Comparison of Automated Ribotyping, spa Typing, and MLST in 108 Clinical Isolates of *Staphylococcus aureus* from Orthopedic Infections

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Abstract: 108 isolates of *Staphylococcus aureus*, belonging to six large ribogroups according to the automated Ribo-Printer® system, were studied with two highly used molecular methods for epidemiological studies, namely multi-locus sequence typing (MLST) and spa typing, followed by BURP and eBURST v3 analysis for clustering spa types and sequence (ST) types. The aim was to evaluate whether automated ribotyping could be considered a useful screening tool for identifying *S. aureus* genetic lineages with respect to spa typing and MLST. Clarifying the relationship of riboprinting with these typing methods and establishing whether ribogroups fit single clonal complexes were two main objectives. Further information on the genetic profile of the isolates was obtained from agr typing and the search for the mecA, tst genes, and the IS256 insertion sequence. Automated ribotyping has been shown to predict spa clonal complexes and MLST clonal complexes. The high cost and lower discriminatory power of automated ribotyping compared to spa and MSLT typing could be an obstacle to fine genotyping analyzes, especially when high discriminatory power is required. On the other hand, numerous advantages such as automation, ease and speed of execution, stability, typeability and reproducibility make ribotyping a reliable method to be juxtaposed to gold standard methods.

Keywords: *Staphylococcus aureus*; orthopedic infections; strain typing; Riboprinting; multilocus sequence typing (MLST); spa typing

1. Introduction

*Staphylococcus aureus* is a leading pathogen in infections associated with orthopedic implants [1–4]. Originally described using multi locus enzyme electrophoresis (MLEE) and pulsed field gel electrophoresis (PFGE), and subsequently confirmed by multi-locus sequence typing (MLST) [5], the highly clonal structure of *S. aureus* population underlines the flexibility of *S. aureus* to evolve into an assortment of different clones.

Sequence-based typing methods, such as MLST and spa typing, characterized by a universal and worldwide nomenclature and by organized databases and networks (e.g.,
http://spaserver.ridom.de, http://www.seqnet.org/, http://saureus.beta.mlst.net/; accessed on 26 January 2022), are becoming the gold standards for epidemiological and for microbial population genetic investigations. In fact, the increase of variations by mutation occurs approximately linearly with time, and the resulting genetic distance tends to be proportional to the time of divergence between alleles, particularly in MLST [6].

At present, MLST is the most diffuse and highly discriminative typing method, based on the sequencing of seven housekeeping genes. The alleles at these seven loci are compared to an online database for each gene and provide an allelic profile represented by a distinct numerical identifier. A given allele’s combination generates an allelic profile which defines the sequence type (ST) that unequivocally identifies a clone. By this technique, linked STs that share 6/7 alleles (single locus variants, SLV) or at least 5/7 alleles (double locus variants, DLV) may be clustered together by eBURST software (http://eburst.mlst.net/; accessed on 26 January 2022) into genetically related groups referred to as ‘clonal complexes’ (CC), useful to assess the population organization and the patterns of evolution [7].

Spa typing is another commonly used sequence-based typing method for S. aureus isolates, which targets the polymorphic variable number tandem repeat (VNTR) region of the staphylococcal protein A gene (spa). The spa repeats are in general 24 bp in length, and with their putative mutations, duplications, deletions, and rearrangements, contribute to the identification of more than 15,100 distinctive patterns identified as ‘spa types’. Relatedness between spa types is analyzed by Based Upon Repeat Patterns (BURP) algorithm. spa CCs obtained by BURP analysis generally display an excellent concordance with MLST CCs [8]. As for MLST, spa typing data is unambiguous, portable and has a database accessibility, which permits intra- and inter-laboratory comparability. Moreover, this technique is cost-effective, involves easy processing, allows a rapid throughput of samples, and is dynamically evolving [9].

Ribo typing is a classic variant of the southern hybridisation-mediated assay that assesses the number of ribosomal gene loci and their collocation in the genome [10,11]. Bacterial ribosomes contain three distinct types of rRNA, the 16S, the 23S, and the 5S rRNA. Their encoding genes are orderly organized into the operon (rrn), which also includes a spacer region between the genes encoding for 16S and 23S rRNAs. The genetic peculiarity of 16S rRNA gene makes it one of suitable tools among phylogenetic markers for the evolutionary relationships between bacteria [12]. More than a copy of the rrn operon can often be found within a single bacterial genome. While the rRNA genes are at least 99.5% identical among multiple copies of the operon of the same microorganism, the spacer regions, including their internally transcribed spacer (ITS) region, can substantially differ in sequence and structure [12].

Automated ribotyping (riboprinting), which is performed using the RiboPrinter® microbial characterization system (Qualicon), consists of the analysis of the genomic fragments generated by the enzymatic restriction of the rrn operon, frequently found in multiple copies (from 1 to 15) in the bacterial genome [13]. Whole genome sequencing analysis has shown as the number of copies of the rrn operon varies not only across species of the same genus, but also within each single species. For instance, in S. aureus the number of copies of rrn operons was found to vary between four and six (http://rrndb.umms.med.umich.edu/; accessed on 26 January 2022), with six copies being the conditions most frequently observed. As aforementioned, the DNA regions encoding ribosomal RNA are highly conserved, but the flanking sequences result in variations that are significant for the diversities of the ribopatterns [14]. The genetic peculiarity of 16S gene makes it one of suitable tools among phylogenetic markers for the evolutionary relationships between bacteria [12]. The restriction enzymes selected by Stoddard et al. [12] for the digestion of chromosomal DNA were EcoRI and HindIII. However, EcoRI was
the best enzyme used, since, producing more bands and ribotypes than HidIII within the same Staphylococcus species, it can discriminate between more genetic types [15]. The number of bands distinguished by manual or automated ribotyping depends on the quantity of recognitions sites of the restriction enzyme and on the polymorphisms that are present within the rrr operons and their flanking regions.

Automated ribotyping is easy to execute and, due to the complete automation and standardisation, the riboprinting patterns have successful typeability, high reproducibility, and stability. In fact, the patterns of repeated isolates were always the same also after one year period [11,15,16]. Moreover, the reliability and rapidity (approximately 2–3-fold greater than manual ribotyping) make this method an ideal system in the clinical laboratory when identification rather than characterization is requested [14].

This study aimed at analysing by spa typing and by MLST 108 S. aureus isolates belonging to six major ribogroups present in our database, identified by the RiboPrinter® microbial characterization system. Moreover, the study aimed at understanding the relation between the most widely used typing techniques and ribotyping, and at detecting the correspondence of ribogroups with MLST and spa CC. Additional information on the genetic profile of the isolates belonging to the same clonal complexes was obtained from agr typing and from the search for the meca, tst and IS256 genes.

2. Results

2.1. Results of the Automated Ribotyping Analysis

The outcomes resulting from the comparative analysis of the three distinct typing genotypic methods, respectively ribotyping, spa typing, and MLST, on the 108 clinical isolates of S. aureus are shown in Tables 1 and 3. Automated ribotyping identified nine different ribotyping patterns. However, three of them were merged in a unique ribocluster, based on their high similarity, as reported in a previous work [17]. In fact, the three ribogroups cra-119-S8, cra-138-S-2 and cra-53-S-7, constituted respectively by 17, six, and four isolates, were merged in a larger one of 27 isolates hence called ribocluster. In the present study, that ribocluster has been further extended to include 21 additional isolates, thus obtaining a ribocluster consisting of 48 strains. The other ribogroups in order of frequency were cra-94-S-7 (16 isolates), cra-137-S-4 (14 isolates), cra-157-S-4 (13 isolates), cra-146-S-8 (9 isolates), and cra-147-S-6 (8 isolates), as reported in Table 1.

Table 1. Relatedness between ribogroups, STs, MLST CCs and groups founded by eBURST v3 analysis.

| Ribogroup | ST (%) | MLST CC Founder (Breaker) | eBURST Groups ** |
|-----------|--------|---------------------------|-----------------|
| Ribocluster (48) |   | ** |  |
| ST30 (64.6%) | ST34 (20.8%) | ST2954 * (6.2%) | ST2957 * (2.1%) |
| ST2960 * (2.1%) | ST untypeable (2.1%) | CC30 (SLV) | Group 1 |
| cra-94-S-7 (16) | ST228 (87.5%) | ST2956 * (6.25%) | ST46 (6.25%) |
| cra-146-S-8 (9) | ST5 (88.9%) | ST2626 (11.1%) |  |
| cra-137-S-4 (14) | ST8 (64.3%) | ST247 (28.6%) | ST241 (7.1%) |
| cc-137-S-4 | CC (69.5%) | CC (28.6%) | CC (7.1%) | Group 2 |
| cc-137-S-4 | CC (69.5%) | CC (28.6%) | CC (7.1%) | Group 3 |
2.2. Results of the MLST Analysis

The MLST analysis generated 19 STs, these including five new, previously unreported, STs, plus an additional non-typeable ST (Table 1). The isolates belonged to five clonal complexes, namely CC30, CC8, CC7, CC5, and CC1, and a sporadic CC, CC45. The population structure analysis based on eBURST v3 algorithm with the limit of 5 SLV produced 4 groups and singletons, namely ST1, ST109, ST101, and ST46.

2.3. Relatedness between Ribogroups, STs, MLST CCs and eBURST Groups

As reported in Table 1, the majority of the 48 strains of the ribocluster belonged to two MLST strain types: ST30 (64.6%) and ST34 (20.8%). The other less represented strains belonged to: ST243, ST2954, ST2957, and ST2960. A further strain resulted a non-typeable ST. All of them formed part of the CC30 (this was perfectly aligned with our previously published findings [17]) and of Group 1 founded by eBURST v3 analysis (DLV limit).

The ribogroup *cra*-146-S-7 consisted prevalently of strains identified as ST228 (87.5%). The 14 strains ST228 and an additional strain belonging to a previously unknown ST, namely ST2956, were all part of the CC5 and of the eBURST Group 2. A further strain ST46 was attributed to CC45 and to the ST46 singleton, as per eBURST identification. A similar ribogroup, *cra*-146-S-8, consisted uniquely of strains belonging to CC5 and eBURST Group 2. In this case, ST5 represented the most frequent ST (87.5%).

All 14 strains of the ribogroup *cra*-137-S-4 belonged to CC8 and eBURST Group 3, with ST8 (64.3%) and ST247 (28.6%) being the two prevailing STs and ST241 being represented by a single strain. The ribogroup *cra*-157-S-4 saw the prevalence of strains ST7 (69.2%) and included one strain, ST2955, a new, previously unreported, strain type. Although most of its strains belonged to CC7, a pair of strains were ST109 and associated to CC1 and one strain was ST101, for which the CC was not delivered. The strains of CC7 were clustered by eBURST in Group 4, while both ST109 and ST101 were classified as singletons. The ribogroup *cra*-147-S-6 consisted uniquely of strains ST1 (CC1), which were grouped in the ST1 singleton by eBURST analysis.

Overall, ribogroups *cra*-94-S-7 and *cra*-146-S-8 were found to be associated to CC5, the ribocluster to CC30, the ribogroup *cra*-137-S-4 to CC8 and the ribogroup *cra*-147-S-6 to CC1. Based on the above data, the ribogroups of the isolates would predict the MLST CC in a proportion of strains ranging from 76.9 to 100%, which is a remarkable result giving the different methodological approach adopted for strain typing.

2.4. Relatedness between Ribogroups, spa Types, spa CCs and BURP Clusters

The characterization by spa typing discriminated 39 spa types, while the BURP analysis clustered 82 isolates in four spa clonal complexes (spa CC), namely spa CC021/012, spa CC166, spa CC002 and spa CC121, and classified 24 isolates as singletons (Table 2 and Figure 1). Two sporadic isolates (t693 and t1252) were excluded, having the sequences shorter than 5 repetitions. Among the 39 different spa types, three spa types (t13129, t13130 and t13132) were discovered as totally new.
In total, three isolates with spa type t1382 belonging to spa CC021/012, were observed in two different ribogroups, respectively in the ribocluster (2 isolates) and in cra-94-S-7 ribogroup (1 isolate) (Table 2).

The clonal complex spa CC021/012, prevalently correlated with the ribocluster, was the most frequent spa CC among all the 108 isolates (34%), followed by spa CC121 (12%), spa CC166 (7%), and spa CC002 (6%). Moreover, two ribogroups were not associated with any specific spa CC, namely: cra-157-S-4, which was structured by only singletons, and cra-147-S-6, which was structured by singletons and along with the two excluded isolates (Table 2).

Differently from what was observed with MLST CCs, ribotyping could less effectively predict spa CCs and spa clusters. For instance, in cra-147-S-6, only 25% of the strains belonged to Cluster 5 and none to a perfectly identified CC. Similarly, cra-137-S-4 consisting of 92.9% of strains CC121 and Cluster 3, and showing up to 87.5% of the strains belonging to CC002(Cluster 2). Thus, ribotyping never reached 100% ability to predict spa CCs. Conversely, MSLT CC could be predicted by ribotyping in 100% of the strains of the ribocluster and other 3 ribogroups: cra-146-S-8, cra-137-S-4, and cra-147-S-6. Further considerations in this regard may be made on the strange segregation of CC021/012 (Cluster 1), which, although prevailing within the ribocluster, was found to segregate into two further ribogroups: cra-94-S-7 and cra-137-S-4. Such misalignment occurred even with the MLST CC identifications as CC021/012 strains were observed to belong to MLST CC30, CC8, and CC5, suggesting that ribotyping and MLST shared greater similarity when predicting the relatedness of the isolates of S. aureus with respect to spa typing. Given the different methodological approaches existing between the three methods, whose two based on gene sequencing and one based on Southern blotting and restriction fragment length polymorphism (RFLP), it is at least in part surprising that MLST results at CC level seem more consistent with ribotyping than with spa typing.

### Table 2. Relatedness between ribogroups, spa types, spa CCs, and clusters as per BURP analysis.

| Ribogroup          | MLST CC | MLST     | spa Type   | spa CC | Cluster |
|--------------------|---------|----------|------------|--------|---------|
| Ribocluster (48)   | CC30 (48) | ST30 (31)| t012 (12)  |       |         |
|                    |         |          | t021 (8)   |       |         |
|                    |         |          | t018 (3)   |       |         |
|                    |         |          | t093 (1)   |       |         |
|                    |         |          | t338 (3)   |       |         |
|                    |         |          | t1382 (2)  |       |         |
|                    |         |          | t5891 (1)  |       |         |
|                    |         |          | t11956 (1) |       |         |
|                    |         | ST2954 ** (3) | t298 (2) | CC021/012 (37) | Cluster 1 |
|                    |         | ST2957 ** (1) | t021 (1)  |       |         |
|                    |         | ST2960 ** (1) | t021 (1)  |       |         |
|                    |         | ST untyp. (1) | t338 (1)  |       |         |
|                    |         | ST34 (8) | t166 (4)   |       |         |
|                    |         |          | t4437 (1)  |       |         |
|                    |         |          | t369 (3)   |       |         |
|                    |         |          | t3906 (1)  |       |         |
|                    |         | ST34 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |
|                    |         | ST228 (7) | t001 (7)   |       |         |
|                    |         | ST228 (6) | t041 (6)   |       |         |
|                    |         | ST30 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |
|                    |         | ST30 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |
|                    |         | ST30 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |
|                    |         | ST30 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |
|                    |         | ST30 (1) | t13129 * (1) |       |         |
|                    |         | ST30 (1) | t13132 * (1) |       |         |

*spa types with missing alignments (2) -

**CC5 (15) ST228 (7) ST228 (6) CC002 (14) Cluster 2
The underlined strains t1382 belonged to different ribogroups and exhibit different STs, with diverse allelic profiles (ST30 and ST228); †: spa type t037 belonging to CC021/012 was found in a different ribogroup and MLST CC; t693 and t1252 were excluded due to the shorter repetitions (<5): t693 (7), t1252 (07-22-13); * new spa types found in this study and submitted to the spa Ridom software; ** new STs founded in this study and to be submitted to the MLST database. In brackets are the number of isolates.
Figure 1. Clusters founded by BURP software analysis.

2.5. Ribogroups Association with agr Type, tst and mecA Genes, IS256 and Phenotypic Antibiotic Resistance

Table 3 shows a high correlation between agr types and the prevalence of mecA, IS256 and tst genes, within respective ribogroups, confirming the results of previous works. Moreover, the phenotypic antibiotic resistance traits followed the same patterns between isolates belonging to the same ribogroup.

As reported in Table 3, most of the strains of the large ribocluster exhibited an agr type III (93%), were endowed with the tst (83%) gene, resistant to the antibiotics PEN and AMP, but lacking IS256. All strains were negative to the mecA gene, thus largely susceptible to OXA. These findings confirm our previous observations [17]. Conversely, the two ribogroups cra-94-S7 and cra-146-S8, whose strains mostly belonged to CC5, exhibited very distinct genotypic and phenotypic traits except for the agr type II, which was highly prevalent (respectively in 94% and 100% of the strains), the tst gene (6% and 22%), and antibiotic resistance to three antibiotics, VAN, SXT, and CHL. Indeed, cra-94-S7 showed a high frequency of strains endowed with IS256 (94%), mecA gene (88%), and resistant to OXA, PEN, AMP, GEN, ERY, CLI and CIP. The high prevalence of its resistant/multiresistant strains was reflected by the MAR index [18,19] that reached its
highest value of 0.66. On the other hand, *cra*-146-S8 exhibited a MAR of 0.11, which was the lowest observed across ribogroups. Even the generally frequent resistance to PEN and AMP concerned less than 50% of the strains. The ribogroup *cra*-137-S4 consisted uniquely of *agr* type I strain, lacking the *tst* gene, and resistant to both PEN and AMP. IS256 was present just in half of its strains, while the *mecA* gene was detected in a much larger proportion (79%). The MAR reached a value of 0.57 reflecting a high prevalence of strains resistant to the different antibiotics assayed, in particular GEN, ERY, and CIP. Similarly, *cra*-157-S4 included prevalently *agr* type I strains, all lacking the *tst* and *mecA* genes and the insertion element IS256. Its MAR value was 0.18 and reflected a particularly low frequency of antibiotic resistant strains, apart from PEN and AMP. Finally, *cra*-147-S6 consisted of *agr* type III strains. A quarter of the strains was endowed with the *tst* and *mecA* genes, while IS256 was detected in half of its strain population. The MAR (0.24) indicated an antibiotic resistance just slightly lower than the average MAR calculated on all 108 strains (0.31).

**Table 3.** Overall prevalence of *agr* type, IS256, *tst*, and *mecA* genes, and phenotypic antibiotic resistance traits within each ribogroup.

| Ribogroup | Prev. *agr* type | *tst* | IS256 | mecA | OXA | PEN | AMP | GEN | ERY | CLI | CHL | SXT | CIP | VAN | MAR |
|-----------|------------------|------|-------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ribocluster | 98%              | 83%  | 0%    | 0%   | 2%  | 94% | 92% | 4%  | 11% | 6%  | 0%  | 0%  | 4%  | 0%  | 0.21|
| *cra*-94-S7 | II 94%           | 6%   | 94%   | 88%  | 88% | 94% | 94% | 94% | 94% | 6%  | 0%  | 94% | 0%  | 0%  | 0.66|
| *cra*-146-S8 | II 100%          | 22%  | 0%    | 22%  | 0%  | 44% | 44% | 0%  | 0%  | 0%  | 0%  | 0%  | 0%  | 0%  | 0.11|
| *cra*-137-S4 | I 100%           | 0%   | 50%   | 79%  | 86% | 100%| 100%| 57% | 57% | 62% | 21% | 7%  | 86% | 0%  | 0.57|
| *cra*-157-S4 | I 85%            | 0%   | 0%    | 0%   | 0%  | 77% | 77% | 8%  | 8%  | 8%  | 0%  | 0%  | 0%  | 0%  | 0.18|
| *cra*-147-S6 | III 100%         | 25%  | 50%   | 25%  | 25% | 75% | 75% | 13% | 25% | 25% | 0%  | 0%  | 0%  | 0%  | 0.24|

Percent frequency of strains resistant to each antibiotic, with the insertion sequence IS256, and endowed with the genes *tst* and *mecA*. The average MAR value for all the 108 strains and all 10 antibiotics was 0.315. Legend: oxacillin (OXA); penicillin (PEN); ampicillin (AMP); gentamicin (GEN); erythromycin (ERY); clindamycin (CLI); chloramphenicol (CHL); trimethoprim-sulfamethoxazole (SXT); ciprofloxacin (CIP); vancomycin (VAN).

2.6. *Uncommon STs and spa Types Atypically Found within Ribogroups*

Table 4 reports the genetic characteristics of some uncommon STs and *spa* types that have been identified within three ribogroups and appear atypical, as they markedly differ from the other more prevalent STs, and *spa* types observed. ST46, detected only once, shows a sequence type different from all the others of the ribogroup *cra*-94-S7 constituted by 16 isolates. In fact, it differed by all six alleles with respect to the most frequent ST228, and its t1646 *spa* type was very dissimilar from both t001 and t041. BURP analysis generated the singleton t1646 *spa* type, whereas the ribogroup was constituted by *spa* CC002.

Other atypical STs were identified in the *cra*-157-S4 ribogroup, i.e., ST109 and ST101, which differed from the prevalent strains ST7 by five and six alleles, respectively. Their *spa* types (t209 and t7956 respectively) were also very dissimilar from the principal t091 *spa* type of that ribogroup.

Some strains such as *cra*2198 with the genetic profile ST241/t037 (Table 2) exhibited ambiguous connotations. In terms of allele succession, the sequence type ST241 showed similarity, with the genetic founder ST8, but its *spa* type t037 clustered into *spa* CC021/012 (Cluster 1).
Table 4. Atypical STs and spa types in relation with the most frequent genetic profile observed in the same ribogroup.

| Ribogroup | STs | MLST Allele Succession (arc-aroe-glpf-gmk-pta-tpi-yqi) | Spa Typing | spa Types-spa CC | spa Repeats Succession |
|-----------|-----|------------------------------------------------------|------------|------------------|------------------------|
| cra-94-S-7 (16) | ST228 (14) | 01-04-01-04-12-24-29 | II | t001-CC002 (7) | 26-30-17-34-17-20-17-12-17-16 |
| | ST46 (1) | 10-14-08-06-14-03-02 | I | t1646-singleton (1) | 09-20-16-34-13-17-34-16-34 |
| cra-157-S-4 (13) | ST7 (9) | 05-04-01-04-04-06-03 | I | t091-singleton (9) | 07-23-21-17-34-12-23-02-12-23 |
| | ST109 (2) | 03-27-01-01-01-01-10 | II | t209-singleton (2) | 07-16-12-34 |
| | ST101 (1) | 03-01-14-15-11-19-03 | I | t7956-singleton (1) | 04-13-21-12-17-20-17-12-17-17 |
| cra-137-S-4 (14) | ST8 (9) | 03-03-01-01-04-04-03 | I | t008 (5 CC121) | 11-19-12-21-17-34-24-34-22-25 |
| | ST241 (1) | 02-03-01-01-04-04-03 | I | t037 (1 CC021/012) | 15-12-16-02-25-17-24 |

STs and spa types atypical for the ribogroup are in bold. MLST allelic profile and spa repeats successions that are unusual within the ribogroup are in italics. In brackets the number of isolates.

2.7. Hunter-Gaston Discriminatory Power Analysis

The Hunter-Gaston discriminatory power of spa typing, ribotyping, and MLST obtained on the 108 S. aureus is shown in Table 5. For all 108 S. aureus, the method with the highest discriminatory index was spa typing (D = 0.955), followed by MLST (D = 0.869), and ribotyping (D = 0.744). Ribotyping discriminatory index indicates a low or moderate ability to create different genotypes. Moreover, considering MSSA isolates, the discriminatory index was superior to MRSA considering both spa typing and MLST. In fact, for spa types, the discriminatory power was of 0.936 vs. 0.879, while for MLST ST was of 0.802 vs. 0.751, and considering the spa CC, the discriminatory power was of 0.746 vs 0.594 while for MLST CC was of 0.590 vs 0.567. Tables 6 and 7 illustrate the genotypic characteristics of MSSA and MRSA strains (spa types and CC, MLST ST and CC) inside the ribogroups. The data obtained clearly confirm the prevalence between MSSA strains of more genetic lineages respect MRSA and then, excluding ribotyping, a major discriminatory power.

Table 5. Hunter–Gaston discriminatory index of spa typing and MLST of 108 S. aureus.

|          | Spa Typing | MLST    | Ribotyping | spa CC | MLST CC |
|----------|------------|---------|------------|--------|---------|
| MSSA (79) | 0.936      | 0.802*  | 0.596      | 0.746  | 0.590** |
| MRSA (29) | 0.879      | 0.751   | 0.636      | 0.594  | 0.567  |
| TOTAL (108) | 0.955      | 0.869*  | 0.744      | 0.817  | 0.721** |

MSSA methicillin-susceptible S. aureus, MRSA methicillin-resistant S. aureus, * Excluding one non typeable ST (Total n° = 107, MSSA n° = 78), ** Excluding one strain without CC (Total n° = 107, MSSA n°=78). † Excluding two strains with no founder CC (due to the shorter repetitions i.e., <5). In brackets the number of isolates.
Table 6. Clonal Complexes and relationship between the genotypic patterns of the 79-methicillin susceptible *S. aureus* (MSSA).

| Ribogroup 1 | MLST CC 3 | MLST ST | spa Type | spa CC | IS256 |
|-------------|----------|---------|----------|-------|-------|
| Ribocluster (48/48) | CC30 (48/48) | ST30 (31), ST34 (10), ST2954 (3), ST2957 (1), ST2960 (1), ST243 (1), ST non-typeable (1) | t012 (13), t021 (10), t018 (3), t093 (1), t166 (4), t298 (2), t338 (4), t369 (3), t1382 (2), t3906(1), t4437 (1), t5891 (1), t11956 (1), t13129* (1), t13132* (1) | CC021/012 (37) | neg (44) |
| **cra-94-S7 (2/16)** | CC5 (8/25) | ST5 (6), ST228 (1), ST2626 (1), ST46 (1) | t1382 (1), t1646 (1), t002 (1), t045 (1), t179(1), t548 (2), t5349 (1), t13130 * (1) | CC002 (5) | neg (8) |
| **cra-146-S8 (7/9)** | CC45 (1/25) | **CC not delivered (1/13)** | **CC not delivered (1/13)** | **CC not delivered (1/13)** | **CC not delivered (1/13)** |
| **cra-137-S4 (3/14)** | CC8 (3/14) | ST8 (3) | t008 (1), t024 (1), t574 (1) | **CC121 (3)** | neg (3) |
| **cra-157-S4 (13/13)** | CC7 (10/13) | ST7 (9)–ST2955 (1) | t091 (10), t209 (2), t7956 (1) | **CC not delivered (1/13)** | **CC not delivered (1/13)** |
| **cra-147-S6 (6/8)** | CC1 (6/8) | ST1 (6) | t127 (3), t693 (1), t1252 (1), t2478 (1) | **CC121 (3)** | **CC121 (3)** |

Numbers between brackets represent the number of strains. The Clonal Complexes of STs and the Clonal Complexes of spa types were inferred by eBURST v3 and BURP analysis, respectively, on the entire collection of 108 *S. aureus*. The main ST type, spa type, and spa CC appear in bold. 1(number of MSSA isolates /number of isolates of the ribogroup).
3. Discussion

In the present study, 108 *S. aureus* isolates belonging to the six larger ribogroups, determined by automated RiboPrinter® System, were investigated by the two molecular methods most used for epidemiological studies, i.e., MLST and spa typing, followed by BURP and eBURST v3 analysis to cluster the *spa* types and STs (Sequence Types) which originated. The strain variety, the discriminatory power, and the typeability were used to assess the efficiency of the genotyping methods.

### 3.1. MLST vs. Automated Ribotyping

Relationships between ribogroups and STs were shown in Table 1. There was a good concordance between the genetic lineages created by MLST and automated ribotyping. MLST evidenced 19 STs, five of them being new STs (ST2954, ST2957, ST2960, ST2956, and ST2955), plus 1 non-typeable isolate. Each ST was characteristic of a specific ribogroup since it was not found in other ribogroups. Ribotyping assigned strains with specific STs as ST30 (64.6%) with to the riboclusters (cra-119-S-8, cra-138-S-2, and cra-53-S-7 ribogroups), ST228 (87.5%) to cra-94-S-7 ribogroup, ST8 (64.3%) to cra-137-S-4 ribogroup, ST7 (69.2%) to cra-157-S-4 ribogroup, ST5 (88.9%) to cra-146-S-8 ribogroup and ST1 (100%) to cra-147-S-6 ribogroup (Table 1).

MLST and ribotyping, referring to highly conserved nature of the housekeeping genes and *rrn* operon examined, could be considered good tools for long-term and large-scale evolution of a vast spectrum of microorganism, being the clock speed of their DNA alterations moderately slow.

Comparative ribotyping and MLST-based dendrograms for *H. influenzae* and *S. pneumoniae*, constructed with large sets of about 500 isolates each, proved to be highly congruent [14].

A fine relation between MLST and automated ribotyping was previously shown by McAleese *et al.* [20] in a group of CA-MRSA strains, coming from skin and intra-abdominal infections of patients in part from USA and from Europe. In that work, ST8 and ST1 were strictly linked respectively to the A2 and G2 ribotypes. In that study, the use of ribotyping was considered a fine tool to group MRSA with similar genetic background. As previously seen, and also in this study, *cra*-94-S-7, and *cra*-137-S-4 ribogroups, constituted for the mostly part by MRSA strains, had particular STs, as ST228 (87.5%) and ST8 (64.3%) (Table 1). Moreover, the other STs assigned to the same ribogroup were correlated and had similar allele succession. On the contrary, four isolates, namely *cra*1143 (ST46-t1646), *cra*2578, *cra*3029 (ST109-t209), and *cra*3164 (ST101-t7956), had

### Table 7. Clonal Complexes and relationship between the genotypic patterns of the 29 methicillin resistant *S. aureus* (MRSA).

| Ribogroup ¹ | MLST CC ¹ | MLST ST | spa Type | spa CC | IS256 |
|-------------|-----------|---------|----------|--------|-------|
| *cra*-94-S7 (14/16) | CC5 (16/25) | ST228 (13) | t001 (7), t041 (6), t109 (1), t102 (1), t985 (1) | CC002 (16) | pos (14), neg (2) |
| *cra*-146-S8 (2/9) | | ST5 (2) | | | |
| | | ST2956 (1) | | | |
| *cra*-137-S4 (11/14) | CC8 (11/14) | ST8 (6) | t008 (4), t051 (4), t024 (1), t037 (1), t121 (1) | CC121 (10) | pos (7), neg (4) |
| | ST247 (4) | | | | |
| | ST241 (1) | | | | |
| *cra*-147-S6 (2/8) | CC1 (2/8) | ST1 (2) | t127 (2) | singletons (2) | pos (2) |

Numbers between brackets represent the number of strains. The Clonal Complexes of STs and the Clonal Complexes of *spa* types were inferred by eBURST v3 and BURP analysis respectively on the entire collection of 108 *S. aureus*. The main ST type, *spa* type, and *spa* CC appear in bold. ¹(number of MSSA isolates /number of isolates of the ribogroup).
ataypical STs compared to the ones of the other isolates assigned to the same ribogroup (Tables 1 and 4).

Although automated ribotyping is considered a reliable method for identifying different bacterial species, easy to perform, with a high reproducibility, high typeability, and stability, its relatively limited discriminatory power is an obstacle to be used as a reference technique in genotyping method [11]. rRNA 16S, 23S, and 5S are encoded by the polycistronic rrn operon which is present in different copies with a range between one (Rickettsia prowazekii and Mycoplasma pneumoniae) and 15 (Clostridium paradoxaum), depending on the bacterial species [21]. Staphylococcus aureus exposes the presence of a range between four and six copies of rrn operons (http://rrndb.umms.med.umich.edu; accessed on 26 January 2022); then, considering that rRNA operons number commonly raises the discriminatory power, a moderate discriminatory power is obtained.

MLST method is considered as the current gold standard method for microbial population dynamics and global genetic lineages analysis and quickly has become the gold standard for epidemiological surveillance. MLST is useful than ribotyping to relate inter-laboratory results with a standardized taxonomy having an online database increased over the time, making possible the sharing and investigation of MLST data [11].

3.2. Spa Typing vs. Automated Ribotyping

Spa typing is recommended as a fine genotyping method for a national and international epidemiological control as well as for short time and local surveillance [22].

As shown in Table 2, spa typing of the 108 S. aureus strains gave 39 spa types and three of these were new types observed for the first time in this study, namely, t13129, t13130, and t13132. Analyzing the relationship between spa typing and ribogroups, each spa type is generally characteristic for a specific ribogroup.

A significant incongruence was found, namely t1382, belonging to spa CC021/012, was observed in three strains assigned to two different ribogroups and to two different MLST CCs. In fact, two of these strains with the genetic profile t1382-ST30 belong to the ribocluster-MLST CC30, mainly constituted by strains assigned to spa CC021/012 (37 isolates out of 48), while the other strain had the different genetic profile t1382-ST228 and was assigned to cra-94-S-7 ribogroup-MLST CC5, mainly constituted by strains assigned to spa CC002 (14 isolates out of 16).

This finding should not be considered eccentric, since “spa type” genotypes are based on highly variable X region sequences of the gene encoding for surface protein A, and therefore it is not obvious that this gene match with the same evolutionary lineages observed using the other molecular epidemiologic methods as ribotyping and MLST. Moreover, a confirmation of this was observed in the isolate carrying the spa type t037 clustered into spa CC021/012 and assigned to a ribogroup (namely cra-137-S-4), whereas all of the other strains had dissimilar spa repeat succession and were clustered into spa CC121 (Tables 2 and 4). In this case, its MLST sequence type (ST241) showed a similarity, in term of allele succession, with the genetic founder ST8, clustered into MLST CC8. This observation validates that the evolutionary histories of isolates, (taking in consideration spa typing and MLST methods) are not always linked and the mutations on the seven housekeeping alleles and that spa repeat succession cannot always be predicted and do not follow any precise scheme.

Furthermore, as previously evidenced, four strains had with unusual STs had in addition atypical spa types respect the other strains assigned to the same ribogroups (Table 4).

Considering spa typing and MLST, more genetic assortments were detected in MSSA isolates. The discriminatory power of spa typing and MLST for MSSA isolates was 0.936 and 0.802, respectively (32 spa types and 16 STs), while for MRSA isolates, the discriminatory power was of 0.879 and 0.751, respectively (11 spa types and 7 STs). This finding was similar to those observed in previous studies [23–26]. In fact, the genetic lineage population of MSSA has in general a diversity in genetic assortment greater than
MRSA lineages [22,26–30]. In some instances, there were not specific MLST STs and spa types for discriminating among MSSA and MRSA strains (Table 6A,B).

3.3. Clonal Complexes

As observed from the results shown in Table 1, automated ribotyping and eBURST v3 analysis had higher concordant results between ribotyping and BURP analysis, since ribogroups corresponded to determine groups and MLST clonal complexes, excluding the CC5 (group 2 from eBURST analysis), which was represented by two different ribogroups (cra-94-S-7 and cra-146-S8) (Tables 1 and 2). The ribocluster matched with CC30 (group 1), cra-137-S-4 ribogroup with CC8 (group 3), cra-147-S-6 ribogroup with isolates corresponded to CC1 (singleton), and cra-157-S-4 ribogroup corresponded to CC7 (group 4 and three singletons).

BURP analysis of spa types generated numerous singletons. The cra-157-S-4 and cra-147-S-6 ribogroups were constituted by only singletons. As for eBURST v3 analysis, where Group 2 (MLST CC5) corresponded to cra-94-S-7 and cra-146-S-8 ribogroups; additionally, BURP analysis created a single spa CC002 from the strains assigned to two cra-94-S-7 and cra-146-S-8 ribogroups. On the contrary, two spa CCs (spa CC021/012 and spa CC166) were merged in the same ribocluster and MLST CC30.

The outcome was that BURP and eBURST v3 analysis were more linked to each other than to with ribotyping.

Spa CC021/012 is found in three ribogroups, i.e., the ribocluster, the cra-94-S-7, and the cra-137-S-4 ones ribogroups, even if, as previously observed, there were only two isolates with spa types t1382 and t037, belonging to the cra-94-S-7 and the cra-137-S-4 ribogroups (Tables 2 and 6). This finding can be explained considering the different nature of genes analyzed with the different genotyping methods, which could group in the same cluster by one method, even if strains were clustered into the same clone by another method.

Therefore, there is a better accordance between MLST CCs and ribogroups than between spa CCs and ribogroups, and it could be explained by the intrinsic nature of spa typing which is a single-locus sequence typing (SLST) pertaining to the S. aureus protein A gene, whose repeats are moderately mutable and variable in number, while for DNA sequences, analyzed with MLST and automated ribotyping, genetic variations were slower than those for protein gene A, since polymorphism evolves gradually in rrs operon and husekeeping genes [11].

While spa typing and MLST are considered gold standard and have a high discriminatory power, the combination of the three typing methods are more instructive tools for epidemiological studies on S. aureus strains and useful to perform a clearer evaluation of the outbreaks. It has been suggested that the use of two or more genotyping methods may attain a better estimation of the genetic diversities of isolates [31].

Automated ribotyping, with respect to the other typing methods, was unable to discern difference between the groups of isolates of this study, then had the ability to group strains with different genetic pattern in congruence with MLST and spa typing.

The discriminatory power of the genotyping methods is the crucial argument to take into consideration in the context of a predominant spreading clone [31]. Despite the disadvantage that automated ribotyping may not be the method for genotyping when discriminatory power is needed, it offers the possibility to correlate clone complexes with virulence factors, i.e., adhesins, toxins, antibiotic resistances, capacity to form biofilm (Table 3), etc. Automated ribotyping could be useful to identify isolates belonging to virulent clones, with particular interest to phylogenetic studies between members of the same taxon [17,32,33].

Ribotyping could therefore be an advantage in a preliminary study where it is required clinical isolates that possess distinct pathogenic characteristics (Table 3). The study of Blasi F et al. [34] exhibits that C. difficile strains belonging to the same ribotypes have the same toxinotypes and antimicrobial resistance profiles. Additionally, it would be an additional value to improve the information obtained from spa typing and MLST in
order to associate the identified clones with probable emerging global clones with special genetic lineages and also to verify the incidence of the same clones in the laboratory databases and to compare their genetic and phenotypic properties.

4. Conclusions

The goal of the current work is to evaluate if automated ribotyping could be considered as a helping screening tool to identify and differentiate S. aureus genetic lineages in relation with other golden methods as spa typing and MLST. For the multitude of spa types generated, spa typing, unlike ribotyping and MLST, proves a strong tool in an outbreak location, since endemic clones request high levels of discrimination.

The automated ribotyping, can predict in some cases the spa clonal complexes and, in a more balanced way, MLST clonal complexes. The high cost, similar to the MLST method one, and lower discriminatory power of automated ribotyping than spa typing and MSLT, could be an obstacle for genotyping analysis especially when high discriminatory power is needed. Otherwise, different advantages as automation, easy to perform, stability, typeability, rapidity (less than 8 h), and reproducibility make ribotyping a reliable method to juxtapose to the gold standard ones [11,33]. Combination of genotyping technique should be better and preferable over single technique if high discriminatory method is required [32]. Spa typing and MLST offer a multitude of genetic lineages which would otherwise have been overlooked by automated ribotyping; both of the methods use a universal nomenclature, which makes them helpful for collaborative networks.

Finally, automated ribotyping, indicating a relatedness among isolates with different genetic background, could be better for small or local analysis and regional or national investigations, instead of studies among internationally collaborative networks and explorations of worldwide disseminated clones and outbreaks.

5. Materials and Methods

5.1. Bacterial Isolates

A total of 108 S. aureus isolates, coming from revisions of surgical wounds and treatment of infected prostheses of patients coming from the entire Italian territory, were collected over a period of 13 years (2000–2012) and stored at −80 °C. The majority (82 samples, 76%) of the isolate was from orthopedic implant-related infections. The strains were deposited in the ISO 9001:2015 certified biobank of the Research Unit on Implant Infections at the Rizzoli Orthopaedic Institute (Bologna, Italy). The strains were identified by either or both Api-Staph and ID 32 Staph test (BioMérieux, Marcy l’Etoile, France) and confirmed by ribotyping analysis.

5.2. Antibiotic Susceptibility

The agar diffusion (Kirby-Bauer) method was utilized to perform the antibiotic susceptibility tests according to Clinical and Laboratory Standards Institute (CLSI) guidelines (NCCLS, 2002) [35]. Antimicrobial susceptibility was tested for a panel of 10 antibiotics: oxacillin (OXA), penicillin (PEN), ampicillin (AMP), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), chloramphenicol (CHL), trimethoprim–sulfamethoxazole (SXT), ciprofloxacin (CIP), and vancomycin (VAN).

5.3. Automated Ribotyping

To be subjected to ribotyping method, each strain was seeded on brain hearth infusion (BHI) agar plate for 24 h at 37 °C. A single colony of each isolate culture obtained on BHI agar plates was transferred to mannitol salt agar (MSA) plate specific for staphylococci growing. A pure colony from MSA agar plates of each isolate was processed by RiboPrinter® microbial characterization system (Qualicon, Wilmington, DE, USA), following the manufacturer’s instructions. EcoRI was the restriction enzyme used for the analysis.
The identification of each isolate was obtained when its ribotype pattern matched the pattern of one of the reference isolates of the DuPont Identification Library with a similarity $\geq 0.85$. Isolates with a pattern similarity $\geq 93\%$ are clustered together in the same ribocluster. When the similarity with other previously analyzed isolates does not reach such a threshold, a new ribogroup is automatically generated. Conversely, the attribution of an isolate to a previously existing ribogroup automatically occurs when the similarity with the ribogroup average profile is $\geq 90\%$.

The RiboPrinter patterns were also imported and analyzed in BioNumerics version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Pairwise similarities were calculated using the Pearson-product-moment correlation coefficient with 0.5% optimization and from the resulting similarity matrix a UPGMA (unweighted pair group method using arithmetic averages) dendrogram was derived. The Cluster Cut-off method of BioNumerics was utilized for the identification of the major clusters [36].

5.4. Bacterial DNA Isolation

The chromosomal DNA used as an amplification template was extracted from the bacterial cultures using QIAmp DNA mini kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturer's instruction, as previously described [37].

5.5. Detection of mecA, tst, IS256, and agr Typing

PCR conditions and primers of mecA, tst and IS256 genes used in this study are previously described [38]. The identification of the agr polymorphisms was performed through PCR, as described by von Eiff et al. [39].

5.6. Spa Typing

The polymorphic X, or short sequence repeat (SSR) region, of the S. aureus protein A gene (spa) was amplified with the forward (5'-TGTAAAACGACGGCCAGT-3') and the reverse (5'-CAGGAAACAGCTATGACC-3') primers according to the protocols previously defined [40,41]. A total of 10 microliters of the amplified products were analyzed on 1.5% agarose gels and 5 µL were purified with EXO SAP-IT (GE Health care, Buckinghamshire, UK). In total, two microliters of the purified amplification products were used for subsequent sequencing using the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and were finally analyzed on ABI Genetic Analyzer 3500 Dx (Applied Biosystems). The chromatograms obtained were analyzed with the Ridom StaphType software version 1.4 (Ridom GmbH) and the Ridom SpaServer database (http://www.spaserver.ridom.de; accessed on 26 January 2022) to determine the spa type of each isolate [41]. spa types were deduced by the difference in number and sequence of spa repeats. For establishing the evolutionary relationship, spa types were clustered into different spa CCs and groups by using the BURP algorithm (Ridom GmbH) and the Ridom SpaServer database (http://www.spaserver.ridom.de; accessed on 26 January 2022).

5.7. MLST

MLST genotyping was performed on all 108 S. aureus isolates as described in [42]. The amplification of a portion of seven housekeeping genes (arc, aroE, glp, gmk, pta, tpi, and yqiL) was performed and successively sequenced. The sequences were analyzed using the bioinformatics software package UGENE 1.13 (Unipro) and the reference seven genes sequences were obtained on the MLST website (https://pubmlst.org/bigsdb?db=pubmlst_sauureus_seqdef; accessed on 26 January 2022). Each allele was acquired by submitting the examined sequence to the S. aureus MLST database (https://pubmlst.org/bigsdb?db=pubmlst_sarueus_seqdef&page=profiles&scheme_id=1; accessed on 26 January 2022), and finally the allelic profiles (sequence types, STs) and the CCs were allocated via the MLST database. With the eBURST v3 software
(https://pubmlst.org/bigsdb?db=pubmlst_saureus_islates&page=plugin&name=BURST; accessed on 26 January 2022), sequence types (STs) were clustered to assign them to groups and to identify the potential ancestral type (AT). S. aureus isolates with STs differing for just one or two housekeeping genes/loci were assigned to a unique group, then a double locus variant (DLV) was the limit set for the analysis.

5.8. Discriminatory Power

MRSA and MSSA strains discriminatory power of ribotyping, spa typing, MLST, spa CC and MLST CC, was obtained using the Hunter-Gaston discriminatory index (HGDI; http://insilico.ehu.es/mini_tools/discriminatory_power/; accessed on 26 January 2022).

\[
D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n(j - 1)
\]

where \(N\) is the total number of examined strains, \(S\) the total number of identified types and \(n\) the total number of isolates belonging to the \(j\)th type.

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