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Comparative genomics of *Staphylococcus aureus* strains from wild birds and pig farms elucidates levels of mobilomes, antibiotic pressure and host adaptation

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ABSTRACT

Objectives: This study characterized the resistome, mobilome and phylogenomic relatedness of *Staphylococcus aureus* strains previously obtained from healthy nestling storks (HNS), pigs (HP) and pig farmers (HPF) to analyse possible transmission pathways of *S. aureus* with implications for the spread of antimicrobial resistance.
Methods: The genomic contents of 52 *S. aureus* strains obtained from the nasal cavity of HNS, HP and HPF in Spain were sequenced using the Illumina NextSeq platform to characterize their resistome, virulome and mobile genetic elements. The relatedness of strains was assessed by core-genome single nucleotide polymorphisms (SNPs).
Results: The frequencies of multidrug-resistance phenotype and transposons were significantly lower in strains from HNS than in those from HP and HPF ($P < 0.005$). However, the presence of human immune evasion cluster genes in *S. aureus* strains from HNS was significantly higher than in those from HP and HPF ($P < 0.005$). Interestingly, the frequencies of plasmids and phages were not significantly associated with the host ($P > 0.05$). The phylogenetic analysis identified a cluster of all the MSSA-CC398 strains carrying φSa3 and *ermT* on *rep13* separately from the two MRSA-CC398 strains (carrying *ermT* on *repUS18*). Highly related MRSA-CC398 strains were detected in some pigs and related farmers (<10 SNPs).
Conclusion: This study confirms high-level antibiotic selection in *S. aureus* in HP and HPF in comparison to HNS. Furthermore, our findings highlight the continuous transmission of MRSA-CC398 in the pig-to-human interface and MSSA-CC398 with human adaptation markers in HNS. Molecular surveillance of *S. aureus* using the One Health model is required to establish appropriate control strategies.
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1. Introduction

Antimicrobial resistance (AMR) constitutes one of the major global health challenges that need a holistic ‘OneHealth’ approach. In this regard, *Staphylococcus aureus* is one of the suitable bacteria, as certain genetic lineages can cross host species barriers and transfer AMR genes among humans, animals and their shared environment [1]. The emergence and spread of methicillin-resistant *S. aureus* (MRSA) is often blamed on the overuse and overprescription of antibiotics in human and veterinary medicine and livestock production [2]. On the other side, wild animals generally carry low-level AMR in commensal bacteria [3,4], but some of them, such as migratory birds that forage in areas close to anthroponotic activities or livestock grazing areas, could be colonized by MRSA [5].

S. aureus is a multi-host bacterium and is generally a significant component of the nasal and skin microbiota of humans and animals (including wild animals) [6–9]. However, it can also causes various infections in humans and animals [10,11]. *S. aureus* is an

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asymptomatic colonizer in pigs, but it can cause disease in other livestock, such as mastitis in dairy bovines [12,13]. AMR, virulence and host adaptation in *S. aureus* are relevant features in its pathogenesis and evolution [14]. Molecular studies have shown that livestock can act as intermittent carriers of *S. aureus* and reservoirs for zoonosis and dissemination of high-level AMR in farmers, the communities close to the farms, and food derived from the animals [15–19]. In this regard, the major livestock-adapted lineage (MRSA-CC398) emerged from human-adapted MSSA-CC398 after the loss of φSa3 and the acquisition of *tet*(M) and SCC*mec* elements and spread in the European pig production [20,21], with subsequent spillover back into humans in the community and healthcare settings [19,22]. Years later, the human-adapted and community-associated MSSA-CC398 reemerged to cause invasive infections in some European countries and China [23–26]. Epidemiologically, the *scn* and *tet(*M) genes are considered molecular markers that can be used to track these lineages [21]. However, fundamental questions that need to be elucidated are the molecular markers of host-switch and virulence level of MRSA-CC398 when they are in non-livestock hosts such as humans and other animals living close to pig farms.

Recently, there has been much interest but scarce data about the global epidemiology of MRSA in wildlife [27]. MRSA could be transmitted by anthropogenic or livestock activities which have been hypothesized to be the primary link to wild animals [8]. As MRSA gets transferred to wild animals, it can be responsible for the spread of AMR genes through mobile genetic elements [27]. Consequently, these underscore the need for global and elaborate studies on wild animals, to direct the best strategies for the control against the spread of MRSA across ecosystems and to limit the global emergence of AMR traits.

Intra-species AMR and lineage diversity (*S. aureus* strains from a single host with more than one AMR profile or lineage at a given point in time) could provide an insight into the dynamics of AMR, inter-host transmission and inter-ecological transmission of *S. aureus* [28]. Few comparative genomic studies have determined the effect of hosts, habitat, ecology and human occupation on antibiotic selection pressure and associated mobile genetic elements. The hosts from the two ecological niches (wildlife and pig farms) were selected in this study due to their distinct differences concerning antimicrobial pressures.

Here, we conducted a comparative genome-based study of *S. aureus* obtained from nestling white storks, healthy pigs and pig farmers to elucidate levels of AMR, mobile genetic elements, and their host adaptation profile to get insight into possible transmission pathways of *S. aureus* with implications for the spread of AMR.

2. Materials and methods

2.1. Staphylococcus aureus strains in this study

Fifty-two *S. aureus* strains obtained in previous studies [29–31] from 87 nestlings of white stork parents foraging in natural areas (n = 5) and landfills (n = 18), and from healthy pigs (n = 17) and pig farmers (n = 12) from four pig farms (A-D), were subjected to whole genome sequencing (Table 1). One *S. aureus* strain from each of the nestling storks was selected, although more than one strain was included when they presented different clonal complexes (CC). Concerning the *S. aureus* strains from the pig farms (A to D), the selection was based on similarity in their sequence type and AMR genes in each farm and the similarity of genetic lineages and AMR genes from strains of pigs and pig farmers as previously determined [29]. Multidrug resistance (MDR) was defined when a strain carried genes that mediate resistance to ≥3 classes of antimicrobial agents [32]. Comprehensive information on the sample collection and processing procedures, bacterial isolation and identification, *S. aureus* antimicrobial susceptibility and AMR genotyping are presented in previous studies [29–31]. All the study protocols have been reviewed and approved by the ethical research committees of the University of Zaragoza, the University of La Rioja and the University of Castilla La Mancha (Spain).

2.2. Genome sequencing, assembly and phylogenetic analyses

Whole genome sequencing of the 52 selected *S. aureus* strains was carried out on the Illumina NextSeq platform. The MagNA Pure 96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA, 4413021) was used to extract genomic DNA according to instructions provided by the manufacturers. The Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) was used for DNA quantification, and sequencing libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA, FC-131-1096) and sequenced on the NextSeq 500 platform (Illumina, San Diego, CA) using a 300-cycle kit to obtained paired-end 150 bp reads, as previously described [33].

All the genomes analysed in this study were *de novo* assembled using SPAdes (v.3.15.5). Core-genome single nucleotide polymorphisms (SNPs) between the 52 *S. aureus* strains in this study were detected with the NASP pipeline (v.1.0.0) [34] and used to reconstruct their phylogenetic relationship. Briefly, the raw sequencing data from all CC398 strains were mapped together with 88 previously published *S. aureus* CC398 genomes (Bioproject number: PRJNA514245) against the chromosome of ST398 strain S0385 (GenBank accession no. AM990992) as a reference to obtain a CC398 phylogeny. GATK (v.4.2.2) was used to call SNPs and excluded positions featuring <90% unambiguous variant calls and <10 depth. IQ-TREE (v.2.1.2) was used to construct the phylogenetic trees using ModelFinder with 100 bootstrap replicates. The graphical data were added to the phylogenies with iTOL (v.6.6) [35]. Supplementary Table S1 shows the SNPs difference between the *S. aureus* CC398 strains from this study (both MRSA and MSSA) and 89 publicly available genomes of *S. aureus* CC398 strains from Price et al. [21].

2.3. Genome annotation, typing and in silico analysis

The sequence types (STs) were determined with MLST v.2.16 [36]. Virulence factors, plasmid replicons and antimicrobial resistance genes were identified using ABRicate (v.0.9.0) (https://github.com/tseemann/abricate) and the respective databases VFDB, Plasmidfinder and Resfinder databases from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). Mutations associated with AMR were identified using ResFinder (v.4.1) [37] and PointFinder [38]. Biocide and heavy metal resistance genes were identified using BACMET [39]. PHASTER was used to identify all prophage elements [40]. Moreover, manual mapping of raw sequencing reads using the KMA algorithm [41] with a minimum of 90% coverage, 80% identity and minimum 6 × depth was performed on sets of prophage integrases and IEC genes and their corresponding GenBank accession numbers: Sa12int (NC_010147), Sa9int (NC_007057), Sa8int (NC_007622), Sa7int (NC_007049), Sa6int (NC_005356), Sa6int (M27965), Sa5int (NC_004615), Sa4int (NC_002953), Sa3int (NC_009641), Sa3int (NC_004617), Sa3int (DQ530361), Sa2int (NC_004616), Sa2int (NC_002321), Sa1int (NC_003288), phiJB_int (NC_028669), SebagoInt (MK618716), IEC_chp (NC_009641), IEC_sak (NC_009641), IEC_scn (NC_009641), IEC_sea (NC_009641) and IEC_sep (BA000018). The *spa*Typer (v.1.0) tool was used to confirm the *spa* types [42]. The SCC*mec* types were assigned using SCC*mec*Finder (v.1.2) (https://cge.food.dtu.dk/services/SCCmecFinder/). The genetic environment of the *ermT* gene was illustrated in comparison with the reference strains; MRSA pUR1902 (GenBank accession

Table 1
Characteristics of *S. aureus* strains from the nestling storks, pigs and pig farmers.

Variables	Nestling storks from parents foraging in landfills (n = 18)	Nestling storks from parents foraging in natural areas (n = 5)	Pigs (n =17)	Pig farmers (n 12)
No. of strains with methicillin resistance trait	0	0	14	12
No. of strains with MDR phenotype	1	0	17	12
spa types	t015, t127, t209, t223, t227, t521, t335, t571, t774, t1094, t1451, t1654, t3380, t7778, t18009	t571, t1451, t2313, t3380 t6220	t011, t034, t1430, t1451	t011, t034, t1451
ST	ST1, ST5, ST15, ST22, ST25, ST26, ST30, ST45, ST97, ST109, ST398	ST97, ST291, ST130, ST398	ST9, ST398	ST398
CC	CC1, CC5, CC9, CC15, CC22, CC25, CC30, CC45, CC97, CC398	CC97, CC130, CC398	CC9, CC398	CC398

number: HF583291.1), MRSA pUR2941 (GenBank accession number: HF583290.1) and MRSA AV4_1 (GenBank accession number: SAMN00828682) using EasyFig software.

2.4. Genome availability

All the raw genome reads generated from this study have been deposited at the European Nucleotide Archive under study accession no. PRJEB66351.

3. Results and discussion

3.1. Genetic relatedness of the S. aureus strains

The genomes of the 52 *S. aureus* strains revealed that pig and pig farmer strains belonged mainly to MRSA-CC398, except for three MSSA-CC9, whereas stork nestling strains belonged to many different CCs and included MSSA-CC398 but not MRSA-CC398 (Table 1). All MSSA-CC9 from pigs clustered into the same cluster, which was different from the MSSA-CC9 strain from a nestling stork (Fig. 1). Furthermore, strains from nestlings of parents feeding in landfills were interspersed compared to those from nestlings of parents feeding in natural areas with no apparent difference in spillover patterns between the two groups.

The phylogenetic analysis of the CC398 strains furthermore showed that all MSSA-CC398 strains from storks (both from natural and landfill areas) clustered within the human clade, whereas the MRSA-CC398 strains from pigs and pig farmers clustered in the LA clade (Fig. 2). Furthermore, closely related MRSA-CC398 strains (<10 SNPs) carrying the same AMR profile were detected between pigs and pig farmers from the same farm (Supplementary Table S1), indicating within-farm transmission of these *S. aureus* strains. This suggests that pig farmers have contracted the LA MRSA-CC398 strains because of their occupation. However, nestling storks carried the MSSA-CC398-*scn*-positive subclade, which is known to frequently circulate among humans [25], and infrequently in livestock. In this regard, nestlings of parent storks that foraged in landfill areas with anthroponotic remains could be hypothesized to be the source of this subclade. However, some CC398- and non-CC398-*scn*-positive strains were obtained from nestlings of parent storks that foraged in natural areas where no or very limited traces of anthropogenic remains can be expected. The reason for this unexpected finding could be complex and related to multiple factors that need to be further studied.

3.2. Association of strains' origin with antibiotic pressure and mobilome levels

Bivariate logistic analysis showed that the frequencies of MDR phenotype and transposons were significantly lower in nesting storks than in pigs and pig farmers ($P < 0.005$) (Supplementary Table S2). However, the presence of the *scn* gene (a marker of the IEC system) in *S. aureus* strains from nestling storks was significantly higher than in those of pigs and pig farmers ($P < 0.005$). The reason for the abundance of the *scn* gene among the storks' strains is not clear, as previously indicated, because it is considered a human adaptation marker. Some authors suggest that various prophages could be relevant determinants for *S. aureus* host switching, transmissibility, infection and adaptation [17]. Future studies focused on this issue could elucidate the mechanisms involved. On the other hand, the frequencies of plasmids and phages were not significantly associated with hosts ($P > 0.05$) (Supplementary Table S2).

3.3. Antimicrobial and metal resistance and associated mobile genetic elements

Generally, most of the *S. aureus* strains from nestling storks were either resistant to only one class of antibiotics (in most cases beta-lactam or macrolides) or entirely susceptible to all antibiotics. In this sense, the resistome profile of the strains was mainly *blaZ, ermT, lnuA* and *tet*(K) as previously detected by PCR [30]. In addition, we here further identified the *ant9′* gene in strain X3799, which was co-localized with *ermA* linked with a Tn*554* transposon (Supplementary Table S3). This was similarly reported in a previous study on *Staphylococcus lugdunensis* [43,44]. To our knowledge, the linkage of the *ermA* gene in Tn*554* has never been reported in MSSA, suggesting the expansion of the ecology of these genes outside methicillin-resistant *S. lugdunensis.* Moreover, *S. aureus* strain X4139 from a nestling of a parent stork foraging in landfills carried the *vga(A)V* gene, which we could not associate with any detectable mobile genetic element (Supplementary Table S3).

Supplementary Table S4 shows the complete resistome of the 35 *S. aureus* strains from pigs and pig farmers. All the *S. aureus* strains presented an MDR phenotype mediated by a large repertoire of resistance markers (often >5 different genes). In this sense, the resistome of these strains mainly consisted of genes that mediate resistance to beta-lactams, macrolide-lincomycin-streptogramin B (MLS_B), tetracyclines, aminoglycosides, sulfamethoxazole-trimethoprim or fluoroquinolones, as previously detected by PCR [29]. In addition, the aminoglycoside gene *str* that mediates resistance to streptomycin was detected in 12 strains. Also, *ant9′*, another aminoglycoside gene, was co-located with the *ermT* gene on plasmid *repUS18* in pig (X4905) and pig farmer (X5473) strains (Supplementary Table S4).

All the *S. aureus* strains from pigs (both MRSA and MSSA) presented a MDR phenotype, contrary to those from the nestling storks, where only one strain presented an MDR phenotype/genotype (X3799). This observation could be associated with the differences concerning antimicrobial pressures (high and low, respectively) between the two ecological niches. All the strains

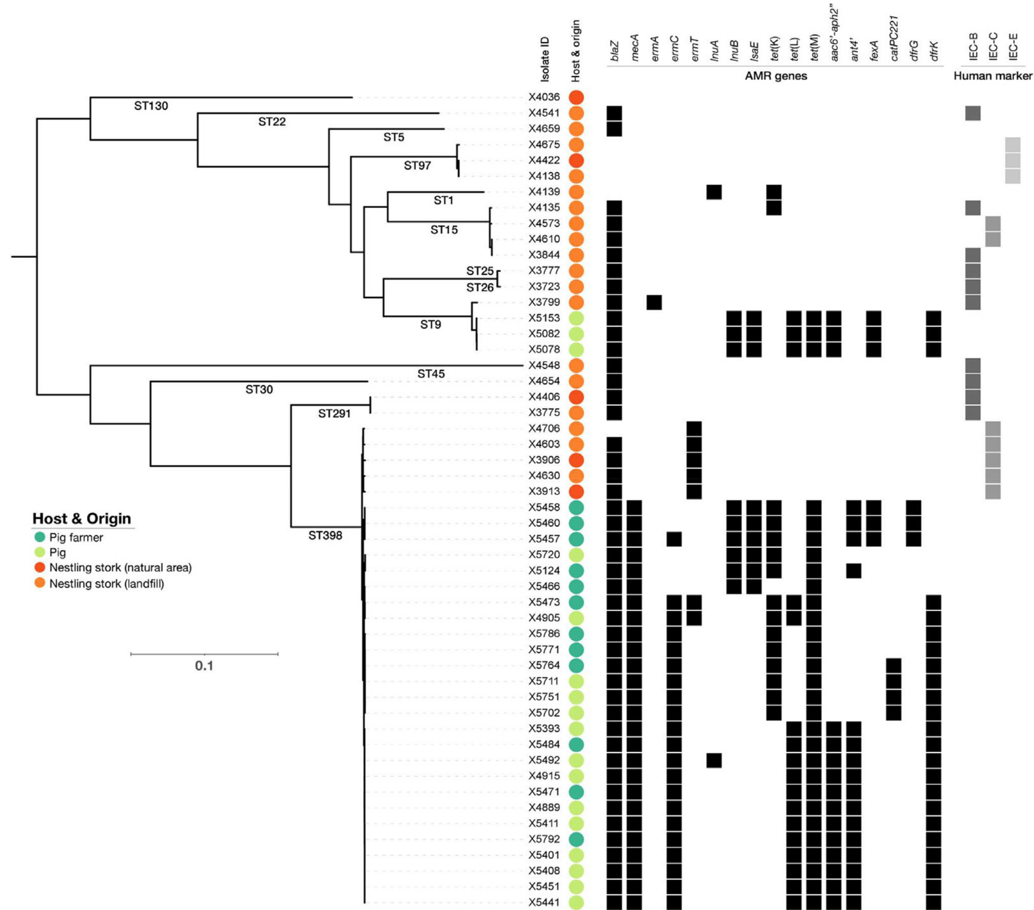

Fig. 1. Phylogenetic tree based on core genome SNP analysis of 52 *S. aureus* strains to demonstrate the influence of antibiotic pressure based on the host.

from pigs and pig farmers presented the characteristic tetracycline resistance where 20 *S. aureus* strains from pigs and pig farmers carried *tet*(M), 12 carried *tet*(K) and 8 carried *tet*(L). It is important to highlight that all the *tet*(L)-carrying *S. aureus* strains were additionally *tet*(M)-positive. Interestingly, the *tet*(M) and *tet*(L) were located in plasmid *rep22* and *repUS43*, respectively, in all the MRSA-CC398 strains harbouring these genes. But *tet*(M)- and *tet*(L)-carrying MSSA-CC9 strains from the two pigs and a pig farmer did not harbour these plasmid replicons. Perhaps these plasmids are emerging mobilomes for *tet*(M)- and *tet*(L) genes in the MRSA-CC398 strains. The *tet*(K) gene in most of the *S. aureus* strains, including the one from a stork nestling (X4139), was located in *rep7a*, while only one strain (X5751) was not associated with this plasmid replicon. The *tet*(M) gene was localized in various transposons such as those detected in this study (i.e., Tn*6009*, Tn*925* and Tn*916*). The Tn*6009* is a member of the Tn916–Tn1545 family and is a conjugative (noncomposite) transposon of Tn*916* [45]. The *tet*(M) has previously been reported to be carried by *repUS43* alongside Tn*6009* in an *E. faecalis* strain [46], but to our knowledge not reported in *S. aureus*. This denotes the diversity of transposons associated with *tet*(M)-mediated tetracycline resistance and suggests their essential role in the evolution and horizontal spread of this resistance marker in *S. aureus* from pigs and pig farmers. However, the *tet*(L) is almost always linked with the *dfrK* gene on small plasmids [47].

Concerning all the strains carrying *tet*(L) and *dfrK* genes, the plasmid *rep22* was always co-located with these genes and is 99% similar to the plasmid pKKS2187 in an MRSA-CC398 strain from a pig (GenBank accession number: FM207105). Moreover, the *dfrK* gene was also found to be located in Tn*558*, which is often integrated into the *radC* gene in MRSA-CC398 strains [48]. Two *S. aureus* strains from nestling storks (X4135 and X4139) presented tetracycline resistance mediated by the *tet*(K) gene carried in circular plasmid *rep7a*, while the *tet*(M) and *tet*(L) genes were absent from all nestling stork strains. It appears that *tet*(M) (in both MRSA and MSSA) was strongly associated with tetracycline resistance in pigs and pig farmers, which is in line with previous findings by Price et al. [21].

Aside from tetracycline, resistance to other clinical antibiotics was also detected, such as to MLS_B antibiotics. AMR to this class of antibiotics was specifically mediated by *lnuB* and *lsaE* in nine *S. aureus* strains from the pigs and pig farmers. However, MLS_B resistance was mediated by *ermA* and *vga(A)V* among the two non-CC398 strains from nestling storks (X3799 and X4139). Also, the *vga(A)LC* gene is located on a *rep7b* in 10 strains (Supplementary Table S3). Furthermore, the *ermT* gene that mediates a peculiar MLS_B resistance (erythromycin-resistant/clindamycin-inducible resistance) was found in five MSSA-CC398 strains from nestling storks (Supplementary Table S3) and two MRSA-CC398 strains from a pig and a pig farmer. the *ermT*-positive-MRSA-CC398 strains are very unusual [49].

Another antibiotic of interest is chloramphenicol, which has long been prohibited for use in pig production, and neither is in use for chemotherapy of human infections in Spain. However, florfenicol (a closely related antibiotic) is approved by the USA Food and Drug Agency and the European Medicines Agencies for use in swine, cattle and sheep [50]. Thus, the detection of *fexA* encoding for chloramphenicol resistance illustrates its persistence due to the use of florfenicol in pig farms, as this was not present in any of the nestling stork strains.

Fig. 2. Circular phylogenetic tree to show the human- and animal-adapted subclades based on SNP analyses of CC398 strains obtained from this study in comparison with publicly available genomes of CC398 lineages from Price et al [21].

Finally, a striking difference in AMR between the strains from the two ecological niches was the ciprofloxacin resistance mediated by DNA topoisomerase IV point mutations at GyrA (S84L) and DNA gyrase at GrlA (S80F) or GrlA (S80Y). These common mutations were found in 16 (55.2%) of the *S. aureus* strains from pigs and pig farmers but not among those from the nestling storks. A similar observation was reported in a previous study on porcine MRSA strains in Germany and Denmark [51,52]. It has been speculated that the mutations in GrlA (S80F or S80Y) might affect recombination between the ΦSa3 phage and *S. aureus* chromosome, which could contribute to the absence of this prophage in MRSA-CC398 from pigs and pig farmers, although an apparent link between fluoroquinolone resistance and integration or stability of ΦSa3 phage could not be demonstrated [52].

Concerning heavy metal resistance genes, certain categorical differences in genes encoding resistance to copper and zinc were observed and widespread among the *S. aureus* strains from pigs and pig farmers, whereas they were absent in the nestling storks' strains. Zinc and copper are frequently used in animal feed formulations, and these metals may co-select for MRSA-CC398 strains that carry the *czrC* and *copA* genes [53,54]. Interestingly, only the *czrC* gene was detected among the MSSA-CC9 strains. The *czrC* gene is part of the SCC*mec* element of MRSA-CC398 [54], so probably the *czrC* in MSSA-CC9 has a different role as these strains do not contain the SCC*mec* elements. Moreover, the *copA* gene appears to be exclusively associated with MRSA strains. Apart from the *cadX* gene that was found only on the MSSA-CC398 subclade with nestling stork strains, the *arsB* gene that encodes for arsenic pump membrane protein was found only in an MSSA-CC130 strain as previously described [55]. However, this gene has also been largely present in MRSA-CC130 strains of wild animals [27]. Moreover, the *cadD* (cadmium transport protein D) gene was carried by 15 (57.7%) MSSA strains (including CC398) from nestling storks. The presence of these genes suggests the existence of metal pollution in the environment where these strains originate from, which could be linked to human activities [56]. It is important to highlight that heavy metal resistance is a matter of public health concern due to its potential hazards in the food chain and the co-selection of AMR [56].

All the MRSA-CC398 strains of pigs and pig farmers carried the SCC*mec* type Vc (5C2&5). The vast majority of MRSA-CC398 strains carry this SCC*mec* type, which often contains the czrC and *copA* genes that detoxify cadmium, zinc and copper [57], of which the *copA* contributes to MRSA-CC398 survival in pig farm environment [58]. Moreover, the use of zinc oxide in livestock production has been found to be associated with the co-selection of MRSA-CC398 [54].

Concerning biocide resistances, only the *qacG* gene was detected in 20 (68.9%) *S. aureus* strains (MRSA-CC398 and MSSA-CC9), all from pigs and pig farmers. The acquisition of *qacG* carried on plasmid *rep*21 could facilitate the persistence of these lineages in the pig/pig farmer environment, as they are resistant to quaternary ammonium disinfectants, thus making their eradication very difficult [59].

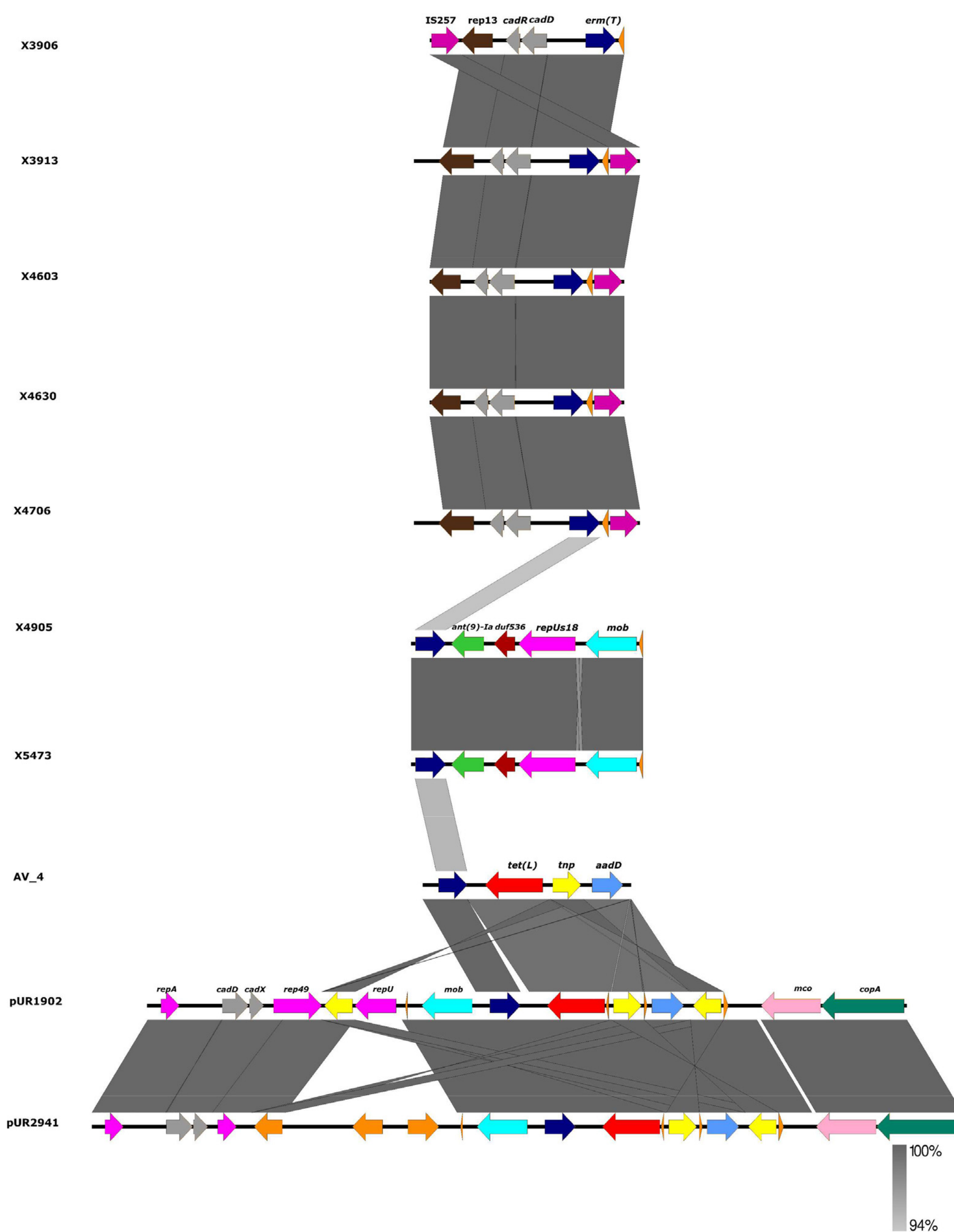

Fig. 3. Schematic comparison between the environment of the *ermT* gene in the five MSSACC398 and two MRSA-CC398 strains with the reference strain MRSA-pURX2941 (HF583290) and pURX1902 (Gene bank accession number: HF583291) and MRSA-AV_4 (Gene bank accession number: SAMN00828682). Colors and arrows indicate the represented genes and their orientation.

3.4. *Genetic environment of the* ermT *gene in MSSA and MRSA strains*

Despite the involvement of the *ermT* gene as a major mediator of inducible MLS_B resistance in MSSA-CC398 strains, its environment and its associated genes and mobilome have rarely been described. The *in silico* analysis of the *ermT* sequences of two MRSA strains from a pig (X4905) and a pig farmer (X5473) and five MSSA strains from nestling storks (X3906, X3913, X4603, X4630, X4703) revealed striking differences in their genetic environment (Figure 3). First, the five *ermT*-carrying MSSA strains were all associated with cadmium-resistance genes, *cadR* and *cadD*, which were absent from the two *ermT*-carrying MRSA strains. The IS*257* was located upstream of the *ermT* gene in MSSA strains, except in one strain in which it was located downstream. Interestingly these markers were absent in the *ermT*-carrying MRSA strains. The *ermT*-gene of the MSSA strains was associated with plasmid *rep13*. Often, the *ermT* gene in MSSA is carried by a plasmid and has recently been recognized as a biomarker of erythromycin-clindamycin inducible resistance in this lineage [25]. Contrary to this observation, the *ermT* gene in MRSA produces erythromycin-clindamycin constitutive resistance, a phenomenon previously reported in the literature [49].

3.5. *Virulome profile of the S. aureus strains from nestling storks and pig farm hosts*

Toxins constitute important virulence determinants of *S. aureus*, with enterotoxins being the most implicated in food safety, especially in meat and dairy products from livestock [60]. More-

over, other virulence factors could be responsible for a range of *S. aureus*-related infectious diseases [11]. The MRSA-CC398 strains were entirely negative for *tst, lukS/F*-PV, *eta, etb, etc, etd* and all genes encoding enterotoxins. This is in line with previous findings [25]. However, the *sem, seo, seu* and *sei* genes were identified in the three MSSA-CC9 strains from pigs. Moreover, *sen, sem, sei, seg, seu* and *seo* genes were also identified in four MSSA strains of the nestling storks belonging to CC5, CC9, CC25 and CC45 (Supplementary Table S3). From our findings, it appears that virulence genes are more predominant in MSSA than in MRSA strains.

Aside from the toxins, some *S. aureus* enzymes were commonly present in all the strains, such as the *adsA* that encodes adenosine synthase A, a cell-wall-anchored enzyme that converts adenosine monophosphate to adenosine and seems to be related to the evasion of host immune responses in *S. aureus* [61]. Furthermore, the *icaABCD* operon and its *icaR* regulatory gene were present in all the strains. This denotes that the *S. aureus* strains easily adhere to the mucosa and serve as a fundamental step in nasal colonization and persistence on environmental surfaces and fomites [62].

3.6. Host adaptation markers of S. aureus

Nineteen (82.6%) strains from the nestling storks carried the *scn* gene mediated by the prophage φSa3 (Supplementary Table S3), whereas all *S. aureus* (MRSA-CC398 and MSSA-CC9) from pigs and pig farmers lacked the prophage φSa3 marker (i.e., *scn*), indicating that they can be considered livestock-associated or originating from nonhuman hosts (Supplementary Table S4), since φSa3 plays a significant role in the host adaptation of S. aureus [14]. For CC398, previous studies divided the lineage into a human-adapted MSSA clade (*scn* positive) and the emerging livestock-associated (LA)-MRSA clade (*scn* negative) [21], even though LA-MRSA CC398, in rare cases, can readapt to the human host through the regaining of an IEC-harbouring φSa3 [63,64]. After a phylogenetic analysis of our CC398 strains, the *scn*-positive stork strains clustered in the human-adapted MSSA lineages, whereas the pig and pig farmer strains clustered in the livestock clade as expected by the absence of *scn* (Fig. 2). Besides prophage φSa3, integrases of other prophages including φSa2, φSa6 and φSa9 were also frequently identified in *S. aureus* strains of the nestling storks, pigs and pig farmers regardless of the methicillin susceptibility and genetic lineages, whereas only MSSA-CC398 strains harboured prophages carrying integrase genes *SebogoInt* and *Sa9int* (Supplementary Tables S3 and S4).

4. Conclusion

Using phylogenetic analysis and the comparison of patterns in host specificity and AMR markers, this study confirms spillover patterns of *S. aureus* between pigs and pig farmers, and from human-associated reservoirs to nestling storks. Furthermore, AMR genes from the pigs and pig farmers were highly associated with mobile genetic elements compared to those from the nestling storks, suggesting increased mobility of AMR genes in pig farms.

A larger sample size could provide more credence to data obtained from the statistical analysis, as some of the observed values were small. However, the idea behind the analyses was to present the relationship of the test variables with the origin or host of the *S. aureus* strains. Collectively, our findings underscore the need to strengthen molecular surveillance of *S. aureus* using the One Health model to direct appropriate control strategies globally.

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Conflict of interest: None declared by the authors.

Ethical Approval: All the study protocols have been reviewed and approved by the ethical research committees of the University of Zaragoza, the University of La Rioja and the University of Castilla La Mancha (Spain).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.12.003.

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