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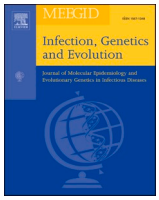

Nasal staphylococci microbiota and resistome in healthy adults in La Rioja, northern Spain: High frequency of toxigenic *S. aureus* and MSSA-CC398 subclade

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ABSTRACT

This study determined the nasal staphylococci diversity and characterized their resistome, with a focus on the mobilome of methicillin-susceptible *Staphylococcus aureus* (MSSA)-CC398 subclade from healthy adults in La Rioja (northern Spain). Nasal staphylococci recovered from 57 healthy individuals (HI) were identified (MALDI-TOF-MS) and their antimicrobial resistance, virulence determinants and genetic lineages were studied. The relatedness of MSSA-CC398 isolates was assessed by core-genome single-nucleotide-polymorphisms (SNPs). One-hundred-forty-three non-repetitive staphylococci were obtained from most HI (98.2%), of which *S. epidermidis* (87.7%) and *S. aureus* (36.8%) were the predominant species. About 15% of the 27 *S. aureus* and 30.1% of the 116 coagulase-negative staphylococci (CoNS) isolates presented a multidrug resistance (MDR) phenotype. All *S. aureus* isolates were MSSA but 30.2% of CoNS isolates were *mecA*-positive and carried SCC*mec* types III, IV, and V. The highest non-beta-lactam resistance (frequency/genes) in *S. aureus* and CoNS were: erythromycin-clindamycin-inducible (25.9%/*ermT, ermC*) and mupirocin (30.1%/*mupA*), respectively. About 85% of *S. aureus* isolates carried relevant virulence genes. Eight clonal complexes (CCs) of MSSA were identified, of which CC398 was the predominant (33.3%). About 78% of the CC398 isolates harboured *rep*13-bound *ermT* gene, however, one carried a *rep*10-bound *ermC* gene. Only the *ermT*-positive MSSA-CC398 isolates were closely related (<50 SNPs) and carried the φ*Sa3*. Diverse MDR-*S. epidermidis* isolates were identified which included the lineages ST59 and ST210. The high rate of toxigenic *S. aureus* and of MSSA-CC398 subclade highlight the ability of HI to carry and transmit virulent isolates. Moreover, the high frequency of MDR-CoNS, often linked with SCC*mec,* needs to be monitored for their potential human health implications.

1. Introduction

Antimicrobial resistance (AMR) in *Staphylococcus* spp. is one of the top-priority health challenges that require genetic characterization to understand its emergence and dissemination pattern. The *Staphylococcus* genus is classified into coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS), of which the former is generally considered to be less pathogenic than the latter (Heilmann et al., 2019).

Staphylococcus aureus (the main representative of the CoPS), as well as many CoNS, are major components of the nasal and skin microbiota of humans (Parlet et al., 2019). However, some CoNS species have been shown to influence the colonization rate of *S. aureus* (Parlet et al., 2019). Certain *S. aureus* lineages exhibit major impacts on public health and in all ecological niches (Howden et al., 2023). In humans, the primary reservoir for staphylococci is the nasal cavity (Raineri et al., 2022). Clinically, *S. aureus* is the most important and has been identified in 30–50% of healthy adults (Abdullahi et al., 2021; Liu et al., 2015). Interestingly, an estimated 30% of bacteremia cases were associated to previous nasal *S. aureus* carriage (Tong et al., 2015). Colonization

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proceeds to infection in some cases (Raineri et al., 2022; Tong et al., 2015).

The virulence potential, MDR trait and host adaptation systems in *S. aureus* are of crucial concern in human health as persistent carriers could be potential vectors of transmission to others, including animals (Raineri et al., 2022; Monecke et al., 2021). Moreover, community-associated *S. aureus* often carries the bi-component *luk-F/S-PV* gene that encodes a cytolytic and toxic substance (Panton-Valentine Leucocidin) that is associated with severe cases (Monecke et al., 2021). Moreover, *S. aureus* causes a series of infectious processes through its ability to elaborate virulence determinants such as toxins, enzymes, haemolysins and other cytolysins (Raineri et al., 2022; Monecke et al., 2021).

The genetic lineage *S. aureus* CC398 has attracted special attention in the last 15 years. Methicillin-resistant *S. aureus* (MRSA)-CC398 is considered a livestock-associated (LA) subclade and is generallly linked to the pig industry and also to humans with professional contact with pigs (Matuszewska et al., 2022). Most isolates of the MRSA-CC398 subclade lack the *scn* gene, a biomarker of the Immune Evasion Cluster (IEC), that seems to be related to the human adaptation process (Price et al., 2012). Another CC398 subclade, that is MSSA-CC398, generally has the *spa*-type t571 and seems to be livestock-independent, more virulent for humans, and often carries the *scn* gene (Tegegne et al., 2022). This subclade has been reported in different countries causing bloodstream human infections (Mama et al., 2021; Bouiller et al., 2016; Vandendriessche et al., 2011) and other types of infections (Arfaoui et al., 2022). Moreover, it has also been sporadically reported as commensal of humans and animals (Gómez-Sanz et al., 2013; Lozano et al., 2011). This MSSA-CC398 subclade is now presenting great epidemiological relevance, so studies that focus on their genomic characteristics are needed.

The CoNS have increasingly gained relevance due to their roles in opportunistic, hospital-associated, prosthetic joint infections and sepsis (Michels et al., 2021). Moreover, some CoNS have been shown to carry the SCC*mec* mobile elements that could be transferred to certain methicillin-susceptible *S. aureus* to become methicillin-resistant (MR) (Rossi et al., 2020). Epidemiologically, the presence of SCC*mec* types I, II, and III are generally linked to hospital-associated MRSA while those that carry SCC*mec* types IV or V are generally thought to be community-associated (Moosavian et al., 2017). Moreover, some CoNS could carry critical and transferable linezolid resistance genes.

Nasal *S. aureus* in supposedly healthy humans can serve as a good indicator of colonization and of potential risk of infecion when they carry important virulence genes. Moreover, within-host (more than one staphylococcal species per host) and intra-species AMR diversity (same staphylococcal species with more than one AMR profile) could provide better information about the dynamics of AMR levels, persistence and potential inter-host interactions and transmission of staphylococci in humans. The concept of the multiple antibiotic resistance (MAR) index has previously been described as an effective and reliable epidemiological marker to track the source of bacteria isolates (Badawy et al., 2022). An isolate with a MAR index of greater than two (>2) could indicate areas with high antibiotic pressure (Badawy et al., 2022).

This study, therefore, evaluated and characterized the diversity of species and the resistome of nasal staphylococci with a focus on virulent *S. aureus* isolates and those of the MSSA-CC398 subclade from healthy adults in La Rioja, Northern Spain.

2. Materials and methods

2.1. Study area, participants, samples processing and staphylococci recovery

Fifty-seven healthy individuals were enrolled in this study in the La Rioja region (Northern Spain) between July 2022 to March 2023, and their nasal samples were collected into Amies transport media and processed for staphylococci recovery. The inclusion criteria for participation in this study were individuals who had not used antibiotics, visited a hospital, had no contact with animals, and had no professional contact with health institutions and/or microbiology laboratories in the last six months.

The protocols used for nasal swab sample processing, the type of bacteriological media used for bacterial isolation and how the bacteria were identified were as follows: the nasal samples were enriched in 6.5% NaCl supplemented brain heart infusion broth and incubated at 37 °C for 24 h. Thereafter, the enriched nasal samples were diluted in sterile Milli-Q water and carefully dispensed onto blood agar (BA), mannitol salt agar (MSA), oxacillin screening agar base (ORSAB), and CHROMagar™ LIN (CHROMagar™ LIN, Paris, France). The inoculated agar plates were then incubated for bacterial recovery at 37 °C for 24 h (BA and MSA) and 48 h (for ORSAB and LIN). After these incubations, between 4 and 12 different colonies per sample were randomly selected based on their morphology, colour, size and haemolysis that are compatible with staphylococci. All the selected colonies were passaged onto BHI agar at 37 °C for 24 h to obtain pure colonies. Pure colonies were identified by the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Bruker Daltonics, Bremen, Germany) using the standard extraction protocol recommended by the manufacturer.

2.2. Antimicrobial resistance profile and genetic characterization of the non-repetitive staphylococci isolates

The susceptibility of all the identified staphylococci to 12 antimicrobial agents was determined by disc diffusion method using the following antibiotic discs (μg/disc): penicillin (1 unit for *S. aureus* and 10 units for CoNS), cefoxitin (30), mupirocin (200), gentamicin (10), tobramycin (10), clindamycin (2), erythromycin (15), ciprofloxacin (5), chloramphenicol (30), tetracycline (30), linezolid (10), and trimethoprim-sulfamethoxazole (1.25 + 23.75). The categorization of results into resistant or susceptible was based on the breakpoints and recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022). Once the antimicrobial resistance phenotype of all staphylococci was determined, non-repetitive isolates were selected (those of different species in each sample, or more than one if they presented different AMR phenotypes) for further characterization. The AMR genes, genetic lineages and virulence factors were determined in non-repetitive staphylococci.

The genes that mediate resistance to the following class of antimicrobial agents were investigated by PCRs: beta-lactams (*blaZ, mecA* and *mecC*), macrolide-lincosamide-streptogramins-b (*mphC, msrA, ermA, ermB, ermC, erm43, ermT, lnuA, lnuB* and *vgaA*), aminoglycosides (*ant4′* and *aac6′-aph2″*), trimethoprim (*dfrA, dfrD, dfrG* and *dfrK*), chloramphenicol (*fexA, fexB, catA,* cat_{pC194}, cat_{pC221} and cat_{pC223}), mupirocin (*mupA*) and tetracycline (*tet*(K), *tet*(L) and *tet*(M)). Moreover, the chloramphenicol-resistant isolates were screened for the presence of *cfr, cfrB, cfrD, poxtA* and *optrA* genes. Furthermore, to study resistance to quinolones, amino acid changes in genes encoding the GyrA and GrlA proteins in *S. aureus* were analyzed. For this, the *gyrA* and *grlA* genes were amplified and sequenced, and the sequences were compared with the reference sequences of *S. aureus* NCTC 8325 (GenBank accession number: CP000253).

Multi-drug resistance (MDR) was defined as isolates with resistance to ≥3 classes of the antimicrobial agents tested (Magiorakos et al., 2012), while the MAR (multiple antibiotic resistance) index was defined as the number of antibiotics to which an isolate was resistant divided by the total number of antibiotics tested (Badawy et al., 2022).

The *spa* types of all the *S. aureus* isolates were determined by PCR/sequencing. The Ridom Staph-Type software (Ridom GmbH, Münster, Germany) was used to analyse the sequences. Subsequently, the CCs of the *S. aureus* isolates were assigned according to their *spa* types. A specific PCR for the CC398 lineage was performed targeting the *sau1-hsdS1*

gene (Stegger et al., 2011). Moreover, multilocus sequence typing (MLST) of *S. aureus* isolates with *spa* types that could not be assigned to definitive CCs was performed.

For the MDR-*S. epidermidis* isolates, the seven MLST genes (*acrC, aroE, gtr, pyrR, mutS, tpi* and *yqiL*) were amplified, and the sequence type (ST) was assigned according to the MLST database (https://pubmlst.org/). Furthermore, the SCC*mec* types of all the non-repetitive methicillin-resistant (MR)-CoNS were determined by multiplex PCRs as previously described (Kondo et al., 2007).

The *tst, luk-F/S*-PV, *eta, etb, etd, sea, seb, sec, sed,* and *see* genes that encode for the toxic shock syndrome, Panton-Valentine leucocidin, exfoliative toxins, and enterotoxins, respectively, were analyzed by PCR, and all *S. aureus* isolates positive for any of these genes were confirmed by sanger sequencing of the PCR amplicons. Furthermore, the presence of the *scn, chp, sak, sea,* and *sep* genes were investigated to enable the classification of the immune evasion cluster (IEC) types into A–G (van Wamel et al., 2006). Primers and conditions of PCRs for the AMR genes tested are included in Supplementary Table S1. Positive controls were included in all the PCR protocols.

2.3. Whole genome sequencing

The whole genomes of seven MSSA-CC398 selected isolates (one per healthy carrier) were sequenced on the Illumina NextSeq platform (San Diego, CA, USA). The genomic DNA extraction and quantification, sequence libraries preparations and whole genome sequencing were carried out following the established protocols previously described (Abdullahi et al., 2023).

All the genomes of the MSSA CC398 isolates obtained in this study were de novo assembled using SPAdes (v.3.15.5), performed using in silico typing with the settings of a minimum 80% identity and 90% coverage. First, genomes of the seven MSSA CC398 isolates in this study were mapped against 47 *S. aureus* CC398 (BioProject number: PRJNA763220) and four MSSA-CC398 isolates from dog owners (GenBank accession numbers: ERX9972359, ERX9972358, ERX9972360, ERX9972361) and the NASP pipeline (v.1.0.0) was used to detect their core-genome single nucleotide polymorphisms (SNPs) (Sahl et al., 2016) and presented on a phylogenetic tree. SNPs calling done using the GATK (v.4.2.2) and position with <90% unambiguous variant calls and < 10 depth were excluded. The IQ-TREE (v.2.1.2) software was used to construct the phylogenetic trees using ModelFinder with 100 bootstraps. Furthermore, graphical data obtained were added to the phylogenies with iTOL (v.6.6) (Letunic and Bork, 2021).

2.4. Genome annotation, typing and in-silico analysis

The genetic lineage (sequence type) of the MSSA-CC398 isolates was reconfirmed with MLST (v.2.16) (Jolley et al., 2018). The AMR genes were identified using ABRicate v.0.9.0 and the various bioinformatic databases such as CARD (https://card.mcmaster.ca/) and ResFinder from the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/services/). Furthermore, virulence factors and plasmid replicons were detected using VirulenceFinder and PlasmidFinder from the CGE database while VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) was used to further search for other virulence genes. The EasyFig Software was used to generate and illustrate the genetic environment of the *ermT* gene in comparison with nine *ermT*-positive MSSA-CC398 isolates (GenBank accession numbers: LNJ000000000, SRR15903552, SRR15903562, SRR15903539, SRR15903541, SRR15903551, SRR15903559, SRR15903563, SRR15903567). Moreover, the genetic environment of the *ermC* gene in one of our MSSA CC398 isolates was illustrated in comparison with two *ermC*-positive MSSA-CC398 isolates (GenBank accession numbers: ERR3306808, SRR445274).

2.5. Genome availability

All genome reads of the MSSA CC398 isolates generated from this study have been deposited at the European Nucleotide Archive under study accession number PRJEB63134.

2.6. Data management and statistics

Data obtained from this study reported frequencies and have been presented on tables and charts. Univariate logistic regression was carried out to compute the odd ratio (OR) at a 95% confidence interval (95%CI) between the co-carriage rate of *S. aureus* and *S. epidermidis* with significant association at $p < 0.05$.

3. Results

3.1. Frequencies and species diversity of nasal staphylococci from healthy humans

Staphylococci were detected in 56 of the 57 healthy humans tested (98.2%), of which a total of 214 isolates were recovered (up to eight isolates of similar or different species per sample). The distribution of *Staphylococcus* species is presented in Supplementary Table S2. After species identification and AMR phenotype determination, 143 non-repetitive isolates (27 *S. aureus* and 116 CoNS) were selected and further characterized in this study. These 143 isolates corresponded to one isolate of each species per sample or more than one if they presented different AMR phenotypes (Supplementary Table S2).

The 27 non-repetitive *S. aureus* isolates were identified from 21 (36.8%) healthy individuals. Moreover, the 116 non-repetitive CoNS isolates identified from 56 healthy individuals were of six species: *S. epidermidis* (87.7%), *S. hominis* (7%), *S. haemolyticus* (5.3%), *S. warneri* (5.3%), *S. lugdunensis* (1.8%), and *S. pasteuri* (1.8%) (Supplementary Table S2).

3.2. Antimicrobial resistance determinants of the non-repetitive nasal staphylococci from healthy humans

Of the 27 non-repetitive *S. aureus* isolates, no methicillin resistance was detected, but 14.8% presented an MDR phenotype. The following AMR rates were found (percentage of isolates/ genes or mutations detected): penicillin (81.5/*blaZ*), erythromycin-clindamycin-inducible (25.9/*ermC, ermT*), erythromycin (7.4/*msrA*), clindamycin (3.7/*lnuA*), ciprofloxacin (14.8/GrlA [p.S80F], GyrA [p.S84L]), tobramycin (7.4/*ant4"*), tetracycline (3.7/*tet(K)*), sulfamethoxazole-trimethoprim (3.7/*dfrG*), and mupirocin (3.7/*mupA*) (Supplementary Fig. S1).

Of the 116 non-repetitive CoNS isolates, 12.9% were susceptible to all antibiotics tested, 28.4% were resistant to only one antibiotic and 30.2% presented an MDR phenotype. The following AMR rates were detected (percentage of isolates/genes detected): penicillin (66.4/*blaZ*), cefoxitin (26.7/*mecA*), erythromycin-clindamycin-constitutive (12.1/*ermA, ermB, ermC, erm43, vgaA, mphC*), erythromycin-clindamycin-inducible (4.3/*ermB, ermC, erm43*), erythromycin (24.1/*msrA, mphC*), clindamycin (6/*lnuA, vgaA*), ciprofloxacin (4.3), tobramycin (6/*ant4"*), gentamicin-tobramycin (7.8/*aac6'-aph2"*), tetracycline (18.9/*tet(K)*), sulfamethoxazole-trimethoprim (14.7/ *dfrA, dfrG*), mupirocin (30.1/*mupA*), and chloramphenicol (0.9/*catA*) (Table 1). No linezolid resistance gene was detected in any of the staphylococci (Table 1). Among the 35 MRCoNS, SCC*mec* type IV and V elements were the predominant (28.6% each), then SCC*mec* type III (8.5%), while all others were non-typeable (34.1%) (Supplementary Fig. S2).

The MAR indices of most *S. aureus* isolates were less than two (70.4%) (range: 0.08–0.5). In addition, the MAR index of the CoNS isolates ranged from 0 to 0.67 (Tables 2 and 3).

Table 1
Number of isolates of the different coagulase-negative staphylococci species from healthy humans and those with distinct AMR and MDR phenotype.

CoNS species	Non-repetitive CoNS isolates[a]				% of antimicrobial resistance/ genes detected												
	Total number	Number isolates susceptible to all antibiotics tested (%)[b]	Number isolates resistant to only one antibiotic (%)[c]	Number isolates with MDR phenotype (%)[d]	PEN	FOX	ERY	CLI	ERY-CLI^{cons}	ERY-CLI^{ind}	TET	TOB	GEN-TOB	SXT	CIP	CLO	MUP
S. epidermidis	103	12 (11.8)	27 (26.5)	31 (30.1)	77.5/ *blaZ*	24.3// *mecA*	22.3/*msrA, mphC*	4.8/*lnuA, vgaA*	12.7/*ermA, ermB, ermC, erm43, vgaA, mphC*	4.9/*ermB, ermC, erm43*	20.9/*tet* (K)	5.8/ *ant4'*	6.9/*aac6'-aph2, ant4'*	14.7/*dfrA, dfrG*	4.9	0.9/ *cat*	40.2/ *mupA*
S. haemolyticus	3	0	1 (33.3)	2 (66.7)	66.7/ *blaZ*	66.7/ *mecA*	66.7/*msrA, mphC*	33.3/*vgaA*	33.3/*ermC*	S	33.3/*tet* (K)	33.3/ *ant4'*	33.3/*aac6'-aph2"*	33.3/*dfrG*	S	S	33.3/ *mupA*
S. hominis	4	0	2 (50)	2 (50)	75/ *blaZ*	50/*mecA*	25/*msrA, mphC*	S	S	S	S	S	25/*aac6'-aph2, ant4'*	25/*dfrA*	S	S	25/ *mupA*
S. warneri	4	2 (50)	2 (50)	0	25/*blaZ*	25/*mecA*	S	25/*vgaA*	S	S	S	S	S	S	S	S	S
S. lugdunensis	1	0	1 (100)	0	100/ *blaZ*	S	S	S	S	S	S	S	S	S	S	S	S
S. pasteuri	1	1 (100)	0	0	S	S	S	S	S	S	S	S	S	S	S	S	S
Total isolates (%)	116	15 (12.9)	33 (28.4)	35 (30.2)	77 (66.4)	31 (26.7)	28 (24.1)	7 (6)	14 (12.1)	5 (4.3)	22 (18.9)	7 (6)	9 (7.8)	17 (14.7)	5 (4.3)	1 (0.9)	43 (30.1)

S: Susceptible.
CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

[a] Non-repetitive isolates: one isolate of each species/sample or more than one if they presented different AMR phenotypes.
[b] MAR index = 0.
[c] MAR index = 0.1.
[d] MDR: resistance to at least 3 families of antibiotics.

Table 2
Molecular typing, AMR and virulence determinant of the 27 *S. aureus* isolates from healthy humans.

Host ID No	Isolate ID number	*spa* type	[a]CC/ST	AMR phenotypes	MAR index	AMR genes or mutations detected	IEC type	Virulence genes detected
H4	X6379	t571	CC398	ERY^{1}-CLI^{ind1}	0.17	*ermT*^{2}	C	Negative
H5	X6417	t571	CC398	ERY^{2}-CLI^{ind2}	0.17	*ermT*^{2}	C	*sec*
H7	X6407	t4390*	CC121/ST51	PEN^{1}-ERY^{1}-MUP^{1}	0.25	*blaZ*^{1}, *msrA*^{1}, *mupA*^{1}	E	*eta, etb, sed*
H8	X6389	t012	CC30	PEN^{2}	0.08	*blaZ*^{2}	E	*sec, sed*
	X6390	t4390*	CC121/ST51	PEN^{1}-CIP^{1}	0.17	*blaZ*^{1}, GrlA (p.S80F) ^{1}, GyrA (p.S84L) ^{1}	E	*eta, etb, sec, sed, see*
H9	X6396	t355	CC152	PEN^{3}	0.08	*blaZ*^{3}	D	*luk-F/S-PV, sea, sed, see*
H14	X6610	t571	CC398	PEN^{4}	0.08	*blaZ*^{4}	C	Negative
H15	X6618	t571	CC398	PEN^{4}-ERY^{4}-CLI^{ind4}	0.25	*blaZ*^{4}, *ermT*^{4}	C	Negative
H17	X6621	t091	CC7	PEN^{4}	0.08	*blaZ*^{4}	G	*sea, sec, sed, sep*
	X6625	t091	CC7	PEN^{2}-ERY^{2}	0.17	*blaZ*^{2}, *msrA*^{2}	G	*see, sep*
H18	X6597	t7521*	CC15/ST15	PEN^{4}	0.08	*blaZ*^{4}	C	*sec*
H19	X6589	t091	CC7	PEN^{3}	0.08	*blaZ*^{3}	A	*sea, sed, see*
H22	X6628	t3200*	CC5/ST7476	PEN^{1}-CLI^{1}-TET^{1}- SXT^{1}-TOB^{1}-CIP^{1}	0.5	*blaZ*^{1}, *lnuA*^{1}, *tet*(K)^{1}, *dfrG*^{1}, *ant4*$^{'1}$, GrlA (p.S80F) ^{1}, GyrA (p.S84L) ^{1}	B	*sec, see*
H25	X6714	t1994	CC159	PEN^{1}-TOB^{1}-MUP^{1}	0.25	*blaZ*^{1}, *ant4*$^{'1}$, *mupA*^{1}	E	*eta, etb, sed, see*
	X6713	t1451	CC398	PEN^{1}-ERY^{1}-CLI^{Ind1}	0.25	*blaZ*^{1}, *ermT*^{1}	C	*sec, see*
	X6715	t1451	CC398	ERY^{1}-CLI ^{Ind1}	0.17	*ermT*^{1}	C	Negative
	X6755	t1077*	CC121/ ST4244	MUP	0.08	*mupA*^{1}	B	*eta, etb, sed, see*
H33	X7056	t1998	CC398	PEN^{3}-ERY^{3}-CLI^{ind3}-CIP^{3}	0.33	*blaZ*^{3}, *ermT*^{3}, GrlA (p.S80F) ^{3}, GyrA (p.S84L) ^{3}	C	Negative
	X7052	t1451	CC398	PEN^{2}-ERY^{2}-CLI^{ind2}	0.25	*blaZ*^{2}, *ermC*^{2}	C	Negative
H41	X8988	t1451	CC398	PEN^{3}-ERY^{3}-CLI^{ind3}	0.25	*blaZ*^{3}, *ermT*^{3}	C	Negative
H43	X9053	t6389	CC121	PEN^{1}	0.08	*blaZ*^{1}	G	*sep*
H46	X9070	t223	CC22	PEN^{3}	0.08	*blaZ*^{3}	F	*tst, sec, sed, see, sep*
H47	X9072	t223	CC22	PEN^{3}	0.08	*blaZ*^{3}	F	*tst, sec, sed, see, sep*
H50	X9069	t223	CC22	PEN^{3}	0.08	*blaZ*^{3}	B	*eta, tst, sec, see*
H51	X9071	t159	CC121	PEN^{3}	0.08	*blaZ*^{3}	B	*sec*
H55	X9062	t005	CC22	PEN^{1}	0.08	*blaZ*^{1}	F	*tst, sec, sep*
H63	X9080	t078	CC5	PEN^{1}	0.08	*blaZ*^{1}	B	*etd, sec, sed, see*

Sequence Type: ST; * new *spa*-types; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; TOB: tobramycin; ERY-CLI^{ind}: erythromycin-clindamycin inducible.
[a] CC assigned according to the *spa*-type, except for CC398 (determined by specific PCR). The ST of isolates with new *spa* types were determined by MLST.

3.3. Genetic lineages, virulence genes and IEC types of the S. aureus isolates

Seventeen different *spa* types were detected among the MSSA isolates that were assigned to 8 clonal complexes (CCs), of which CC398 (*spa* types t571, t1451 and t1998) was the predominant (33.3%), followed by CC121 (18.5%), then CC22 (14.8%) and CC7 (11.1%) (Table 2). Four new *spa* types were identified (t4390-ST51, t7521-ST15, t3200-ST7476 and t1077-ST4244). About 85% of the *S. aureus* isolates carried one or more of the *luk-F/V-PV, tst, eta, etb, etd, sea, seb, sec, sed, see* and *sep* virulence genes. All seven IEC types were identified among the MSSA isolates. Interestingly, the IEC-type C was the only one detected in the MSSA-CC398 isolates (Table 2). The predominant virulent factor (genes, frequencies, and genetic lineages) detected were those that encode: enterotoxin (*sea* to *see* and *sep*, 81.5%, all the CCs), followed by exfoliatin (*eta, etb, etd,* 22.2%, CC5, CC22, CC121, CC159), then toxic shock syndrome (*tst,* 11.1%, CC22). Moreover, one isolate carried the genes encoding the Panton-Valentine Leucocidin (*luk-F/S-PV,* 3.7%, CC152) (Table 2).

3.4. Genetic diversity of nasal staphylococci from healthy humans

Of the 21 *S. aureus* carriers, 4 (19%) of them harboured isolates with different AMR genes and/or genetic lineages in the same host (Table 2, Supplementary Fig. S3).

Intra-host CoNS species diversity (more than one CoNS species in a sample) was detected in 12.5% of healthy human staphylococci carriers (Supplementary Fig. S3). Moreover, healthy human carriers of CoNS with similar species but with diverse AMR phenotypes/genotypes (2–6 AMR profile) were detected in 76.8% (Supplementary Fig. S3). For instance, in one of the *S. epidermidis* carriers (CG26), six different AMR genotypes were identified, viz.: (i) *blaZ, mphC, msrA, dfrA, dfrG, ant4', mupA;* (ii) *mphC, msrA, dfrA;* (iii) *blaZ, ermB, tet*(K), *dfrA;* (iv), *blaZ, mecA, msrA, mupA;* (v) *blaZ, mphC, msrA, dfrA, mupA;* and (vi) *blaZ, mecA, ermC, erm43, dfrA* (Table 3).

About 1/3 of healthy humans with MSSA-CC398 isolates had *S. epidermidis* nasal co-carriage, whereas 60% of those with non-CC398-MSSA isolates had *S. epidermidis* co-carriage (Supplementary Table S2). Collectively, 52.4% of those with *S. aureus* (both CC398 and non-CC398) had *S. epidermidis* co-carriage. However, there was no significant association between the presence of nasal MSSA, MSSA-CC398 and *S. epidermidis* co-carriage in healthy individuals ($p > 0.05$) (Supplementary Table S2).

3.5. Genetic environment and phylogenetic relatedness of the MSSA-CC398 carriers

About 78% of the CC398 isolates had the *ermT* gene that was in plasmid *rep*13 flanked by IS257, which were upstream of *cadR* and *cadD* genes (Fig. 1). However, one of the MSSA-CC398 isolates carried the *ermC* gene in plasmid *rep*10 (Fig. 2). Interestingly these markers were absent in one of the MSSA-CC398 isolates (X6610) (Table 2). Only the MSSA-CC398-*ermT*-positive isolates were highly related (<50 SNPs) and carried the φ*Sa3 prophage* (IEC type-C) (Fig. 3, Supplementary Table S3). Analyses with other publicly available genomes revealed the relatedness of the isolates X6417 and X6379 with SRR15903565 in France (SNP < 200) (Fig. 3, Supplementary Table S3).

3.6. Genetic characteristics of the 35 MDR-CoNS isolates from healthy human carriers

Of the 35 non-repetitive MDR-CoNS isolates (*S. epidermidis, S.*

Table 3
Antimicrobial resistance determinants identified in CoNS carrying multi-drug resistant (MDR) phenotype of healthy humans.

Host ID number	Isolate/ code	*MDR phenotype	MAR index	AMR genes detected	SCC*mec* type	Genetic lineage[a]
H1	*S. epidermidis*/ X6411	PEN-ERY-CLI-MUP	0.33	*blaZ, ermA, msrA, vgaA, mupA*	–	NT
H4	*S. epidermidis*/ X6380	PEN-FOX-ERY-CLI-MUP	0.42	*blaZ, mecA, msrA, mupA*	IV	ST59
H5	*S. epidermidis*/ X6412	PEN-FOX-ERY-SXT-MUP	0.42	*blaZ, mecA, msrA, dfrA, dfrG, mupA*	IV	ST59
	S. epidermidis/ X6473	PEN-FOX-ERY-MUP	0.33	*blaZ, mecA, msrA, mupA*	IV	ST5
H6	*S. epidermidis*/ X6414	PEN-FOX-CLI-SXT-CIP	0.42	*blaZ, mecA, dfrA, dfrG, vgaA*	V	ST2
H7	*S. epidermidis*/ X6406	PEN-FOX-ERY-CLI-SXT	0.42	*blaZ, mecA, erm43, vgaA*	IV	ST22
H13	*S. haemolyticus*/ X6461	PEN-FOX-CLI-MUP	0.33	*blaZ, mecA, vgaA, mupA*	V	NT
H16	*S. epidermidis*/ X6630	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	–	NT
	S. epidermidis/ X6827	PEN-FOX-TET-TOB-GEN	0.42	*blaZ, mecA, tet*(K), *aac6'-aph2", ant4'*	IV	ST87
H19	*S. epidermidis*/ X6590	PEN-ERY-CLI- MUP	0.33	*blaZ, ermC, lnuA, vgaA, mupA*	–	NT
H21	*S. epidermidis*/ X6602	PEN-FOX-ERY-CLI-TET	0.42	*mecA, erm43, vgaA, tet*(K)	III	ST49
H22	*S. epidermidis*/ X6599	PEN-ERY-CLI-SXT-TOB	0.42	*blaZ, ermC, lnuA, dfrG, ant4'*	–	ST210
	S. epidermidis/ X6601	PEN-FOX-SXT-CIP-CHL	0.42	*blaZ, mecA, dfrA, dfrG, catA*	V	ST210
H24	*S. epidermidis*/ X6752	PEN-ERY-TET	0.25	*blaZ, mphC, msrA, tet*(K)	–	NT
	S. epidermidis/ X6711	PEN-FOX-ERY-SXT-MUP	0.42	*blaZ, mecA, dfrA, mupA*	IV	ST969
H25	*S. hominis*/ X6712	PEN-FOX-ERY-MUP	0.33	*mecA, mphC, msrA, mupA*	IV	NT
H26	*S. epidermidis*/ X6759	PEN-ERY-CLI-TOB-CIP	0.42	*blaZ, mphC, msrA, dfrA, dfrG, ant4', mupA*	–	ST24
	S. epidermidis/ X6737	ERY-SXT-CIP	0.25	*mphC, msrA, dfrA*	–	NT
	S. epidermidis/ X6738	PEN-ERY-CLI^{ind}-TET-SXT	0.42	*blaZ, ermB, tet*(K), *dfrA*	–	ST59
	S. epidermidis/ X6739	PEN-FOX-ERY-MUP	0.33	*mecA, mphC, msrA, mupA*	V	ST59
	S. epidermidis/ X6740	PEN-ERY-SXT-MUP	0.33	*blaZ, mphC, msrA, dfrA, mupA*	–	–
	S. epidermidis/ X6741	PEN-FOX-ERY-CLI^{ind}-MUP	0.42	*blaZ, mecA, ermC, erm43, dfrA*	V	ST5
H29	*S. epidermidis*/ X6837	PEN-ERY-TOB-GEN	0.33	*blaZ, msrA, aac6'-aph2"*	–	NT
	S. epidermidis/ X6821	PEN-ERY-MUP	0.25	*blaZ, mphC, msrA, mupA*	–	NT
H32	*S. epidermidis*/ X6832	PEN-ERY-TOB	0.25	*blaZ, mphC, msrA, ant4'*	–	NT
H33	*S. haemolyticus*/ X7059	PEN-FOX-ERY-TET-TOB-GEN-SXT	0.58	*blaZ, mecA, msrA, mphC, tet*(K), *aac6'-aph2", ant4', dfrG*	V	NT
H35	*S. epidermidis*/ X8987	PEN-FOX-TOB-GEN MUP	0.42	*blaZ, mecA, aac6'-aph2", mupA*	IV	ST8
H39	*S. epidermidis*/ X8993	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	–	NT
H46	*S. epidermidis*/ X9066	PEN-FOX-CLI-TET-SXT-TOB-GEN-CIP	0.67	*blaZ, mecA, lnuA, tet*(K), *aac6'-aph2", ant4'*	V	ST173
	S. epidermidis/ X9097	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	–	NT
	S. epidermidis/ X9092	PEN-ERY-CLI^{ind}-MUP	0.33	*blaZ, ermC, vgaA, mupA*	–	–
H47	*S. hominis*/ X9052	PEN-FOX- SXT-CIP	0.33	*blaZ, mecA, dfrA*	IV	–
H60	*S. epidermidis*/ X9084	PEN-ERY-CLI^{ind}- TET-TOB-MUP	0.5	*blaZ, erm43, aac6'-aph2", mupA*	–	ST5
	S. epidermidis/ X9085	ERY-TET-TOB	0.25	*msrA, tet*(K), *ant4'*	–	NT
H64	*S. epidermidis*/ X9086	PEN-ERY-TOB-GEN-MUP	0.42	*blaZ, vgaA, aac6'-aph2", mupA*	–	ST22

CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

* MDR was defined by ≥3 AMR phenotypes for different families of agents.

[a] MLST of all MDR-MR-*S. epidermidis* with an MDR phenotype of ≥3 classes were conducted. Moreover, the genetic lineages of methicillin susceptible-*S. epidermidis* with an MDR phenotype of ≥4 classes of antibiotics were also determined.

haemolyticus and *S. hominis*) from 22 carriers (38.6%), the predominant genetic lineage among *S. epidermidis* was ST59 (12.1%), followed by ST5 and ST210 (6.1% each). Other genetic lineages detected but in low frequencies include ST2, ST8 ST24, ST49, ST87 and ST173 (Table 3). Of the 22 MDR-CoNS carriers, 8 (36.4%) had varied AMR genotypes and/or SCC*mec* types in the same or different *Staphylococcus* species (Table 3). AMR patterns and genetic lineages were detected in three households (11.1%) (Table 3).

4. Discussion

Many studies all over the world have previously reported the nasal carriage of *S. aureus* in healthy individuals with or without occupational contact with animals, as systematically reviewed by Abdullahi et al. (2021). However, studies on non-*aureus* staphylococci of nasal origin are seldom reported. To our knowledge, none of these studies characterized the intra-host and intra-species genetic and AMR diversity of staphylococci in healthy humans.

The detection of *S. epidermidis* at a very high frequency in healthy humans is not surprising, as it is the major colonizer of the skin and nasal cavity of individuals. This finding is similar to a German study that also reported a very high recovery rate of *S. epidermidis* in healthy volunteers (Marincola et al., 2021). Usually, previous studies have focused mainly on CoNS in clinical samples or on humans who had contact with animals, likely aimed at determining the influence of healthcare facilities, sickness or contact with animals in the acquisition of antibiotic-resistant isolates (Asante et al., 2021). Moreover, a high nasal carriage rate of *S. aureus* (36.8%) was detected in healthy individuals in this study. The frequency of nasal *S. aureus* in healthy individuals without any risk of prior colonization varies depending on the region and/or country. Lower carriage rates (19.1% and 22.2%) were reported in Spain by Falomir et al. (2014) and Lozano et al. (2011), respectively. The variation in the frequencies of *S. aureus* carriers could be due to difference in detection methods with or without enrichment before cultural plating for bacterial isolation. Moreover, other factors such as the study methodologies, differences in timescale (i.e., the year of study), specific biodata of the participants or level of previous antimicrobial use could be responsible for the variations (Abdullahi et al., 2021). Following these two *Staphylococcus* species, *S. warneri* and *S. haemolyticus* were also detected but in low frequency (6.8% and 5.3%, respectively). Concerning *S. warneri*, a closely similar frequency (11.1%) was previously reported in nasal samples of healthy children in Valencia (Spain) (Falomir et al., 2019).

No MRSA carriage was detected in our study, as this could be because our participants did not have any contact with high-risk locations (such as hospitals and livestock farms) or animals that could predispose to MRSA colonization in humans. The majority of our MSSA isolates were susceptible to most of the antibiotics tested except penicillin. Aside from penicillin, resistance to macrolides-lincomycin-streptogramins B (MLS_b) predominates and this could be explained by the wide use of MLS_b in the treatment of Gram-positive bacterial infections. The detection of some mupirocin-resistant MSSA isolates in this study could render the use of this nasal de-colonizer or topical treatment of superficial skin infections less useful (Nong et al., 2023).

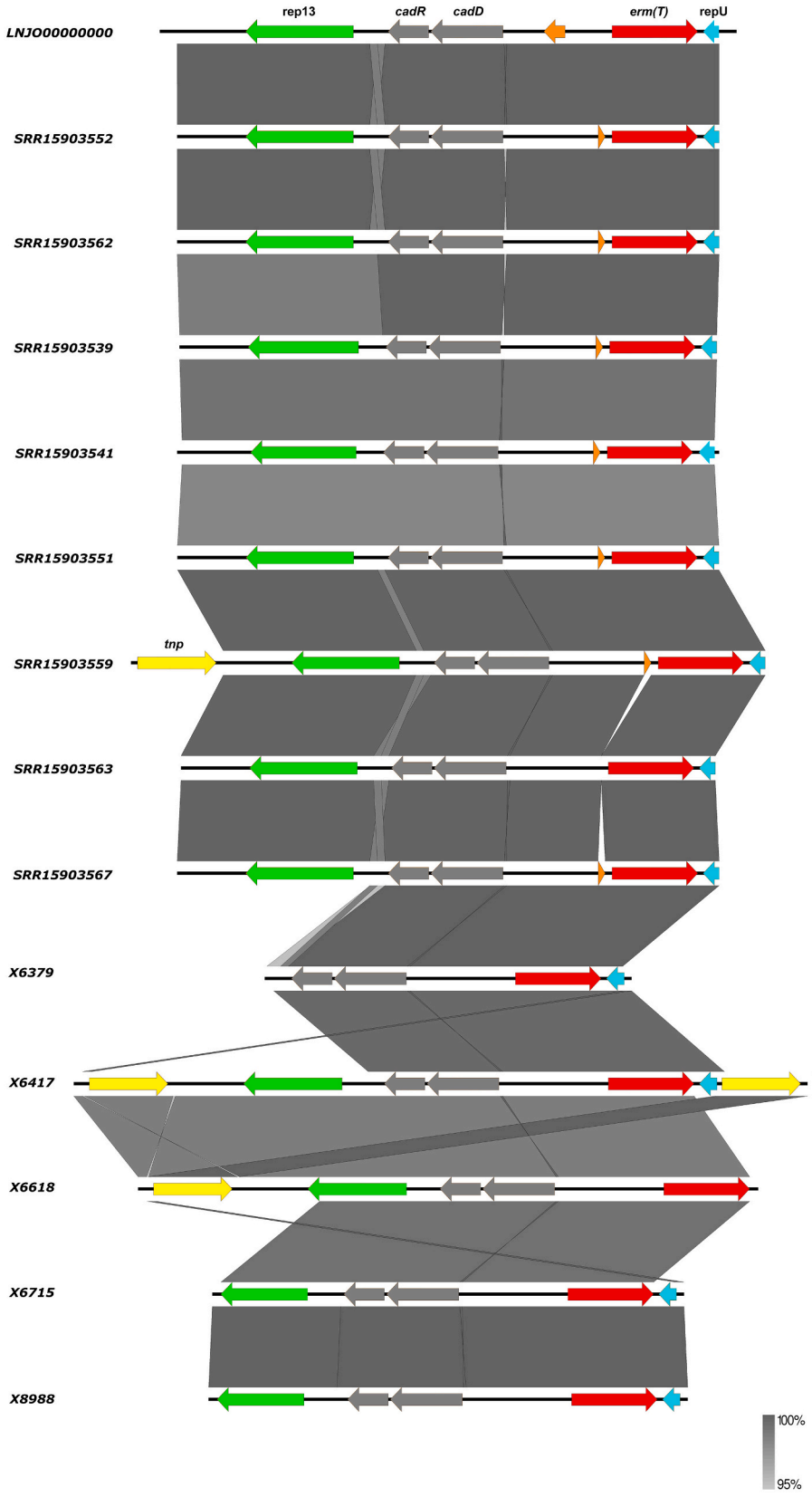

Fig. 1. Genetic environment of the *ermT* gene of the five MSSA-CC398 strains in this study in comparison with nine publicly available *ermT*-positive-MSSA-CC398 genomes. Shown in the figure are *ermT* gene located in the same contigs and frames with their corresponding mobile genetic elements. The percentage of identity and scale bar legends are presented on the right side of the image.

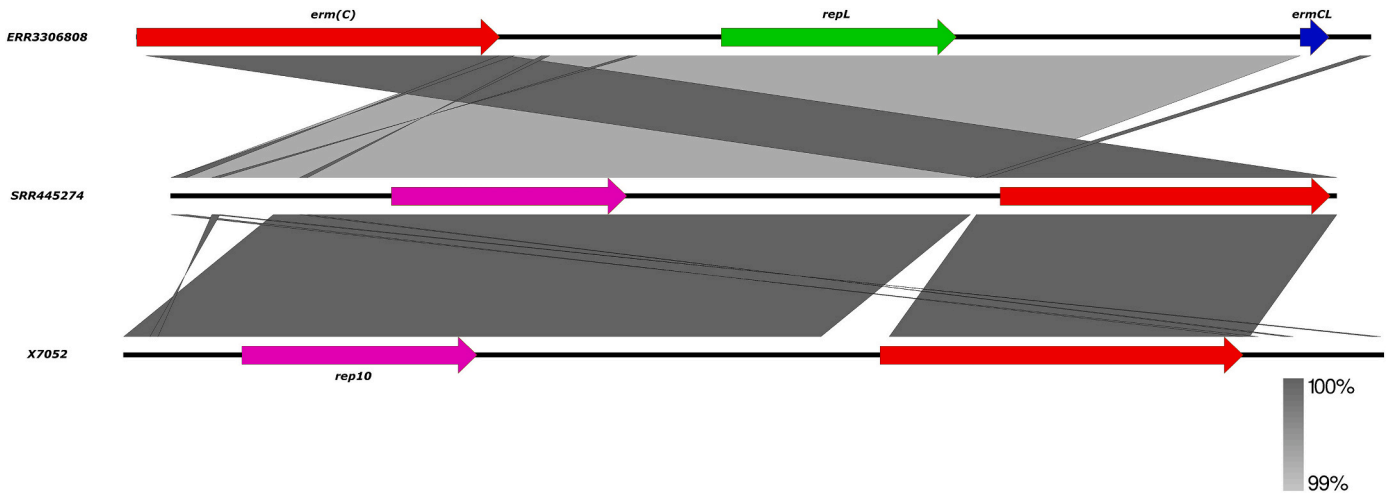

Fig. 2. Genetic environment of the *ermC* gene of one MSSA-CC398 (X7052) in comparison with two publicly available *ermC*-positive-MSSA-CC398 genomes. Shown in the figure are *ermC* gene located in the same contigs and frames with their corresponding plasmid replicon. The percentage of identity and scale bar legends are presented on the right side of the image.

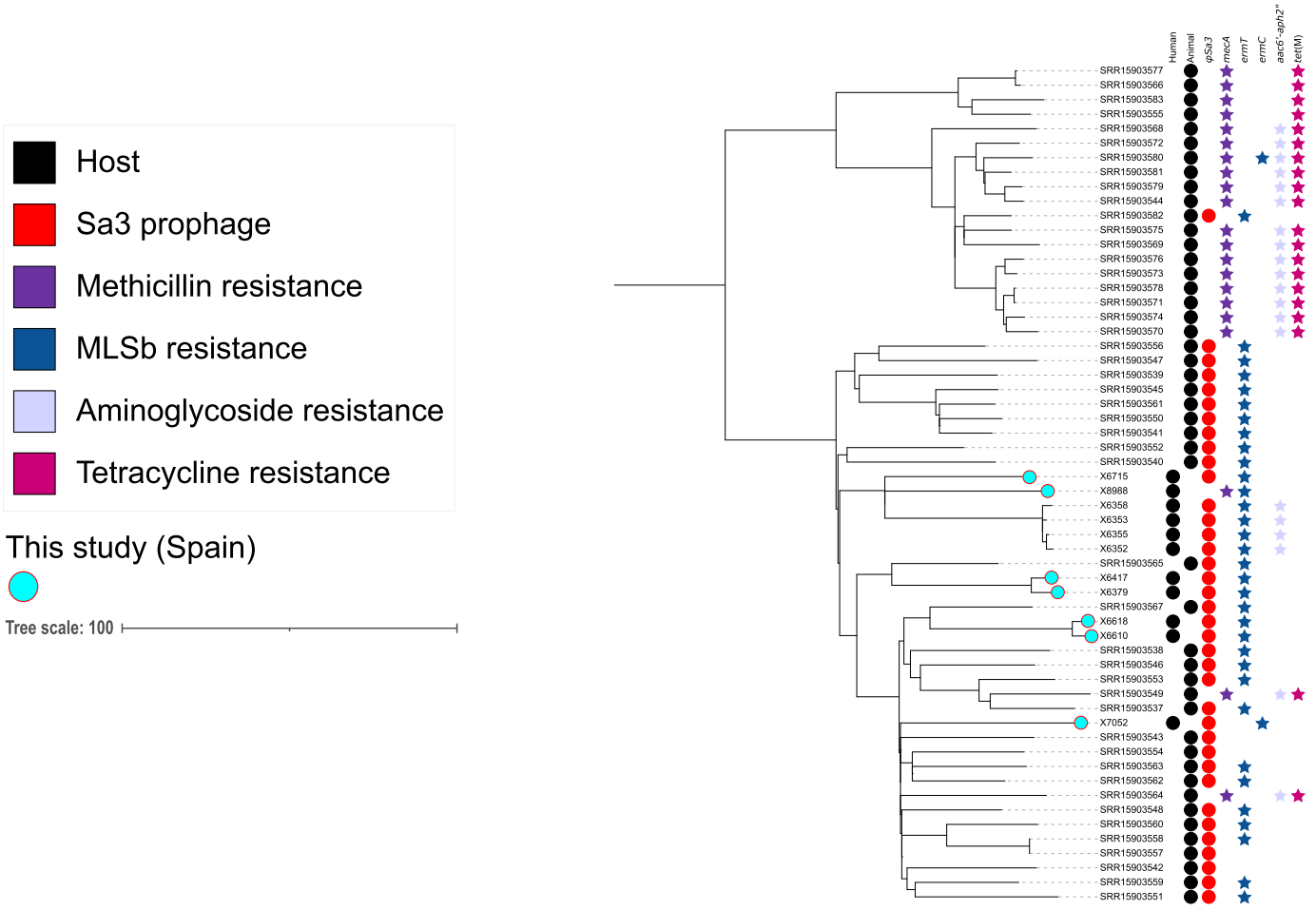

Fig. 3. Phylogenetic tree based on core genome SNP analysis of seven MSSA-CC398 isolates from healthy humans with 51 publicly available MSSA-CC398 and MRSA-CC398 genomes.
Colors (in circles) of the hosts and φSa3 prophage, while those in starts are the AMR genes.

The highly diverse *spa* types among the MSSA isolates recovered, depict that most of the *S. aureus* carriers were sparsely related. However, it is important to remark on the predominance of the MSSA-CC398 and MSSA-CC121 lineages. All the CC398 isolates recovered in our study were MSSA and *scn*-positive, predominantly of the *spa*-type t571. It has been previously reported that the MSSA-CC398 subclade is more virulent for humans and has been implicated in invasive human infections (Mama et al., 2021). Nevertheless, all the MSSA-CC398 isolates

recovered in our study were from healthy individuals who had no prior known risk factors of colonization or infection. In addition, most of them harboured the *ermT* gene which appears to be a very useful biomarker for MSSA-CC398 subclade. As previously indicated, it seems that two subclades of CC398 *S. aureus* isolates exist: the LA-MRSA-CC398 isolates (generally lacking the *scn* gene) and the livestock-independent MSSA-CC398 (mostly of *spa*-t571 and *scn* positive). According to the data from our study, it seems that this livestock-independent MSSA-CC398 (*scn*-positive) subclade could be a frequent colonizer of healthy individuals who had no contact with livestock or with healthcare facilities. Moreover, it is important to remark on the first detection of MSSA-CC398 with erythromycin-clindamycin-inducible resistance mediated by *ermC* in Spain. Consequently, we report for the first time the genetic environment with a focus on the associated plasmid replicons of *ermT* and *ermC* genes of the MSSA-CC398 isolates from healthy individuals. The genetic environment of the contigs that harbour the *ermT* gene was similar to those previously reported but in human and animal isolates (Tegegne et al., 2022; Price et al., 2012). The *ermT* gene was often associated with cadmium-resistant genes (*cadR* and *cadD*) and plasmid *rep*13, whereas these features were absent in the *ermC* genetic environment that was solely associated with plasmid *rep*10. Collectively, it appears that the *ermT*-positive MSSA-CC398 subclade is fast expanding across some European countries and more hosts. Whereas the *ermC*-carrying MSSA-CC398 is silently evolving even in healthy individuals. Although all our MSSA-CC398 isolates carry very few or no enterotoxin genes, more studies should be conducted in the future to unravel the mechanism(s) by which MSSA-CC398 acquires virulence and causes clinical infections in humans.

MSSA-CC121 has evolved into a hypervirulent etiological agent of staphylococcal scalded skin syndrome mediated by the exfoliative toxin genes (*eta* and *etb*). Conversely, *etd*-carrying *S. aureus* are primarily associated with mild cutaneous infections, particularly the MSSA-CC5 lineage has been implicated in paediatric skin abscesses many years ago (Bukowski et al., 2010; Yamasaki et al., 2006). The MSSA-CC152 lineage is often associated with PVL, especially in Africa and Europe causing community-acquired pneumonia (Lawal et al., 2022; Baig et al., 2020). Concerning the *tst*-carrying MSSA, all were of the CC22 lineage that was previously reported in Iran (Goudarzi et al., 2020). The detection of the high frequency of enterotoxin, re-emerging exfoliative and other virulent MSSA isolates underscores the potential roles of healthy humans in the transmission of *S. aureus* infection to vulnerable individuals such as children, the critically ill and immunosuppressed persons who can have personal contact with these carriers via kissing or from their nasal discharge (Raineri et al., 2022).

It is now recognized that CoNS could exchange AMR genes within the same group and even to *S. aureus* through horizontal gene transfer (Frosini et al., 2020). Our findings in this study showed that MR- and MDR-CoNS are carried in the nasal samples of healthy individuals in the community. Methicillin resistance trait in staphylococci is a major cause for concern especially when they are carried by the SCC*mec* mobile genetic elements. In our study, MRCoNS isolates were high (26.7%) while 30.2% presented the MDR phenotype. Specifically, 48.6% of the MDR-CoNS isolates were methicillin-resistant. This high MR-CoNS rate agrees with previous reports on CoNS in healthy individuals (He et al., 2020; Lebeaux et al., 2012). However, the frequency may vary considerably, as one study reported as high as 50% (Kateete et al., 2020). Relatively high erythromycin, mupirocin and tetracycline resistance rates were also found among the CoNS isolates. For the macrolide resistance, it was not surprising, as it is among the top classes of antibiotics that are frequently prescribed (Fan et al., 2020), whereas mupirocin has been used to decolonize nasal MRSA (Lord et al., 2022). Thus, resistance to these categories of antibiotics might be associated with high selective pressure due to their frequent use (Marincola et al., 2021).

Fortunately, linezolid resistance was not detected in any of the *Staphylococcus* isolates. However, very low (only one) resistance to chloramphenicol was recorded. This strongly indicates that our study participants do have no contact with livestock as resistance to these antibiotics is relatively more common in livestock farmers than in healthy individuals who had no previous contact with animals.

MDR was high in our CoNS carriers, as even some isolates presented resistance against five or six classes of antibiotics (MAR index >5). Therefore, MDR-CoNS may limit the available chemotherapeutic options against staphylococcal and many other Gram-positive bacterial infections. Another important phenomenon to remark on is the high intra-host species and intra-species AMR diversity. To our knowledge, this is one of the few studies on healthy individuals to determine this phenomenon. Being heterogeneous, many *S. epidermidis* carriers had genetically diverse isolates with varied AMR genes and/or lineages. In the future, this may pose a difficulty in eradicating *S. epidermidis* when they cause infections such as prosthetic joint infections (Widerström et al., 2022). The detection of methicillin-resistant *S. epidermids*-ST59, a known community-associated lineage highlights its versatility and ease of transmission in the human population (Liu et al., 2022). Also, methicillin-resistant *S. epidermidis*-ST2, a well-established hospital-associated genetic lineage (Ruiz-Ripa et al., 2021), was identified from one of our participants. It is important to remark on the detection of multi-resistant *S. haemolyticus* (MAR index = 0.58) that carried multiple genes that mediate MDR phenotype (*blaZ, mecA, msrA, mphC, tet(K), aac6'-aph2", ant4', dfrG*). In recent times, *S. haemolyticus* has been a major cause of neonatal sepsis (Westberg et al., 2022), consequently, this species deserves genome-base surveillance to track its relevance in clinical infectious diseases.

5. Conclusion

The high rate of toxigenic, diverse *S. aureus* and clonally related MSSA-CC398 isolates highlight the ability of healthy individuals to carry and transmit pathogenic isolates of *S. aureus*. Since the majority of the MSSA-CC398 isolates presented the characteristic erythromycin-clindamycin-inducible resistance, this phenotypic feature and/or the detection of the *ermT* gene could be useful as biomarkers for the surveillance of this subclade. Moreover, MDR-CoNS needs to be closely monitored to determine their potential implications in non-*aureus* staphylococcal infections.

Consent to participate (include appropriate statements)

All the human subjects freely gave informed consent to participate in the study prior to their enrolment.

Ethics approval (include appropriate approvals or waivers)

All the procedures and experimental protocols used in this study were approved by the Ethical Committee of the University of La Rioja (Spain).

Data statement (data transparency)

The data generated from this study has been fully presented in the manuscript. However, further requests can be made through the corresponding author.

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CRediT authorship contribution statement

Idris Nasir Abdullahi: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Carmen Lozano:** Validation, Formal analysis, Data curation, Writing – review & editing, Supervision. **Myriam Zarazaga:** Validation, Formal analysis, Writing – review & editing, Funding acquisition. **Islem Trabelsi:** Validation, Data curation, Writing – review & editing. **Rine Christopher Reuben:** Validation, Data curation, Writing – review & editing. **Marc Stegger:** Validation, Data curation, Writing – review & editing. **Carmen Torres:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None declared by authors.

Data availability

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2023.105529.

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