was positive for both *scn* and *sak* genes, and other two different isolates were positive for *tsst* (6%) and *mecA* (6%), respectively.

Fourteen isolates out of 17 (82.3%) were enterotoxigenic, harbouring at least 1 of the genes coding for A, C, D, G, I and J enterotoxins. The most frequently detected genes were *sed* (82.3%) and *seg* (70.5%), followed by *sej* (64.7%), *sea* (58.8%) and *sei* (47%). Six isolates harboured 5 enterotoxin genes (combination of *sea*, *sed*, *seg*, *sei* and *sej,* or *sea*, *sed*, *seg*, *sec* and *sej*); 4 other isolates

206 harboured 4 enterotoxin genes (combination of *sea, sei, sed* and *seg*, or *sei, sed*, *seg* and *sej* or *sea,*
207 *sed*, *sej* and *seg*). Moreover, 2 isolates harboured 3 different enterotoxins (*sea*, *sed* and *seg*) and 2
208 isolates, from the same farm, a combination of *sed* and *sej*.
209
210 *NewYork State*
211 As reported in Table 7, all the New York State isolates were positive for *lukE-lukD*, but negative for
212 *chp*, *scn*, *sak, tsst*, *eta*, *mecA* and *sec*, *sel*, *seh*, *sej*. Out of 17 isolates, 15 (88.2%) were positive for
213 *cna* and *lukE*, while 13 (76.4%) and 9 (53%) were positive for *lukM* and *clfA* genes, respectively. In
214 addition, 6 isolates (35.2%) and 2 (12%) had the *fmtB* and *lukSF-PV* genes, respectively. Only one
215 isolate was not enterotoxigenic; the remaining 16 isolates (95%) harboured at least one of the genes
216 encoding SEA, SED, SEG, SEI enterotoxins. Five isolates, collected from 5 different farms, had all
217 the enterotoxin genes (*sea*, *sed*, *seg*, *sei*); 6 isolates, from 6 different farms, harboured 3 genes
218 (combination of *sea*, *sed* and *seg* or *sea*, *seg* and *sei* or *sed*, *seg* and *sei*). Five isolates, from 4
219 different farms, had 2 enterotoxin genes (combination of *sed* and *seg* or *seg* and *sei* or *sed* and *sei*).
220
221 *South Africa*
222 As reported in Table 8, all the South African isolates were positive for *sak*, *cna*, *lukE-lukD*, *lukE*
223 genes. All the isolates were negative for *chp*, *mecA*, *tsst* and for *sec*, *sed*, *seg*, *sej* and *sel*. In
224 addition, 10 (90.9%) out of 11 isolates were positive for *fmtB*, 7 (63.7%) for *clfA*, 3 (27.3%) for
225 *lukSF-PV*, 2 (18.2%) for *lukM* and 1 (9%) for *eta* genes, respectively. Ten isolates, recovered in 9
226 different farms, were enterotoxigenic and positive for both *sea* and *seh* genes; out of them, 3
227 isolates from 2 different farms, harboured also the *sei* gene.
228
229 *Tunisia*
230 The Tunisian isolates were all positive for *fmtB*, *cna* and *clfA* genes, but negative for *eta*, *mecA*,
231 *lukSF-PV* and *sea*, *sed*, *seg*, *sei*, *sej* (Table 9). Out of 12 isolates, 11 (91.6%) harboured leucocidin

genes (*lukM*, *lukE*, *lukE-lukD*). Six isolates (50%) were positive for at least one gene of the immune evasion cluster with the combination of *chp*, *scn* and *sak* for 2 isolates, *scn* and *sak* or *chp* and *scn*, respectively, while the remaining 2 isolates harboured only the *chp* gene. Moreover, 4 isolates from 4 different farms, were enterotoxigenic harbouring *sec* and *sel* (2 isolates) or *seh* genes (2 isolates).

Discussion

Pathogenic factors of *S. aureus* enable this bacterium to attach, colonize, invade and infect the host tissue. In this study, *S. aureus* isolates, collected from eight different countries, were investigated using RS-PCR genotyping and PCR analysis for the carriage of different virulence factors to examine the epidemiology of this microorganism.

The samples were obtained from collections of the collaborators, allowing a first overview about the presence of the various staphylococcal subtypes among countries. Three new genotypes were observed for South Africa whereas new variants were found in Argentina, Brazil, Colombia, Italy and New York State. As previously described [14], GTB was observed only in Europe (Italy) while CLR and CLC clusters were observed throughout America, Europe and Africa; particularly CLR was detected in each country involved, except for Brazil.

And more, as previously described [15], *S. aureus* isolates harbouring genes coding for clumping factor (*clfA*), a cell wall-associated protein (*fmtB*), and collagen-binding protein (*cna*) have a greater capability to adhere to extracellular matrix proteins, essential for colonization and the establishment of infections. Our results indicated that, except for the American isolates with a lower presence of *fmtB* and *clfA* genes, in the other seven countries these genes were widely present in the circulating isolates particularly in Brazilian and Tunisian ones.

And more, according to previous studies [11-13-15], except for Brazil, Germany and USA, the remaining countries showed isolates encoding at least 2 virulence factors out of staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein of *S. aureus* (*chp*) and staphylokinase (*sak*). These virulence factors show activity prevalently against the human innate immune system

but their presence among isolates recovered in herds with high prevalence of *S. aureus* mastitis suggests their involvement also in bovine mammary gland immune response [16], and should be further studied, especially in Colombia and Tunisia where this gene cluster is quite common [17]. In a previous study [18], human strains were grouped in 7 immune evasion cluster (IEC) types, depending on the presence of 2 out of the 3 genes, in association or not with *sea* or *sep*. Unlike Colombian, Italian, South African strains and Tunisian isolates, the Argentinian ones carried only one gene, *sak*, showing a clear distance from human strains. Among the isolates from the other countries, uniquely the Tunisian strains testing positive for a IEC, did not harbor *sea*.

Superantigens, especially enterotoxins, have been suggested to play a role in the development of mastitis, for instance by creating an attractive environment for colonization [19] since they are more often identified in *S. aureus* isolated from cows with mastitis than in isolates from healthy cows or from the environment [20]. As a result, enterotoxins support the pathogenesis of *S. aureus* compromising mammary gland immune response and susceptibility to antibiotics resulting in the onset of many diseases [21]. In this study, *sea* and *sei* were the main enterotoxins genes present in all countries except for Tunisia (prevalence between 50 and 90 %). While *seh* gene had a frequency higher than 90% in Argentinian, Brazilian, Colombian and South African isolates, *sej* and *sel* genes were carried only by Italian and Tunisian isolates, respectively. Among the 120 isolates analyzed, only 17 (14%) were not enterotoxigenic (1 from Argentina, 1 from Colombia, 2 isolates from Germany, 3 from Italy, 1 from New York State, 1 from South Africa, and 8 from Tunisia). The remaining 103 isolates (86%) harboured a combination of at least 2 up to 5 enterotoxins with the linkages between *sea*, *sed*, *seg* and *seh* confirming their predominance in cows, as previously described [22-23-24-25]. The absence of the enterotoxin genes *seb* and *see* in our isolates was in accordance with previous results [15-23-26-27].

Here, among all the isolates we did not find the presence of *etb* exfoliative gene and only one isolate from South Africa was positive for *eta* gene. These results agree with previous studies conducted in different countries [28-29-30], showing that *S. aureus* isolates from animals with

284 mastitis were rarely positive for exfoliative toxins. On the contrary, in Europe, Kot and coworkers
285 reported a 14.5% of *S. aureus* harbouring the *eta* gene from bovine mastitis [31]. In our study, the
286 presence of *tsst* gene was more relevant, being carried by 37% of Argentinian, 23% of German,
287 16% of Tunisian and 6% of Italian isolates. All these isolates were also positive at least for a
288 combination of *sec* and *sel*, or *sec*, *seg*, and *sei* or *sec*, *seg* and *sej* or *sec*, *seg* and *sel* genes located
289 on the same bovine staphylococcal pathogenicity island (SaPIbov), confirming a positive
290 correlation between *sec, sei* or *sej* and *tsst*, as previously reported [32].
291 Panton-Valentine leucocidin, encoded by 2 co-transcribed genes located on a prophage, causes
292 leukocyte destruction and tissue necrosis [33]. The presence of PVL-encoding genes in *S. aureus* is
293 reported to be associated with increased disease severity [34]. In the present study, the presence of
294 PVL gene was lower than 20% in South Africa and New York State, higher than 80% in Argentina,
295 Colombia and Brazil, while in Germany, Italy and Tunisia none of the *S. aureus* isolates carried the
296 gene. For European countries, previously published results were in accordance with this study [35-
297 36]. Additionally, genes encoding the bicomponent leucotoxin *lukE-lukD* were observed in all
298 isolates, and, except for South Africa with only 2 isolates, most of the other isolates harboured
299 *lukM*, a gene encoding one operon like the one of PVL. The high rates of *lukE-lukD* and *lukM* found
300 in this study agree with other reports [35-37]. Additionally, only 2 isolates, one from Germany and
301 one from Italy were positive for *mec*A, confirming the low diffusion of MRSA among bovine
302 mastitis isolates [38-39]; interestingly, they are both GTS, in accordance with previous results [13].
303

304 **Materials and methods**

305 *Sample collection and bacteriological analysis*

306 A total of 120 *S. aureus* isolates from eight countries Argentina, Brazil, Colombia, Germany, Italy,
307 New York State, South Africa, Tunisia, (Figure 1), were selected for this study (Table 10). Isolates
308 of *S. aureus* were taken from the authors' bacterial culture collections (**BC**) and they included

309 isolates previously collected (between 2012 and 2017) from clinical mastitis quarters (**Q**) or
310 composite samples (**C**), or high somatic cell count samples (**H**). The isolates were stored at -20°C
311 until they were transported to the Italian laboratory (University of Milan) where storage was
312 continued at −20°C until further use. During transport to the laboratory, they were kept frozen using
313 styrofoam boxes and dry ice (for long distances) or wet ice (for short distances).

314 After samples thawing, 10 µl were streaked on blood agar plate. The plates were then incubated
315 aerobically at 37°C and examined after 24 h. The colonies were provisionally identified based on
316 morphology and hemolysis patterns and confirmed by coagulase test.

317 **Figure 1**. American, African and European countries contributing isolates of *S. aureus* to the
318 present study. Bacterial isolates were obtained from milk samples (clinical mastitis quarters,
319 composite samples, and high somatic cell count samples) taken in the indicated country (●)
320 (www.d-maps.com).

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321

322 **Table 10.** World survey on *S. aureus* cow isolates: participating countries, total isolates analyzed
323 per country, number of isolated from clinical mastitis or high somatic cell count (SCC) samples,
324 and type of sample collection (C = composite milk sample; Q = quarter milk sample).

Country	Total isolates analyzed per country			
	Clinical mastitis	High SCC	Number of farms	Sample collection
Argentina	16		10	C
Brazil	15		12	Q
Colombia		15	11	Q
Germany	17		17	Q
Italy	17		15	Q
New York State (USA)	17		13	Q
South Africa	11		9	Q
Tunisia		12	10	C
Total	**93**	**27**	**97**	

325

326 *DNA extraction*

327 DNA was extracted from isolates using the protocol previously described by Cremonesi and co-
328 workers [40]. The amount and quality of DNA were measured using a NanoDrop ND-1000
329 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and DNA was stored at
330 −20°C until use.

331

332 *Genotyping*

333 All the 120 *nuc* positive isolates (= *S. aureus*) were then genotyped by RS-PCR and a miniaturized
334 electrophoresis system (Agilent Technologies, Santa Clara, USA) as previously described [23; 41]
335 where a detailed working protocol is given. The method is based on amplification of the 16S–23S
336 rRNA intergenic spacer region. Each reaction contained (total volume 25 µl) 1x HotStarTaq Master
337 Mix (Qiagen), 800 nM of each primer (G1 and L1 primer) [23] and 7 µl of DNA (originally
338 extracted DNA diluted 1:100 in water). The PCR profile was: 95 °C for 15 min, followed by 27
339 cycles comprising 94 °C for 1 min, followed by a 2 min ramp and annealing at 55 °C for 7 min.
340 After a further 2 min ramp, extension was done at 72 °C for 2 min. PCR was terminated by
341 incubating at 72 °C for 10 min followed by cooling down to 4 °C. One µl of each of the PCR
342 products was then used for the miniaturized electrophoresis (Agilent) performed as described by the
343 manufacturer of the system. New genotypes were named and extended according to Fournier and
344 co-workers [23] leading to the genotypes GTA to GTZ, followed by the genotypes GTAA to
345 GTAZ, GTBA to GTBZ, and GTCA. An electrophoretic pattern differing in one band from the one
346 of a known genotype was considered as a genotypic variant. It was indicated with roman numerals
347 superscripted after the name of the genotype (e.g. GTR^{I}, GTR^{II}). To identify the genotypes and their
348 variants of the present strains, a freely available, in-house computer program was applied [42].

349

350 *Molecular isolates characterization*

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The DNA was amplified to investigate the presence of 26 factors that can contribute in different ways to *S. aureus* pathogenicity and therefore influence the management of the disease. In this study genes encoding enterotoxins (from *sea* to *sel*), leucocidins (*lukE*, *lukS–lukF/PV*, *lukE-lukD*, *lukM*), the acquisition of methicillin resistance (*mecA*) and genes related to host invasion (*clfA*, *fmtB*, *cna, eta*, *etb*) or to factors that have the potential to interfere with host defense mechanisms (*tsst*, *scn*, *chp*, *sak*) were analyzed using primers and protocols described in literature and listed in Table 11. The amplified PCR fragments were visualized on 2% agarose gel electrophoresis (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0.05 mg/ml; Sigma Aldrich, Milan, Italy), and visualized by UV transilluminator (BioView Ltd, Nes Ziona, Israel). A 100 bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel.

Table 11. Primer used in this study for *S. aureus* isolates characterization.

Target gene	Primer sequence (5'-3')	Amplification size	Reference
Invasion			
*clf*A	GGCTTCAGTGCTTGTAGG TTTTCAGGGTCAATATAAGC	1000 bp	[43]
coa	CCGCTTCAACTTCAGCCTAC TTAGGTGCTACAGGGGCAAT	204 bp	[44]
nuc	AGTTCAGCAAATGCATCACA TAGCCAAGCCTTGACGAACT	400 bp	[44]
*luk*E	AATGTTAGCTGCAACTTTGTCA CTTTCTGCGTAAATACCAGTTCTA	831 bp	[23]
*luk*M	TGGATGTTACCTATGCAACCTAC GTTCGTTTCCATATAATGAATCACTAC	780 bp	[45]
*luk*E-*luk*D	TGAAAAAGGTTCAAAGTTGATACGAG TGTATTCGATAGCAAAAGCAGTGCA	269 bp	[45]
lukSF-PV	ATCATTAGGTAAAATGTCTGGACATGATCA GCATCAAGTGTATTGGATAGCAAAAGC	433 bp	[46]
scn	ATACTTGCGGGAACTTTAGCAA TTTTAGTGCTTCGTCAATTTCG	320 bp	[10]
chp	TTTTTAACGGCAGGAATCAGTA TGCATATTCATTAGTTTTTCCAGG	404 bp	[10]
fmtB	AATGAAGATGCGAATCATGTTG CATCCATTTTTGTTTGCGTAGA	725 bp	[10]
sak	TGAGGTAAGTGCATCAAGTTCA CCTTTGTAATTAAGTTGAATCCAGG	403 bp	[10]
cna	AAAGCGTTGCCTAGTGGAGA AGTGCCTTCCCAAACCTTTT	192 bp	[47]
Interfere with host defence mechanism			

tsst	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	300 bp	[43]
eta	CTAGTGCATTTGTTATTCAA TGCATTGACACCATAGTACT	120 bp	[43]
etb	ACGGCTATATACATTCAATT TCCATCGATAATATACCTAA	200 bp	[43]
sea	TAAGGAGGTGGTGCCTATGG CATCGAAACCAGCCAAAGTT	180 bp	[44]
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478 bp	[45]
sec	ACCAGACCCTATGCCAGATG TCCCATTATCAAAGTGGTTTCC	371 bp	[44]
sed	TCAATTCAAAAGAAATGGCTCA TTTTTCCGCGCTGTATTTTT	339 bp	[44]
see	TACCAATTAACTTGTGGATAGAC CTCTTTGCACCTTACCGC	170 bp	[48]
seg	CCACCTGTTGAAGGAAGAGG TGCAGAACCATCAAACTCGT	432 bp	[44]
seh	TCACATCATATGCGAAAGCAG TCGGACAATATTTTTCTGATCTTT	463 bp	[44]
sei	CTCAAGGTGATATTGGTGTAGG CAGGCAGTCCATCTCCTGTA	529 bp	[44]
sej	GGTTTTCAATGTTCTGGTGGT AACCAACGGTTCTTTTGAGG	306 bp	[44]
sel	CACCAGAATCACACCGCTTA CTGTTTGATGCTTGCCATTG	240 bp	[44]
Antibiotic resistance			
mecA	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310 bp	[46]

364

365 **Conclusions**

366 Knowledge about the epidemiology of *S. aureus* genotypes in dairy species and herds might help to
367 formulate strategies for reducing the infection spreading and for focused treatments. In our work we
368 found that CLR and CLC clusters and some virulence factors related to host invasion, such as *fmtB*,
369 *cna*, *clfA* or immune defense impairment such as leukocidin genes, were the most frequent ones.
370 Further, *fmtB* gene has been shown to be related to the resistance of *S. aureus* to β-lactam
371 antibiotics [10]. Therefore, due to the prevalence of these genes worldwide, it might be useful
372 screening them in *S. aureus* isolates to help predicting clinical outcomes and specially to identify
373 harmful strains. Meanwhile, our work demonstrated also that each country had a specific genotypic
374 pattern and in some countries the isolates harboured some virulence factors, such as PVL-encoding
375 genes, with high prevalence, recommending a close surveillance of *S. aureus* isolates in the animals
376 of these countries to avoid the wide spreading of these genes. Finally, it is notable that most of the

isolates worldwide were negative for *mecA*, confirming the evidence of the low diffusion of MRSA among bovine mastitis isolates, as previously described [38-39].

In conclusion, this study confirms the wide variety of *S. aureus* genotypes found in dairy cattle worldwide and that genetic differences are related to geographical origin of the isolates, suggesting that considering the region of interest might help to formulate strategies directed to reduce the infection spreading and to set up control measures according to pathogen and host features.

Author Contributions

Valentina Monistero performed and analyzed the data; Hans Graber analyzed the RS-PCR genotypes; Claudia Pollera contributed to obtain samples and isolated from Italy; Paola Cremonesi performed and designed experiments, analyzed the data and wrote the manuscript; Bianca Castiglioni designed experiments and wrote the manuscript; Enriqueta Bottini contributed to obtain samples and isolated from Argentina; Alejandro Ceballos-Marquez contributed to obtain samples and isolated from Colombia; Laura Lasso-Rojas contributed to obtain samples and isolated from Colombia; Volker Kroemker contributed to obtain samples and isolated from Germany; Nicole Wente contributed to obtain samples and isolated from Germany; Inge-Marie Petzer contributed to obtain samples and isolated from South Africa; Carlos Santisteban contributed to obtain samples and isolated from New York State; Jeff Runyan contributed to obtain samples and isolated from New York State; Marcos Veiga Santos contributed to obtain samples and isolated from Brazil; Bruna Gomes Alves contributed to obtain samples and isolated from Brazil; Renata Piccinini contributed to obtain samples and isolated from Italy; Valerio Bronzo contributed to all logistic and project organization; Mohamed Salah Abbassi contributed to obtain samples and isolated from Tunisia; Meriam Ben Said contributed to obtain samples and isolated from Tunisia; Paolo Moroni designed experiments, analyzed the data and wrote the manuscript.

Conflicts of Interest

403 The authors declare that they have not conflict of interest.

404

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557 **Table 1.** Distribution of genotypes in the eight countries.

Country	Genotype (isolate No.)	New genotypes or variants	Total strains
Argentina	GTC (6, 15) GTI^{I} (1, 4, 5, 7) GTI^{II} (10, 11, 14) GTI^{V} (9) GTI^{VI} (12) GTP (8) GTR^{I} (2, 3, 16) GTR^{VI} (13)	GTI^{V}, GTI^{VI}	16
Brasil	GTAQ (31) GTAQ^{I} (30) GTBA (17) GTBN (29) GTBN^{I} (20) GTBN^{II} (23) GTBY (18, 19, 21, 28) GTBY^{I} (24, 25) GTC^{III} (26) GTS^{I} (22) GTZ (27)	GTBN^{I}, GTBN^{II}, GTBY^{I}, GTAQ^{I}	15
Colombia	GTA^{I} (33) GTAO (39, 40, 41) GTAO^{I} (38, 43, 44, 46) GTAO^{II} (32, 42) GTBY (45) GTI^{I} (35, 36, 37) GTR (34)	GTAO^{I}, GTAO^{II}	15
Germany	GTC^{I} (54, 55, 56, 57, 59) GTR (47, 48, 49, 51)		17

	GTR^{I} (58, 60, 61, 63) GTR^{II} (50, 62) GTR^{VI} (52) GTS (53)		
Italy	GTB (64, 65, 66, 78, 80) GTBG (70) $GTBQ^{I}$ (73, 79) GTC^{I} (69, 75) GTC^{II} (76) GTR^{I} (67, 68) GTR^{XIII} (72) GTR^{VI} (71) GTS (77) GTZ (74)	GTR^{XIII}	17
New York State	GTAI (93) GTC (82, 83, 85, 86, 88, 94, 96) GTC^{I} (81, 87, 91) GTC^{III} (90) GTC^{V} (95) GTI^{I} (89) GTI^{V} (97) GTR^{I} (84)	GTC^{V}, GTI^{V}	17
South Africa	GTAR (101) GTBH (98) GTBZ (99, 100, 105) GTCA (103) GTR (102, 104, 107, 108) GTR^{VI} (106)	GTAR, GTBZ, GTCA	11
Tunisia	GTAJ (111) $GTBW^{II}$ (110)		12

GTCA (113, 114)
GTCB (119)
GTR^{I} (109)
GTR^{VI} (112, 115, 116, 117, 118, 120)

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559

560 **Table 2**. Molecular characteristics of strains isolated in Argentina.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
1	GTI^{I}	+	+	+	+	-	+	+	-	-	-	-	+	*sea, seg, sei*	-
2	GTR^{I}	+	+	+	+	+	+	+	-	-	+	-	-	*sed, seg, seh, sei*	-
3	GTR^{I}	+	-	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	-
4	GTI^{I}	-	-	+	+	+	+	+	-	-	-	-	-	*sea, seg, sei*	-
5	GTI^{I}	+	+	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	-
6	GTC	+	+	+	+	+	+	-	-	-	+	-	+	*sea, sec, seg, seh, sei*	-
7	GTI^{I}	+	+	+	+	+	+	-	-	-	+	-	+	*sea, seg, seh, sei*	-
8	GTP	+	+	+	-	+	+	+	-	-	+	-	-	*sea, sed, seg, seh, sei*	-
9	GTI^{V} *	+	+	+	+	+	+	+	-	-	-	-	-	*sed, seg, seh, sei*	-
10	GTI^{II}	+	+	-	+	+	+	+	-	-	+	-	-	*sea, seg, seh, sei*	-
11	GTI^{II}	+	+	-	+	-	+	+	-	-	-	-	-	*sea, seg, seh, sei*	-
12	GTI^{VI} *	+	+	+	+	+	+	+	-	-	-	-	+	*seg, seh, sei*	-
13	GTR^{VI} *	+	+	-	+	+	+	+	-	-	-	-	-	*seg, seh, sei*	-
14	GTI^{II}	+	+	+	+	-	+	+	-	-	-	-	-	*sea, sed, seg, seh, sei*	-
15	GTC	+	+	+	+	+	+	+	-	-	-	-	+	*sea, seg, seh, sei*	-
16	GTR^{I}	+	+	+	+	+	+	-	-	-	-	-	-	*seh, sei*	-

561 * new genotypes or new variants.

562

563

564 **Table 3.** Molecular characteristics of strains isolated in Brazil.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
17	GTBA	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
18	GTBY	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
19	GTBY	+	+	+	+	+	+	+	-	-	-	-	-	*seh*	-
20	GTBN^{I*}	+	+	+	+	+	+	+	-	-	-	-	-	*seh*	-
21	GTBY	+	+	+	+	+	+	+	-	-	-	-	-	*seh*	-
22	GTS^{I}	+	+	+	+	+	+	+	-	-	-	-	-	*seh*	-
23	GTBN^{II}	+	+	+	+	+	+	+	-	-	-	-	-	*seh*	-
24	GTBY^{I}	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
25	GTBY^{I}	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
26	GTC^{III}	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
27	GTZ	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seg, seh, sei*	-
28	GTBY	+	+	+	+	+	+	+	-	-	-	-	-	-	-
29	GTBN	+	+	+	+	+	+	+	-	-	-	-	-	-	-
30	GTAQ^{I}	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-
31	GTAQ	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh*	-

565 * new genotypes or new variants.

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571 **Table 4.** Molecular characteristics of strains isolated in Colombia.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
32	GTAO^{II} *	-	+	+	+	-	+	+	-	-	-	-	-	-	-
33	GTA^{I}	+	+	+	+	+	+	+	+	-	+	-	-	*sea, seh*	-
34	GTR	+	+	+	+	+	+	+	+	-	+	-	-	*seh*	-
35	GTI^{I}	+	+	+	+	+	+	+	+	-	+	-	-	*sea, seh*	-
36	GTI^{I}	+	+	+	+	-	+	-	+	-	+	-	-	*sea, seh*	-
37	GTI^{I}	+	+	+	+	+	+	-	+	-	+	-	-	*sea, seh*	-
38	GTAO^{I} *	+	+	+	+	+	+	+	+	-	+	-	-	*sea, seh*	-
39	GTAO	+	+	+	+	+	+	+	-	-	+	-	-	*sea, seh*	-
40	GTAO	+	+	+	+	+	+	+	-	-	+	-	-	*sea, seh*	-
41	GTAO	+	-	+	+	-	+	+	+	-	+	-	-	*sea, seh*	-
42	GTAO^{II} *	+	+	+	+	+	+	+	-	-	-	-	-	*sea, sed, seg, seh, sei*	-
43	GTAO^{I} *	+	+	+	+	+	+	+	-	-	+	-	-	*sea, seg, seh, sei*	-
44	GTAO^{I} *	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seg, seh, sei*	-
45	GTBY	+	+	+	-	-	+	+	-	-	-	-	-	*sea, seh*	-
46	GTAO^{I} *	+	+	+	+	+	+	+	-	-	-	-	-	*sea, seh, sei*	-

572 * new genotypes or new variants.

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577 **Table 5.** Molecular characteristics of strains isolated in Germany.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*sak*	*chp*	*eta*	*tsst*	Enterotoxins positive	*mecA*
47	GTR	+	+	+	+	+	+	-	-	-	-	-	-	*sea*	-
48	GTR	+	+	+	+	+	+	-	-	-	-	-	-	*sea*	-
49	GTR	+	+	+	+	+	+	-	-	-	-	-	-	*sea, seg*	-
50	GTR[II]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, seg*	-
51	GTR	+	+	+	+	+	+	-	-	-	-	-	-	*sea, seg*	-
52	GTR[VI]	+	+	+	+	-	+	-	-	-	-	-	-	-	-
53	GTS	-	+	+	+	-	-	-	-	-	-	-	-	-	+
54	GTC[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sec, seg, sei*	-
55	GTC[I]	+	-	+	+	+	+	-	-	-	-	-	+	*sea, sec, seg*	-
56	GTC[I]	+	-	+	+	+	+	-	-	-	-	-	+	*sea, sec, seg*	-
57	GTC[I]	+	-	+	+	+	+	-	-	-	-	-	+	*sea, sec, seg, sei*	-
58	GTR[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sei*	-
59	GTC[I]	+	-	+	+	+	+	-	-	-	-	-	+	*sea, sec, seg*	-
60	GTR[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, seg*	-
61	GTR[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, seg*	-
62	GTR[II]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sei*	-
63	GTR[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sei*	-

578 * new genotypes or new variants.

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581 **Table 6.** Molecular characteristics of strains isolated in Italy.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
64	GTB	+	+	+	+	-	+	-	-	-	-	-	-	-	-
65	GTB	+	+	+	+	-	+	-	-	-	-	-	-	*sed, sej*	-
66	GTB	-	+	+	+	-	+	-	-	-	-	-	-	*sed, sej*	-
67	GTR^{I}	+	+	+	+	-	+	-	-	-	-	-	-	-	-
68	GTR^{I}	+	+	+	+	-	+	-	-	-	-	-	-	-	-
69	GTC^{I}	+	+	+	+	+	+	-	-	-	-	-	-	*sed, seg, sei, sej*	-
70	GTBG	+	+	+	+	+	+	-	-	-	-	-	-	*sed, seg, sei, sej*	-
71	GTR^{VI}	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sei, sej*	-
72	GTR^{XIII} *	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
73	GTBQ^{I}	+	+	+	+	-	+	-	-	-	-	-	-	*sea, sed, seg, sei, sej*	-
74	GTZ	+	+	+	+	-	+	-	-	-	-	-	-	*sea, sed, seg, sei, sej*	-
75	GTC^{I}	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg*	-
76	GTC^{II}	+	+	+	+	+	+	-	-	-	-	-	+	*sea, sec, sed, seg, sej*	-
77	GTS	-	+	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg*	+
78	GTB	+	+	+	+	+	+	-	+	-	+	-	-	*sea, sed, seg, sei, sej*	-
79	GTBQ^{I}	+	+	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sej*	-
80	GTB	-	+	+	+	-	+	-	-	-	-	-	-	*sea, sed, seg, sei, sej*	-

582 * new genotypes or new variants.

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587 **Table 7.** Molecular characteristics of strains isolated in New York State.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
81	GTC[I]	+	-	+	+	-	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
82	GTC	-	-	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
83	GTC	+	+	+	+	+	+	-	-	-	-	-	-	*sed, seg*	-
84	GTR[I]	+	+	+	+	-	+	-	-	-	-	-	-	*sed, seg*	-
85	GTC	+	-	+	+	+	+	-	-	-	-	-	-	*sed, seg, sei*	-
86	GTC	+	-	+	+	+	+	-	-	-	-	-	-	*seg, sei*	-
87	GTC[I]	+	-	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg*	-
88	GTC	+	-	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
89	GTI[I]	+	+	+	+	+	+	-	-	-	-	-	-	*sed, seg, sei*	-
90	GTC[III]	-	-	-	-	+	+	-	-	-	-	-	-	*sea, sed, seg*	-
91	GTC[I]	-	-	+	+	+	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
92	GTI[V *]	-	+	+	+	+	+	-	-	-	-	-	-	*sed, sei*	-
93	GTA[I]	+	-	+	-	-	+	-	-	-	-	-	-	*sea, sed, seg, sei*	-
94	GTC	-	-	+	+	+	+	-	-	-	-	-	-	*sea, seg, sei*	-
95	GTC[V *]	-	-	-	+	+	+	+	-	-	-	-	-	*sea, seg, sei*	-
96	GTC	-	+	+	+	+	+	-	-	-	-	-	-	*seg, sei*	-
97	GTI[V *]	-	+	+	+	-	+	+	-	-	-	-	-	-	-

588 * new genotypes or new variants.

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592 **Table 8.** Molecular characteristics of strains isolated in South Africa.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
98	GTBH	+	+	+	+	-	+	+	-	-	+	-	-	*sea, seh*	-
99	GTBZ *	-	+	+	+	-	+	-	-	-	+	-	-	*sea, seh, sei*	-
100	GTBZ *	-	+	+	+	-	+	-	-	-	+	-	-	*sea, seh, sei*	-
101	GTAR *	-	+	+	+	-	+	-	-	-	+	-	-	*sea, seh, sei*	-
102	GTR	+	+	+	+	+	+	-	-	-	+	-	-	*sea, seh*	-
103	GTCA *	+	+	+	+	-	+	-	+	-	+	+	-	*sea, seh*	-
104	GTR	+	+	+	+	-	+	-	-	-	+	-	-	*sea, seh*	-
105	GTBZ *	-	-	+	+	-	+	-	-	-	+	-	-	*sea, seh*	-
106	GTR^{VI}	+	+	+	+	+	+	-	-	-	+	-	-	*sea, seh*	-
107	GTR	+	+	+	+	-	+	+	-	-	+	-	-	*sea, seh*	-
108	GTR	+	+	+	+	-	+	+	-	-	+	-	-	-	-

593 * new genotypes or new variants.

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598 **Table 9.** Molecular characteristics of strains isolated in Tunisia.

Isolates	RS-PCR	*clfA*	*fmtB*	*cna*	*lukE*	*lukM*	*lukE-lukD*	*lukSF-PV*	*scn*	*chp*	*sak*	*eta*	*tsst*	Enterotoxins positive	*mecA*
109	GTR[I]	+	+	+	-	-	+	-	-	-	-	-	-	-	-
110	GTBW[II]	+	+	+	+	+	+	-	-	-	-	-	+	*sec, sel*	-
111	GTAJ	+	+	+	+	+	+	-	-	-	-	-	+	*sec, sel*	-
112	GTR[VI]	+	+	+	+	+	+	-	+	+	+	-	-	-	-
113	GTCA	+	+	+	+	+	+	-	-	+	-	-	-	*seh*	-
114	GTCA	+	+	+	+	+	+	-	+	-	+	-	-	*seh*	-
115	GTR[VI]	+	+	+	+	+	+	-	-	-	-	-	-	-	-
116	GTR[VI]	+	+	+	+	+	+	-	-	+	-	-	-	-	-
117	GTR[VI]	+	+	+	+	+	+	-	-	-	-	-	-	-	-
118	GTR[VI]	+	+	+	+	+	+	-	-	-	-	-	-	-	-
119	GTCB	+	+	+	+	+	+	-	+	+	-	-	-	-	-
120	GTR[VI]	+	+	+	+	+	-	-	+	+	+	-	-	-	-

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