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Detection of Methicillin-Susceptible *Staphylococcus aureus* ST398 and ST133 Strains in Gut Microbiota of Healthy Humans in Spain

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Abstract Fecal samples of 100 healthy humans were tested for *Staphylococcus aureus* recovery. Fifteen samples (15 %) contained *S. aureus*, all methicillin-susceptible (MSSA), being one isolate/sample further studied. These 15 isolates were characterized by *spa* and *agr* typing as well as multilocus sequence typing. High diversity of *spa* types (*n*=11) and sequences types (*n*=8) was detected. Two *S. aureus* of lineages ST398 or ST133 were detected, and six isolates were ascribed to clonal complex 30 (CC30). Strains were susceptible to most of the 17 antimicrobial agents tested with exceptions: erythromycin/clindamycin (three strains, containing *erm*(C) and/or *erm*(A) + *mph*(C) genes) and tobramycin and mupirocin (one strain containing *ant*(4′)-Ia + *mup*(A) genes). The presence of 18 staphylococcal enterotoxin genes was studied by PCR, and isolates were negative for *lukF*/*lukS*-PV genes, although strain ST133 harbored the *lukD*-*lukE* + *lukM* genes. Other virulence genes detected were (number of strains): *tsst-1* (6), *hla* (15), *hlb* (9), *hld* (15), *hlg* (6), *hlgv* (9), *cna* (2), *aur* (14), and *egc*-like cluster (3). Analysis of immune evasion cluster genes showed six types, highlighting their absence in two strains of lineages ST133 and ST5. A high clonal diversity of MSSA strains was identified in the intestinal microbiota of healthy humans, being CC30 the most frequent one. This is the first report of MSSA ST133 and ST398 isolates in gut microbiota of healthy humans.

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Introduction

Staphylococcus aureus is an opportunistic pathogen commonly found as part of the normal human microbiota, especially of the oropharynx, nose and skin, although it can also colonize the intestine and the perineal region. Most studies that analyze the *S. aureus* fecal colonization are referred to hospitalized patients [1, 18, 19, 38, 49], with different percentages reported depending on the studies, and very few reports are focused on fecal samples of healthy individuals [12]. In addition, very scarce data do exist on the molecular characterization of fecal *S. aureus* isolates of healthy humans. It is known that *S. aureus* colonization can precede infection and that it can adapt to different body sites [45]. *S. aureus* can also acquire different resistance mechanisms, highlighting the acquisition of the *mecA* gene, given that it confers resistance to almost all available beta-lactams [22]. Methicillin-resistant *S. aureus* (MRSA) can affect both hospitalized patients as well as patients from the community (CA-MRSA). In the last years, a new MRSA lineage (namely ST398, which is associated with farm animals, especially pigs) [14, 16] has emerged. Some other *S. aureus* genetic lineages are also associated with animals, such as ST133, which is mainly related to ungulates and ruminants [15, 41]. There is an increasing interest in the analysis of the circulating *S. aureus* genetic lineages in different ecosystems in order to determine the flux of lineages among different niches and their potential evolution.

S. aureus is also important due to its capacity to produce a large variety of virulence factors that can exacerbate the clinical situation of staphylococcal infections [25]. Some of these toxins are Panton–Valentine leukocidin (PVL), toxic

shock syndrome toxin 1 (TSST-1), different hemolysins, enterotoxins, exfoliatins, and other virulence factors which facilitate cellular invasion, bacterial growth, and reduction of immune system human cells [9, 25, 40, 47].

S. aureus can acquire genes that seem to be associated with the capacity of this microorganism to evade the human immune system. This set of genes is called immune evasion cluster (IEC). The IEC system is located on an 8-kb region at the conserved 3′ end of β-hemolysin-converting bacteriophages. This region contains the genes *scn*, *chp*, *sak*, and *sea* or *sep*, which have different functions to achieve the survival of *S. aureus* within our innate defenses. The presence of different combinations of these genes in *S. aureus* allows its classification into different IEC types [39, 47].

The aims of this study are to determine the rate of *S. aureus* fecal carriage in healthy humans and to analyze the genetic lineages, the resistance genes and virulence determinants of recovered isolates. Furthermore, we focused on the determination of the IEC types to hypothesize on the possible human or animal origin of isolates.

Materials and Methods

Bacterial Isolation

Fecal samples of 100 healthy human volunteers were obtained during September 2010 and March 2011 in La Rioja (northern Spain). Healthy humans (age range 2–89 years; 53 % women and 47 % men) had neither received antibiotics nor had relation with the hospital environment for at least 3 months prior to sampling, and all gave their informed consent (or their parents in case of underage) to participate in this study. Direct suspension of the fecal swabs was performed in sterile saline solution, and 100 μL was inoculated into brain–heart infusion broth containing 6.5 % NaCl and incubated at 37 °C for 24 h. Then, 100 μL of this culture was seeded on mannitol–salt agar (MSA, Beckton–Dickinson), and oxacillin resistance screening agar base (ORSAB), OXOID) supplemented with 2 mg/L of oxacillin, and the plates were incubated at 37 °C for 24–36 h. Presumptive *S. aureus* and MRSA colonies were selected in MSA (yellow colonies) and ORSAB plates (blue colonies), respectively (two per positive plate). Identification of *S. aureus* and MRSA was performed by conventional methods (DNAse assay, catalase, and Gram staining) and confirmed by specific duplex PCR of the *nuc* (*S. aureus* specific thermonuclease) and *mecA* (staphylococci methicillin-resistance determinant) genes [30]. *S. aureus* isolates were stored at −80 °C until further analysis. All isolates were tested for their capacity to coagulate the bovine plasma (Sigma–Aldrich) following standard methodology [20].

Molecular Typing of Isolates

S. aureus isolates were characterized by different molecular methods. Single-locus DNA sequencing of the hyper variable region of *S. aureus* protein A (*spa*) [21] was performed, and the obtained sequences were analyzed using the Ridom StaphType software version 1.5.21 (Ridom GmbH). Detection of *agr* allotypes—accessory gene regulatory loci—was carried out by two multiplex PCR [43] in all isolates. Multi-locus sequence typing (MLST) was also performed in all *S. aureus* isolates to determine the sequence type (ST) and the clonal complex (CC) of each isolate (www.saureus.mlst.net).

Susceptibility Testing and Detection of Antimicrobial Resistance Genes

Susceptibility testing was carried out by disk-diffusion agar method for 17 antimicrobials (penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, ciprofloxacin, gentamicin, streptomycin, kanamycin, tobramycin, tetracycline, chloramphenicol, trimethoprim–sulfamethoxazole, linezolid, vancomycin, mupirocin, and fusidic acid) following the CLSI recommendations [7, 8], except for fusidic acid, mupirocin, and streptomycin for which SFM guidelines were followed [6]. In addition, the D-test was performed for the detection of inducible clindamycin resistance [7]. The presence of the *erm*(A), *erm*(B), *erm*(C), *erm*(T), *msr*(A), *msr*(B), *mph*(C), *ant*(4′)-Ia, *mup*(A), and *blaZ* resistance genes was studied by PCR (and sequencing for some of the amplicons) [17, 30, 42, 44].

Detection of Virulence Genes

The presence of the genetic determinants of the Panton–Valentine leukocidin (*lukF/lukS*-PV), toxic shock syndrome toxin (*tsst-1*), exfoliative toxin A (*eta*), B (*etb*), and D (*etd*), as well as alpha-(*hla*), beta-(*hlb*), delta-(*hld*), gamma (*hlg*), and gamma-hemolysin variant (*hlgv*) was investigated by PCR. In addition, the genes encoding leukocidins DE (*lukD-lukE*) and M (*lukM*), aureolysin (*aur*), the biofilm-associated protein *bap*, and the adhesion factor *cna* were also studied by PCR [9, 25, 28, 30, 31, 40, 50, 51]. Eighteen staphylococcal enterotoxin genes (SEs) (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) were analyzed by PCR and PCR multiplex [23].

Immune Evasion Cluster

The five genes of the IEC cluster (*scn*, *chp*, *sak*, and *sea* or *sep*) were studied by PCR and sequencing; according to their presence or absence, *S. aureus* isolates were classified into seven IEC types following previously described patterns [47].

Results

Fecal Carriage and Molecular Typing of *S. aureus*

Fifteen of the 100 tested fecal samples (15 %) were positive for *S. aureus* on MSA plates. As both isolates of each positive sample showed similar resistance phenotype and *spa* type, only one isolate was maintained and further studied. All 15 isolates were susceptible to oxacillin and cefoxitin, and were negative for *mec*A gene; therefore, they were MSSA. No MRSA isolates were recovered from ORSAB medium.

Complete characterization of the 15 MSSA isolates is shown in Table 1. A high diversity of *spa* types was detected (t002, t012, t021, t084, t136, t166, t209, t216, t571, and t3495), including a new *spa* type registered as t7866 in Ridom SpaServer. The detected *agr* allotypes were I, II, and III, grouping all isolates. The *S. aureus* isolates belonged to eight different sequence types that were included into seven different clonal complexes, highlighting the presence of strains typed as ST398 (*spa*-t571), ST133 (*spa*-t3495), and ST5 (*spa*-t002) ascribed to the clonal complexes CC398, CC133, and CC5, respectively. Other sequence types identified were (ST(*spa* type)/CC ascribed): ST30(t012 or t021)/CC30, ST34(t136, t7866 or t166)/CC30, ST59(t216)/CC59, ST15(t084)/CC15, and ST109(t209)/CC9. Forty percent of the strains were included within clonal complex CC30. These strains fell into two sequence types, ST30 and ST34. MSSA isolates lacked the ability to coagulate bovine plasma except the isolate typed as ST133(t3495)/CC133.

Susceptibility Testing and Detection of Antimicrobial Resistance Genes

Few antimicrobial resistance traits were identified among our strains. Nine of them showed penicillin resistance (carrying the *bla*Z gene), three strains erythromycin resistance (carrying the *erm*(A), *erm*(C), *erm*(T) or *mph*(C) genes], and one strain exhibited tobramycin and mupirocin resistance (carrying *ant*(4′)-Ia and *mup*(A) genes). Four strains were susceptible to all tested antimicrobial agents (Table 1).

Virulence Genes Detected

A high diversity of virulence genes was identified among the 15 *S. aureus* strains (Table 1). Interestingly, six strains contained the *tsst-1* toxin gene (40 %) and one strain harbored the exfoliative toxin A gene (*eta*). All MSSA strains carried the *hla* and *hld* genes. Other hemolysin genes detected were as follows (number of strains): *hlb* (nine), *hlg* (six), and *hlgv* (nine). The *lukD-lukE* genes were detected in five strains, and the *lukM* gene was identified in the strain CC133. All strains except the one ascribed to CC133 carried the *aur* gene, while only two strains (both belonging to CC30) were positive to *cna* gene. All strains were negative for the presence of the genes *lukF/lukS*-PV and *bap*. None of our strains harbored the enterotoxin gene cluster named *egc* (*seg*, *sei*, *sem*, *sen*, and *seo*); however, 20 % presented the enterotoxin genes *seg*, *sei*, *sem*, *sen*, *seo*, and *seu*, included in the enterotoxin gene cluster like (*egc*-like) operon [4, 24]. All *S. aureus* strains lacked *see* and *ser* enterotoxin genes (Table 1).

Analysis of Immune Evasion Cluster Genes

All *S. aureus* strains except two harbored the genes of the IEC system. Only the strain of lineage CC133 and one ascribed to CC5 did not present the IEC genes. The following IEC types were obtained in the remaining 13 MSSA (IEC type/ST–CC): IEC type A/ST30–CC30; IEC type B/ST34–CC30, ST59–CC59, or ST109–CC9; IEC type C/ST15–CC15, ST59–CC59 or ST398–CC398; IEC type E/ST34–CC30; IEC type F/ST5–CC5; and IEC type G/ST34–CC30. Six strains were ascribed to the IEC type A/ST30-CC30, IEC type B/ST34-CC30 and IEC type C/ST15-CC15. The IEC type D was not detected among our strains (Table 1).

Discussion

A moderate rate of *S. aureus* fecal carriage (15 %) was detected among the healthy humans in this study. Few reports of this type have been previously performed, and most of them were focused on intestinal *S. aureus* carriage in hospitalized patients, with occurrences ranging from 8 to 31 % [18, 19, 49]. There are very few studies on healthy individuals, and as far as we know, none on genetic lineages of *S. aureus* from the intestinal ecosystem of healthy people.

The few available studies that analyzed the prevalence of intestinal colonization by *S. aureus* found values ranging from 6 % in healthy children [12] to 20 % in people with various clinical situations [1]. The absence of MRSA detection in our study is of relevance, although low percentage of MRSA carriage rates (0–5 %) has been reported in previous studies in healthy children [12], or in people at the time of hospital admission [3, 13, 26]. However, a higher prevalence rate was detected among hospitalized patients in other study (24 %) [27]. One question would be if *S. aureus* is a passenger or a real colonizer of the intestinal tract. A previous study evaluated the frequency of nasal and extranasal MRSA carriage on patients admitted at a hospital, identifying a strong association of extranasal carriage with nasal MRSA colonization [3].

The high genetic diversity of *spa* types detected among our strains (11 different *spa* types, one of them new) is remarkable. Most of the *spa* types identified were related

Table 1 Characteristics of the 15 MSSA isolates from fecal samples of healthy humans recovered in this study

Isolate	Molecular typing				IEC		Antimicrobial resistance		Virulence factors	
	CC	ST	*spa*	*agr*	IEC type	Genes	Phenotype	Genes	SEs	Other genes
C3965	CC398	ST398	t571	I	C	*scn*, *chp*	ERY, CLI[a]	*erm*(T)	*sed*, *sen*	*hla*, *hld*, *hlgv*, *aur*
C3968	CC133	ST133	t3495	I	–	–	–	–	*sec*, *sej*, *sel*, *sen*	*tsst-1*, *hla*, *hlb*, *hld*, *hlgv*, *luk-DE*, *luk-M*
C3972	CC59	ST59	t216	I	B	*scn*, *chp*, *sak*	–	–	*sej*, *sen*	*tsst-1*, *hla*, *hlb*, *hld*, *hlgv*, *aur*
C3973	CC59	ST59	t216	I	C	*scn*, *chp*	PEN	*blaZ*	*seb*, *sej*, *sek*, *sen*, *seq*	*hla*, *hlb*, *hld*, *hlgv*, *aur*
C3969	CC30	ST30	t012	III	A	*scn*, *chp*, *sak*, *sea*	PEN	*blaZ*	*sea*, *sej*, [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][b]	*hla*, *hlb*, *hld*, *hlg*, *cna*, *aur*
C3970	CC30	ST30	t021	III	A	*scn*, *chp*, *sak*, *sea*	PEN	*blaZ*	*sea*, *sej*, [*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][b]	*tsst-1*, *hla*, *hlb*, *hld*, *hlg*, *cna*, *aur*
C3974	CC30	ST34	t7866[c]	III	E	*scn*, *sak*	PEN, TOB, MUP	*blaZ*, *ant*(4′)-Ia, *mup*(A)	*seg*, *seh*, *sei*, *sem*, *seu*	*hla*, *hld*, *hlg*, *aur*
C3975	CC30	ST34	t136	III	G	*scn*, *sak*, *sep*	PEN	*blaZ*	*seg*, *seh*, *sei*, *sem*, *sep*, *seu*	*tsst-1*, *hla*, *hlb*, *hld*, *hlg*, *aur*
C3966	CC30	ST34	t136	III	B	*scn*, *chp*, *sak*	PEN	*blaZ*	*seh*, *sei*, *sem*, *seu*	*tsst-1*, *hla*, *hlb*, *hld*, *hlg*, *aur*
C4239	CC30	ST34	t166	III	B	*scn*, *chp*, *sak*	PEN	*blaZ*	*seg*, *seh*, *sei*, *sem*, *seu*	*tsst-1*, *hla*, *hld*, *hlg*, *aur*
C3962	CC15	ST15	t084	II	C	*scn*, *chp*	ERY, CLI[a]	*erm*(C)	*sen*	*hla*, *hld*, *hlgv*, *luk-DE*, *aur*
C3964	CC15	ST15	t084	II	C	*scn*, *chp*	–	–	*sen*	*hla*, *hld*, *hlgv*, *luk-DE*, *aur*
C3963	CC9	ST109	t209	II	B	*scn*, *chp*, *sak*	PEN, ERY, CLI[a]	*blaZ*, *mph*(C), *erm*(A), *erm*(C)	[*seg*, *sei*, *sem*, *sen*, *seo*, *seu*][b]	*hla*, *hlb*, *hld*, *hlgv*, *eta*, *aur*
C3971	CC5	ST5	t002	II	–	–	PEN	*blaZ*	*seb*, *sei*, *sej*, *sem*, *sen*, *seo*	*hla*, *hlb*, *hld*, *hlgv*, *luk-DE*, *aur*
C3967	CC5	ST5	t002	II	F	*scn*, *chp*, *sak*, *sep*	–	–	*sej*, *sen*, *seo*, *sep*	*hla*, *hld*, *hlgv*, *luk-DE*, *aur*

IEC immune evasion cluster, *PEN* penicillin, *ERY* erythromycin, *CLI* clindamycin, *TOB* tobramycin, *MUP* mupirocin, *SEs* staphylococcal enterotoxins

[a] Inducible

[b] *egc*-like cluster

[c] New *spa* type

to the clonal complex CC30, a lineage that seems to have become a successful colonizer of humans [2, 35] and is also considered one of the most frequent *S. aureus* lineages detected in human infections [45]. The detection of a MSSA strain of the sequence type ST398, which is traditionally associated with farm animals (especially pigs), and a MSSA strain belonging to sequence type ST133, related to ungulates and ruminants, suggests the incipient ability of this microorganism to colonize different animal or human hosts away from their usual niches [15, 31]. In addition, as far as we know, *S. aureus* of lineage ST133 has not previously detected in healthy humans. Some authors have hypothesized about the ancestral human origin of genetic line CC133 and the possible evolutionary leap to ruminants many centuries ago [20]. Moreover, recent studies have suggested that the CC398 lineage could be originated in humans as MSSA and then spread to livestock, where it subsequently acquired the *mecA* gene and became well adapted to these animals [36]. It is of interest to indicate that the healthy individual who carried the ST133 isolate worked in a food–animal farm, but the one with ST398 had no relation with farm animals. The two healthy individuals who carried MSSA of the lineages ST398 and ST133 were negative for *S. aureus* nasal carriage (data not shown). We cannot discard the implication of the food chain in the acquisition of these S. *aureus* clones.

The presence of the IEC system, especially of the *scn* gene, could indicate the possible human origin of a *S. aureus* strain, encoding staphylococcal complement inhibitor (SCIN) [39]. SCIN is a staphylococcal inhibitor of human complement

system, in fact, is a C3 convertase inhibitor, blocking the formation of C3b on the surface of the bacterium and the ability of human neutrophils to phagocyte *S. aureus*. In our case, the presence of the genes of IEC system and the absence of *mecA* gene in the detected strain ST398/CC398 indicate that it might belong to the human-associated subclone. By contrast, given that the strain typed as ST133/CC133 does not contain the genes of IEC system and presents capacity to coagulate bovine plasma, it is estimated to belong to the well-adapted ungulates and ruminants lineage. Moreover, the detection of strains of lineage ST5 with and without IEC genes is also of interest. According to some authors, *S. aureus* strains of ST5 lineage could have evolved in time jumping from different hosts and acquiring different adaptability mechanisms [29]. It is interesting to underline the wide variety of IEC types detected among our strains CC30 (A, B, E, and G).

All our *S. aureus* strains were PVL-negative. The *lukF/lukS*-PV genes encoding the Panton–Valentine leukocidin are located in phages, and it is believed that these genes could be transferred from MSSA into CA-MRSA [10]. A high proportion of *S. aureus* strains (40 %) carried the *tsst-1* toxin gene, most of them of the clonal complex CC30. Similar results have been found in other studies of nasal carriers (close to 30 %) [30, 34]. TSST-1 is a toxin related to the toxic shock syndrome. The association between TSST-1 and CC30 has been previously described [10]. One strain (6 %) ascribed to CC9 carried the *eta* gene, encoding the exfoliative toxin A, associated with the staphylococcal scalded skin syndrome and impetigo [33]. Previous studies remark a high association between the clonal complex CC9 and its ability to produce exfoliative toxins [2].

All strains were positive for *hla* and *hld* genes, and high percentage of them were also positive for other hemolysin genes (*hlb*, *hlg*, and *hlgv*). These toxins are produced by a large amount of *S. aureus* strains [11] and have the ability to destroy red blood cells, especially when *S. aureus* reaches the blood and causes bacteremia. One-third of the strains harbored the *lukD-lukE* genes, and only the strain CC133 contained the *lukM* gene encoding a leukocidin, prevalent in isolates of ungulates and ruminants [15, 41].

All MSSA strains except the one of the lineage ST133 carried the *aur* gene encoding the metalloproteinase aureolysin. This fact points again to the animal origin of our strain ST133, due to the capacity of this enzyme to activate prothrombin in human plasma [37, 40]. The absence of the *cna* gene and the tetracycline-susceptible phenotype in strain CC398 might be also a good marker of human origin, given that presence of the *cna* gene and tetracycline resistance are frequently detected in livestock-associated MRSA CC398 strains [32]. Moreover, none of the *S. aureus* strains harbored the biofilm-associated *bap* gene. It has been previously suggested that the prevalence of the gene *bap* in *S. aureus* strains is very low [9, 48].

Only three strains harbored the *egc*-like operon and belonged to ST109/CC9 and ST30/CC30. This cluster is normally found on strains of these clonal complexes [2]. The remaining 12 strains were positive at least for one SE gene; 2 of them belonged to ST34/CC30 and harbored incomplete *egc*-like cluster, lacking the *seo* gene. Incomplete *egc* or *egc*-like clusters have been detected in previous studies [5].

Regarding the antimicrobial resistance pattern of investigated strains, only 60 % showed resistance to penicillin and harbored the *blaZ* gene. Higher rates are normally detected among the nasal *S. aureus* population of healthy individuals [30]. In addition, three strains were erythromycin- and clindamycin-resistant and presented the resistance genes *erm*(A), *erm*(C), *erm*(T) or *mph*(C); these strains belonged to CC9, CC15, and CC398 lineages. The resistance profile of human-associated CC398 lineage frequently includes resistance to macrolides and lincomycins and the presence of *erm*(T) gene [17, 31, 46], similar to our case. One strain belonging to CC30 harbored the *ant*(4′)-Ia and *mupA* genes encoding resistance to tobramycin and mupirocin, respectively. It is important to remark the detection of a mupirocin-resistant strain in the human intestinal microbiota because this antibiotic is the treatment of choice to decolonize the human nasopharynx [30]. The narrow antimicrobial resistant phenotype and genotype found among the studied strains fall within the normal low range typically identified on MSSA strains.

A moderate rate of *S. aureus* fecal carriage has been detected in this study (15 %) with a wide variety of genetic lineages identified. The detection of MSSA strains of the lineages ST398(t571)/CC398 and ST133(t3495)/CC133 is of relevance. Data from our study suggest that strain ST398(t571)/CC398 belongs to the subclone associated with humans, given that it preserves the genes of IEC system, remains negative for the *mecA* gene, and is tetracycline-susceptible. Nevertheless, more studies should be performed in the future to analyze the genetic lineages of big collections of MSSA of healthy humans and animals to deepen the knowledge of the origin and evolution of this microorganism in different niches.

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