Matched-Cohort DNA Microarray Diversity Analysis of Methicillin Sensitive and Methicillin Resistant Staphylococcus aureus Isolates from Hospital Admission Patients

Ulla Ruffing¹, Ruslan Akulenko², Markus Bischoff¹, Volkhard Helms², Mathias Herrmann¹, Lutz von Müller¹*¹

¹Institute of Medical Microbiology and Hygiene, Saarland University Medical Center, Homburg/Saar, Germany, ²Center for Bioinformatics, Saarland University, Saarbrücken, Germany

Abstract

As genotyping of S. aureus is important for epidemiologic research and for hygiene management, methods are required for standardized fast and easily applicable evaluation of closely related epidemic strains with high prevalence in hospitals. In this single centre matched control study we compared a new commercially available DNA microarray (IdentiBAC) with standard spa-typing for S. aureus genotyping. Included in the study was a subgroup of 46 MRSA and matched 46 MSSA nasal isolates of the Saarland University Medical Center collected during a state-wide admission prevalence screening. Microarray (MA) and also spa-typing could easily differentiate the genetically diverse MSSA group. However, due to the predominance of CC5/003 in the MRSA group a sufficient subtyping required analysis of more complex genetic profiles as was shown here by the MA comprising a total number of 334 different hybridization probes. The genetic repertoire of the MRSA group was characterized by more virulence genes as compared to the MSSA group. The standard evaluation of MA results by the original software into CCs, agr-, SCCmec- and capsule-types was substituted in the present study by implementation of multivariate subtyping of closely related CCs isolates using three different bioinformatic methods (splits graph, cluster dendrogram, and principal component analysis). Each method used was applicable for standardized and highly discriminative subtyping with high concordance. We propose that the identified S. aureus subtypes with characteristic virulence gene profiles are presumably associated also with virulence and pathogenicity in vivo; however, this remains to be analyzed in future studies. MA was superior to spa-typing for epidemiologic and presumably also provide functional respectively virulence associated characterization of S. aureus isolates. This is of specific importance for the hospital setting. In future, MA could become a new standard test for S. aureus typing in combination with multivariate bioinformatic analysis.

Citation: Ruffing U, Akulenko R, Bischoff M, Helms V, Herrmann M, et al. (2012) Matched-Cohort DNA Microarray Diversity Analysis of Methicillin Sensitive and Methicillin Resistant Staphylococcus aureus Isolates from Hospital Admission Patients. PLoS ONE 7(12): e52487. doi:10.1371/journal.pone.0052487

Editor: Dongsheng Zhou, Beijing Institute of Microbiology and Epidemiology, China

Received August 10, 2012; Accepted November 13, 2012; Published December 20, 2012

Copyright: © 2012 Ruffing et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by the Deutsche Forschungsgemeinschaft (He 1850/9-1 to M.H.) and the Federal Ministry for Education and Science (BMBF grant #01KI1014B to MH and MB). Alere Inc. contributed to the study by supplying the IdentiBAC ArrayMateTM Reader and the imaging software Iconoclust. The isolates were collected in the context of the admission prevalence screening carried out by the MRSA Network Saarland (www.mrsaar.net) funded by the State of Saarland and the German Ministry of Health (IIA5-2509NIK008/321-4532-04/21). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have the following interests: Alere Inc. supported this study by supplying the IdentiBAC ArrayMateTM Reader and the imaging software Iconoclust. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: lutz.mueller@uks.eu

Introduction

Staphylococcus aureus is a major human pathogen associated with invasive disease such as deep abscess formation, endocarditis, osteomyelitis, and sepsis [1]. The unabated global presence of methicillin-resistant S. aureus (MRSA) is a challenge for healthcare systems worldwide. Epidemic highly abundant MRSA strains of clonal origin have been characterized based on genetic profiles for healthcare associated (haMRSA), community associated (caMRSA) [2] and also for livestock associated infections (laMRSA) [3,4]. Attempts have been made to associate S. aureus gene profiling [5–7] of clonal lineages with either ecological success [8] or clinical disease [9] yet, it remains to be determined which genetic traits render a given S. aureus clone to be clinical successful.

The focus to combat MRSA in hospitals must be on the reduction of MRSA transmission. Efficient transmission control, however, requires information on source and spread of nosocomial pathogens. Yet, this information is limited with regard to prevalent healthcare associated MRSA strains, as the typically clonal albeit regionally divergent phylogenetic traits of prevalent isolates [10] often preclude in-depth transmission pattern analyses. Moreover, the lack of routinely accessible information on the virulence gene equipment prevents any attempt for differentiated therapeutic or infection control approach as a function of pathogen equipment.
Genomic analysis of the variable X-region of the S. aureus protein A gene (spa) [11,12] by single locus sequencing (spa-typing) has become very popular owed to its ease and standardized processing with easily applicable software tools and international databank functions [13], yet, the discriminatory power of spa analysis is limited in an epidemiological setting. It can be applied as a frontline tool for S. aureus typing; however, only in combination with additional discriminatory markers as e.g. SCCmec typing, lineage-specific genes or specific gene polymorphisms [12,14]. Multiplex sequence typing (MLST) and to some extent also DNA macrorestriction appear to result in even smaller numbers of genotypes distinguishable. Multiple-locus variable-number tandem-repeat analysis (MLVA) [15–17] has provided added distinction even within similar genotypes, yet, MLVA includes multiple sequencing steps requiring expensive consumables and equipment optimized for this purpose. Complete genome analysis by next generation sequencing albeit successfully applied for outbreak analysis [10] will in the next future still remain an application for specialized laboratories. If applied to a specific cluster (e.g., the 003 type) analysis of single nucleotide polymorphism (SNP) is able to further differentiate with a high discriminatory power, yet, in general each SNP probe is unique and restricted to respective clonal complexes [19].

Clonal lineage evolution in S. aureus has also been successfully analyzed by application of a microarray (MA) concept [6]. Moreover, a comprehensive approach through MA genomic hybridization has suggested that isolates from complicated infection may be differentiated from commensals as a result of virulence gene repertoire [20].

As a promising development towards ease-of-application, cost, and turnaround time, a commercial diagnostic DNA-based MA panel (Alere IdentiBAC® StaphType Microarray [IdentiBAC MA]) has been developed for S. aureus genotyping [21]. The method is based on the comprehensive analysis of the S. aureus genome by hybridization to 334 different genetic probes [22,23], and allows for highly reproducible simultaneous analysis of 174 genes dispersed over the complete S. aureus genome [24–26]. Genes analyzed can be grouped into lineage specific S. aureus genes, resistance and virulence genes [27]. As a result, agr-, capsule- and SCCmec typing as well as a highly accurate discrimination of S. aureus lineages is implemented [28,29]. Crude IdentiBAC MA results are available in one working day and MA analysis has been already successfully applied for a broad collection of MRSA isolates [24], demonstrating 34 MRSA lineages and more than 100 different strains in human as well as veterinary isolates.

In this study, we have now employed IdentiBAC MA for a first time in a subgroup of MRSA and matched MSSA isolates collected during a large, state-wide admission prevalence screening in the State of Saarland (manuscript in preparation). Isolates of MSSA colonized patients matched for gender, age and previous hospital admissions were included as a control group of patients with similar predisposition and exposition to healthcare associated infections. MA analyses were complemented by spa-typing for independent lineage attribution, and results were subjected to advanced bioinformatic analysis. The study strived to address the following questions: i) What is the clonal lineage distribution of MSSA and MRSA isolates during a time and region-restricted hospital admission screening? ii) Can a difference in the accessory gene equipment of MRSA and MSSA hospital admission-associated isolates be observed? iii) Are there differences between bioinformatic models in respect to phylogenetic lineage delineation, and does bioinformatic analysis help to further differentiate between predominant clones indistinguishable by spa-typing and clonal complex (CC) attribution?

Materials and Methods

Patients and Clinical Isolates

Clinical isolates were collected in a 4 weeks interval during routine hospital entry screening from patients with nasal S. aureus colonization admitted to the Saarland University Medical Center. 46 MRSA isolates and 46 matched isolates of the MSSA colonized control group were included. Matched controls were selected according to gender, age (<70 vs. ≥70 years), previous hospitalizations in general and in the last 6 months (Table 1). Criteria were selected to match patients with a similar risk exposure for community and healthcare associated S. aureus contacts. The study was approved by the ethic commission of Saarland (registration # 127/10).

Sspa-typing

DNA of clinical isolates was prepared by boiling (95°C for 10 minutes) followed by amplification of the polymorphic X region of the protein A gene (spa) using standard primers spa-1113f (5’ TAA AGA CGA TCC TTC GGT GAG C 3’) and spa-1514r (5’ CAG CAG TAG TGC GTG TTG CTT 3’). Before sequencing (Tseq, Kaiserslautern, Germany) the PCR product was digested by ExoSAP IT® (Affymetrix, Cleveland, United States) at 37°C (15 minutes), and the reaction was terminated at 80°C (15 minutes). Sequences were assigned into spa types using the Ridom StaphType software version 2.1.1 and BURP algorithm (Ridom GmbH, Munster, Germany), as described previously [30].

DNA Microarray-based Genotyping

DNA extraction and hybridization to the IdentiBAC MA (Alere Technologies GmbH, Jena, Germany) was performed as described in the manufacturer’s instructions [21,27]. In brief, genomic DNA was purified using the cell lysis components of the assay in

| Risk factors | MRSA, n (%) | MSSA, n (%) | p-value |
|--------------|------------|------------|---------|
| Male         | 18 (39.13%) | 18 (39.13%) | #       |
| Female       | 28 (60.87%) | 28 (60.87%) | #       |
| <70 years    | 24 (52.17%) | 24 (52.17%) | #       |
| ≥70 years    | 22 (47.83%) | 22 (47.83%) | #       |
| Hospitalisations <6 months | 21 (45.65%) | 21 (45.65%) | #       |
| Inter-hospital transfer | 5 (10.64%) | 1 (2.17%) | ns       |
| Previous MRSA colonization | 3 (6.52%) | 1 (2.17%) | ns       |
| MRSA contacts | 8 (17.39%) | 4 (8.70%) | ns       |
| Long-term care | 11 (23.91%) | 2 (4.26%) | 0.014    |
| Retirement home | 3 (6.52%) | 0 (0.00%) | ns       |
| Diabetes mellitus | 9 (19.57%) | 8 (17.39%) | ns       |
| Antibiotic therapy | 21 (45.65%) | 8 (17.39%) | 0.007    |
| Dialysis      | 3 (6.52%)  | 0 (0.00%)  | ns       |
| Medical devices | 8 (17.39%) | 0 (0.00%) | 0.006    |
| Skin lesions  | 6 (13.04%) | 2 (4.26%)  | ns       |

Statistical analysis was not performed for clinical criteria applied for selection of matched MSSA cases, ns = not significant.

doi:10.1371/journal.pone.0052487.t001
combination with DNeasy blood and Tissue kit (Qiagen, Hilden, Germany). The test principal is based on a linear multiplex primer elongation using one primer for every single target and DNA labeling by incorporation of biotin-16-dUTP. Following DNA hybridization, microarray probes were washed, then horseradish-peroxidase-streptavidin precipitation reaction was performed resulting in visible grey spots in case of a positive reaction. Spot signals were recorded, and automatically analyzed using the designated ArrayMate reader and the corresponding software (Iconoclave, Alere Technologies) [21]. As result, the MA readings of 334 target sequences corresponding to 174 distinct genes were classified into species markers, genes encoding virulence factors, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), antimicrobial resistance genes or SCCmec, capsule- and agr- typing markers. As part of the IdentiBAC MA results in conjunction with the Iconoclave analysis, array profiles are attributed to a specific clonal complex (CC) and sequence type (ST) based on a proprietary algorithm provided by the manufacturer. Similarly, SCCmec types are attributed as a result of array signals obtained.

**Clonal Complex Affiliation**
Upon application of the original MA evaluation software (Iconoclave, Alere Technologies), isolates could be assigned to MLST clonal complexes (CCs) based on the hybridization profiles, except for two untypeable MSSA isolates (S19, S27) (Figure 1B). The MRSA isolates clustered into only five different CCs, while MA analysis of MSSA revealed twelve different CCs. MRSA isolates were dominated by CC5 (41, 89.1%) whereas the predominant MSSA types were found to be CC45 (12, 28.6%) and CC30 (10, 23.8%). Isolates of CC5, CC8, CC22, CC45 and CC398 were found both in the MRSA and the MSSA group, whereas CC30, CC15, CC7, CC1, CC78 and CC101 were present only in the MSSA group. CCs attributed to the MRSA group only were not found.

**Analysis of Gene Equipment**
Microarray results of MRSA and MSSA isolates were analyzed for individual genes associated with e.g. antibiotic susceptibility, toxin production, adhesion and immune evasion. An overview of the most relevant genes in the investigated isolate cohort was provided for MRSA as compared to MSSA (Figure S1). Genes respectively gene components which were not detected in any cohort isolate were not displayed (ermB, mefA, mph(C), vat(A), vat(B), vga, aphA3, sat, dfrAS, spa, cfr, conA/B/C, qacA/B/C, sbh, shv, mtr, mvtr, sul, qnr, qnrS, rac, cmlA/B/C, cmlM, cmlE, cmlF, kaiA, kaiB, iutA, iucA, ent, ade, cmeA, cmeB, qepA, qepB, tetA, tetB). For more detailed analysis of selected gene profiles of individual isolates we refer to the supporting information (Table S1).

**Agr-typing**
All CC5 isolates (n = 41, 89.13%) affiliated with agrII (accessory gene regulator type II). The remaining 5 MRSA isolates of CC8, CC22, CC45, CC398 (10.9%) as well as MSSA of CC7, CC22, CC45, CC97, CC101, CC398 (n = 26, 52.2%) were associated
with agrI, 12 MSSA isolates of CC1, CC30, CC78 with agrIII (26.1%), and 7 isolates of CC5 and CC15 with agrII (15.2%). The agr type of three MSSA isolates could not be determined using MA.

SCCmec Typing

SCCmec types were identified based on hybridization patterns. Corresponding to the predominant clonal complex of the MRSA isolates all except four isolates of CC5 (37 of 41, 90.2%) comprised a SCCmec-cassette of type II. Isolates of the CC3 (n = 2), CC22 (n = 1), CC45 (n = 1) and one isolate of CC5 harbored the SCCmec type IV while the CC390 isolate was characterized by SCCmec type V. The SCCmec types of three isolates could not be determined by MA.

Resistance Genes

MRSA isolates were defined and characterized by the detection of mecA in the SCCmec cassette. 39 MRSA isolates (84.8%) and also 29 (63.0%) MSSA isolates were positive for the β-lactamase operon (blaZ, blaA, blaB, blaE), 43 (93.3%) MRSA yet only 20 (43.5%) MSSA isolates carried fosB, a putative marker for fosfomycin and bleomycin resistance (p < 0.001); the detection of the fosB gene was limited to CC5, CC8, CC15, CC30, CC101. The macrolide, lincosamide and streptogramin (MLSB) resistance gene ermB was detected with significantly higher rates in the MRSA (41, 89.1%) as compared to the MSSA group (3, 6.5%) (p < 0.001). Other MLSB resistance genes enmC was detected with significantly higher rates in the MRSA group (41, 89.1%) as compared to the MSSA group (3, 6.5%) (p < 0.001). Only one (2.2%) MSSA isolate was positive for enmC. The aminoglycoside resistance gene aadA was detected more frequently in MRSA (27, 58.7%) than in MSSA isolates (1, 2.2%) (p < 0.001). Most isolates (84/92 [91.3%]) carried the unspecific efflux pump gene (sdrM, formally spoIIA) which was equally distributed among MSSA and MRSA isolates. The tetracycline resistance gene tet(K) was detected in only one MRSA (2.2%) and two MSSA (4.3%) isolates, respectively.

Virulence Genes

Panton-Valentine leukocidin (pvl) genes (pvlF/S-PV) were not detected in the total study cohort. Only 9/46 (19.6%) MSSA isolates were tst1 (toxic shock syndrome toxin) positive, most of them clustering into CC30 (8, 17.4%). The genetically linked leukocidin components (lukD and lukE as well as lukS, lukF and hlgD) were found more frequently in MRSA than in MSSA (p < 0.001).

Among the haemolysin gene family, high abundance was detected among MRSA and MSSA for hla, hlb, hld and hlgIII, whereas differences between groups were detected for hlb (p < 0.001).

The immune evasion gene cluster of sak (staphylokinase), sdp (chemotaxis-inhibiting protein), or serrr (staphylococcal complement inhibitor) was abundantly found both in the MRSA and the MSSA group.

Hybridization signals for exfoliative toxin eta, etb, etD and epidermal cell differentiation inhibitor ednA, ednB, ednC were detected only in a minority of strains.

The enterotoxin gene cluster (egcg comprising seg, sei, sem, set, see, seu) was frequently identified both in MRSA (43/46, 93.5%) and MSSA (29/46, 63%) (p < 0.001), yet, the gene cluster was restricted to isolates of CC5, CC22, CC30, CC45. Enterotoxin genes sea, sed, sej and sees were significantly more frequent in the MRSA group while all isolates were negative for seh, sef, sek and ses [35]. Interestingly, the 16 isolates of CC7, CC15, CC78, CC97, CC101 and CC398 (one MRSA and 15 MSSA) did not contain any hybridization signal for enterotoxin genes.

The serineprotease genes, splA and splB, were predominantly found in the MRSA group (p < 0.001), and this gene cluster was restricted to clonal complexes CC1, CC5, CC7, CC8, CC15 and CC97. The aureolysin gene (aur) was detected in 43 MRSA (93.5%) and 30 MSSA isolates (65.2%) (p < 0.001). Other protease genes such as sypD (glutamyldipeptidase), sypB and sypP (staphopain B and A) were detected in the entirety of isolates tested. The ACME gene cluster, which had been brought to attention during analysis of caMRSA outbreak strains, was found in our population in the ST5-MRSA-II group (3, 6.5%).

Microbial surface components recognizing adhesive matrix molecule genes (MSCRAMM) comprising cna (collagen-binding adhesin), sasG (S. aureus surface protein G), seb (van Willebrand factor binding protein) and sfb (fibrinogen binding protein) are abundantly expressed, however, with higher proportions of cna positive isolates in the MSSA group, and higher rates of sfb, sasG and seb in the MRSA group. Other MSCRAMM genes such as bhp (bone sialoprotein-binding protein), clfA (clumping

Figure 1. Diversity analysis of all MSSA (S1–S46) and MRSA (R1–R46) isolates by splits graph. (A) Splits graph constructed based on cost distance matrix produced by Ridom StaphType and (B) on default settings of the IdentiBAC microarray hybridization profiles of 334 genes and alleles. Clonal complexes (CC) as well as the most abundant spa-types t003 (circles) and t012 (quadrates) were highlighted. doi:10.1371/journal.pone.0052487.g001
Table 2. Differences of spa-types and clonal complexes in MSSA and MRSA isolates.

| Clonal complex | Spa-type | MSSA, n (%) | MRSA, n (%) |
|----------------|----------|-------------|-------------|
| CC1            | t8864    | 1 (2.17%)   | 0 (0%)      |
| CC5            | t003     | 0 (0%)      | 29 (63.04%) |
|                | t504     | 0 (0%)      | 4 (8.70%)   |
|                | t010     | 0 (0%)      | 2 (4.35%)   |
|                | t002     | 1 (2.17%)   | 1 (2.17%)   |
|                | t045     | 0 (0%)      | 1 (2.17%)   |
|                | t481     | 0 (0%)      | 1 (2.17%)   |
|                | t493     | 1 (2.17%)   | 0 (0%)      |
|                | t887     | 0 (0%)      | 1 (2.17%)   |
|                | t1079    | 0 (0%)      | 1 (2.17%)   |
|                | t3195    | 0 (0%)      | 1 (2.17%)   |
| CC7            | t091     | 2 (4.35%)   | 0 (0%)      |
| CC8            | t008     | 1 (2.17%)   | 2 (4.35%)   |
| CC15           | t084     | 2 (4.35%)   | 0 (0%)      |
|                | t018     | 1 (2.17%)   | 0 (0%)      |
|                | t306     | 1 (2.17%)   | 0 (0%)      |
|                | t8786    | 1 (2.17%)   | 0 (0%)      |
| CC22           | t005     | 1 (2.17%)   | 0 (0%)      |
|                | t022     | 0 (0%)      | 1 (2.17%)   |
|                | t310     | 1 (2.17%)   | 0 (0%)      |
|                | t625     | 1 (2.17%)   | 0 (0%)      |
| CC30           | t012     | 6 (13.04%)  | 0 (0%)      |
|                | t019     | 1 (2.17%)   | 0 (0%)      |
|                | t273     | 1 (2.17%)   | 0 (0%)      |
|                | t584     | 1 (2.17%)   | 0 (0%)      |
|                | t8831    | 1 (2.17%)   | 0 (0%)      |
| CC45           | t015     | 5 (10.90%)  | 1 (2.17%)   |
|                | t026     | 1 (2.17%)   | 0 (0%)      |
|                | t040     | 1 (2.17%)   | 0 (0%)      |
|                | t050     | 1 (2.17%)   | 0 (0%)      |
|                | t073     | 1 (2.17%)   | 0 (0%)      |
|                | t339     | 1 (2.17%)   | 0 (0%)      |
|                | t620     | 1 (2.17%)   | 0 (0%)      |
|                | t1689    | 1 (2.17%)   | 0 (0%)      |
|                | t2239    | 1 (2.17%)   | 0 (0%)      |
| CC78           | t8863    | 1 (2.17%)   | 0 (0%)      |
| CC97           | t267     | 3 (6.62%)   | 0 (0%)      |
|                | t131     | 1 (2.17%)   | 0 (0%)      |
|                | t8831    | 1 (2.17%)   | 0 (0%)      |
| CC101          | t0444    | 1 (2.17%)   | 0 (0%)      |
| CC398          | t011     | 0 (0%)      | 1 (2.17%)   |
|                | t571     | 1 (2.17%)   | 0 (0%)      |
| unknown        | t078     | 1 (2.17%)   | 0 (0%)      |

doi:10.1371/journal.pone.0052487.t002

factor gene A), clfB (clumping factor gene B), ebpS (cell surface elastin binding protein), fnbA (fibronectin-binding protein A) and sdrC (serine aspartate repeat fibrinogen binding protein) were found in the majority of strains without clear association to the methicillin resistance profiles.

As expected, the most obvious genetic differences in the highly abundant CC5 MRSA group (bla operon, aadD, sea, sed, sej, ser, hlb and ild) were associated with altered mobile genetic elements.

More detailed characteristics of individual isolate in respect to spa-type, repeat succession, CC, SCCmec-type, age-type, toxin profile, resistance profile, strain assignment and relation analyzed by hierarchical cluster dendrogram was shown in the supporting information (Figure S1).

Microarray and spa-type Based Subclassification of CC5 Isolates

Most MRSA isolates were attributed to a genetic group of healthcare associated strains clustering into the CC5 (41, 89.1%). Except for two isolates of unidentified strain assignment, all isolates of CC5 referred to ST5-MRSA-II. This phylogenetically related and epidemiologically important CC5 was then selected for more detailed subtyping using MA hybridization as compared to classical spa-typing.

A more detailed subtyping of spa-sequence data beyond the spa-type level was not possible as was demonstrated by splits graph distance matrix analysis (Figure 2A). Using the standard IdentibiAC MA software, subtyping of the MA results was not straight-forward. Instead, three alternative bioinformatics methods were found to be very helpful in subdividing genetically related strains by analysis of comprehensive genetic signatures determined by the MA. Results obtained by splits graph analysis (Figure 2B), cluster analysis using dendrograms (Figure 3), and principal component analysis (PCA) based on MA hybridization signals were evaluated (Figure 4). Splits graph of the MA results allowed subclassification of the 41 CC5 isolates into 5 different clusters (A-E), including subclassification of spa-type t003 and of both t010 isolates. Interestingly the t504 isolates with regional cumulation clustered exclusively into the subgroup B. Clusters A (ldh negative), C (ACME locus positive) and D (β-lactamase negative) were characterized by indicated specific genetic groups, whereas the genetic repertoire of cluster B and E was more heterogeneous. Cluster dendrogram of CC5 isolates revealed similar subclustering as compared to splits graph analysis except for few isolates (R1, R2, R11, R15, R16, R17). All CC5 cohort isolates were agrII and the majority of CC5 isolates with MRSA resistance profile were SCCmec type II positive strains of the Rhine-Hesse clone (95%).

Using PCA, 39 CC5 strains (90.9%) could be discriminated in two major clusters; additionally, four singleton isolates without clustering were found (9.1%) (Figure 4A). For more detailed information, the predominant cluster I (30 isolates) could be subdivided by focused PCA into four different subclusters (Ia-Id) (Figure 4B) resembling similar subtypes as compared to splits graph and cluster analysis (Figure 3).

Discussion

In the present single centre study, the novel IdentibiAC MA platform was applied to the genotypic characterization of matched nasal methicillin sensitive and resistant S. aureus isolates collected upon patient admission to a tertiary care university hospital. We could demonstrate that within the colonizing MSSA population tested, a large diversity of CCs was found in contrast to MRSA isolates with limited numbers of CCs and over-representation of CC5/1003. Low lineage diversity in the MRSA in contrast to the MSSA group was found very similarly also in clinical setting e.g. in cystic fibrosis patients [36]. Despite limited number of isolates the IdentibiAC MA revealed significant
differences in the genetic repertoire of MRSA vs. MSSA isolates. Genetic differences were found to be distributed among various types of gene families including antimicrobial resistance genes, \textit{agr} types and capsule type. In the present study the MRSA population was characterized by a significantly higher abundance of virulence genes attributed to the leukocidin, enterotoxin, haemolysin, protease and adhesion gene families, whereas only few single virulence genes (\textit{tst}, \textit{entL} and \textit{cna}) were found more frequently in the MSSA group. Certainly, the genetic profile of the MRSA group was dominated by the genetic repertoire of one single epidemic MRSA clone (Rhine-Hesse); however, it may be also hypothesized that the Rhine-Hesse virulence gene repertoire was relevant for epidemic spreading of this successful epidemic MRSA clone. Of note, all isolates tested in this study were of commensal nature precluding association of virulence gene equipment with disease, yet, MA may become a regular diagnostic tool if specific clinical features could be associated with virulence gene patterns in subsequent studies.

In this study, it was demonstrated for the first time that evaluation of the raw IdentiBAC MA hybridization data by three independent bioinformatic methods allowed for in-depth phylogenetic MRSA isolate typing even beyond the prevalent CC5/t003 MRSA genotype. Poor diversity of MRSA with predominance of CC5 isolates could be assumed as a limitation of this study; however, discrimination of these closely related strains is the most important challenge for analysis of healthcare-associated MRSA isolate cohorts obtained from geographically confined studies. In fact, it is the challenge for MA as a new alternative to established typing systems to overcome these limitations.

\textit{Spa}-types and MA results were clustered into the same CCs; however, subclustering of the \textit{spa}-types into STs [37] and also MA associated subtyps was not compelling. While genetic signatures of MA allow direct assignment to CCs and STs an assignment to \textit{spa}-types cannot be achieved due to the heterogeneous genetic repertoire in the same \textit{spa}-type. Single run IdentiBAC MA analysis in conjunction with appropriate software tools may now answer

\textbf{Figure 2. Subclassification analysis of 41 MRSA (R1–R41) and two MSSA (S42, S43) of CC5.} (A) Splits graph based on cost distance matrix computed by Ridom StaphType software. (B) Splits graph based on MA hybridization profiles. Characteristic gene profiles for isolate cluster assignment were arbitrarily stated into group A-E. The most common MRSA \textit{spa}-types t003 (circles), t504 (quadrates) and t010 (hexagons) were highlighted.

doi:10.1371/journal.pone.0052487.g002
detailed questions both of epidemiologic as well as of infection control character.

Splits graph analysis by neighbor joining clustering, cluster dendrogram using hierarchical agglomerative clustering and also principal component analysis (PCA) formed very similar subgroups of the closely related CC5 isolates. In general, for more detailed strain assignment it has to be amended that a clearcut nomenclature discriminating strains and clones is still missing. In

Figure 3. CC5 isolates (n=43) characterized by spa-typing and comprehensive MA subgroup analysis using three different bioinformatic modes (principal component analyses, splits graph and cluster dendrogram).
doi:10.1371/journal.pone.0052487.g003
the present study, the CC5 subgroups characterized by a different lineage specific accessory gene repertoire were arbitrarily named group A-E. These predominant subgroups differed for specific gene families encoding β-lactamase resistance (blaZ/blaI/blaR) [38], the arginine catabolic mobile element (ACME) [39–41], the K+‐transporting ATPase A-C chain, or the sensor histidine kinase, i.e. the kdp operon [42,43]. ACME positive ST3-MRSA-II isolates have been identified before also in Hong Kong and USA (California) [24] which could be the base for new clone/substrain assignment by MA analysis. MRSA strains of the same CC can be attributed to characterized epidemic strains based on the presence/absence of characteristic genes. Thereby, the highly abundant toxic shock gene (tst) negative ST5-MRSA-II isolates were identified as Rhine-Hesse clone [44] whereas the CC8-MRSA-IV isolates were attributed to the Lyon clone [45,46] due to their carriage of enterotoxin A (sea) with or without sed/sej/ser. The tst positive New-York Japan clone [47,48] of ST5-MRSA-II was not detected in our population. By implementation of MA into routine diagnostics more detailed subtyping with elaborate techniques as e.g. whole genome sequencing [19] can be restricted to few closely related isolates with identical MA profiles clustering in the same genetic subgroup. Differences in characteristic gene families could result in altered metabolism and biologic activity. However, there is still limited evidence that genetically different subgroups may act differently according to S. aureus virulence in vivo [6,49–51]. Additionally, also single nucleotide mutations beyond the resolution of the MA may influence the biologic behaviour of S. aureus strains which remains undetectable by MA [52]. Correlation between genotypic variants and clinical phenotype remains to be confirmed in future clinical studies.

While splits graph and cluster dendrogram evaluation are abundantly used for phylogenetic analysis [53,54], PCA is a dimension reduction model becoming popular in recent years for genome-wide association studies [33,34,55,56]. Thereby most of the original variability in the data can be retained without organizing them in a hierarchical format.

Comparing the three independent bioinformatic methods, a very similar sub-clustering of closely related CC5 isolates was demonstrated although each model may have its specific strengths for clinical application [55,56]. The optimal choice between the three methods may indeed depend on the number of samples to be visualized and on the degree of diversity. For example, PCA enables a direct simple overview of an almost unlimited amount of isolates as shown here in the 2-dimensional graph. However, simple assignment of each point in the graph to the corresponding isolate is difficult in the case of densely overlapping samples. On the other hand, cluster dendrogram analysis reveals a more detailed isolate relationship with direct assignment of each isolate to the corresponding subgroup. Yet, this representation is most useful for sample sizes of less than a hundred. In the present case, splits graph analysis appeared to be most appropriate for diversity analysis during routine diagnostics due to ease-of-applicability, open-source software tools and direct assignment of each isolate to the branched subgroups in the 2-dimensional graph [31]. For future application of MA as an internationally accepted diagnostic tool it is important that a common standardized database-associated software tool is implemented independent of universally applicable bioinformatic tools investigated in the present study.

In conclusion, the present matched control study demonstrated a high genetic diversity for MSSA, either directly by spa-typing or by MA. However, differentiation of the predominant epidemic CC5 MRSA isolates was limited for spa-typing whereas detailed subtyping was achieved by bioinformatic-assisted MA analysis. The IdentiBAC MA could fulfil a number of criteria required for a new standard test for S. aureus typing including standardisation, ease of performance, low turn-around time (<24 hours), appropriate costs and superiority to established typing methods as was shown here for spa-typing. Based on the IdentiBAC MA concept, and as goal for the future development, standardized and easily applicable software tools based on the bioinformatic approaches with set highly differentiated strain assignment would then allow for comprehensive strain differentiation and global data exchange.

**Supporting Information**

**Figure S1** Detailed characteristics of individual isolates (n = 92) in the cohort. Spa-type, repeat succession, CC, SCCmec-type, agr-type, toxin and resistance profile, strain assignment and hierarchical clustering was of 46 MSSA (R1–R46) and 46 MSSA (S1–S46) was shown. Additionally also both major isolate groups were displayed (CC5 vs. others).

**Table S1** Genetic repertoire of MRSA and MSSA isolates.

[DOCX]
Acknowledgments

The help of C. Petit, S. Freis and S. Loibl is appreciated. Special thanks to S. Monecke for critical advice (Technical University of Dresden and Alere Technologies, Jena).

Author Contributions

Conceived and designed the experiments: UR RA MB VH MH LvM. Performed the experiments: UR LV MH. Analyzed the data: UR RA MB VH MH LvM. Contributed reagents/materials/analysis tools: UR RA MB VH MH LvM. Wrote the paper: UR VH MH LvM.

References

1. Lowy FD. (1998) Staphylococcus aureus infections. The New England Journal of Medicine 339: 520–532.
2. Gorwitz RJ. (2008) A review of community-associated methicillin-resistant staphylococcus aureus skin and soft tissue infections. The Pediatric Infectious Disease Journal 27: 1–7.
3. Kock R, Becker K, Gisakon B, van Gemert-Pijnen JE, Harbarth S, et al. (2010) Methicillin-resistant staphylococcus aureus (MRSA): Burden of disease and control challenges in Europe: Euro Surveillance : Bulletin European Sur Les Maladies Transmissibles = European Communicable Disease Bulletin 15: 19688.
4. Kock R, Sian K, Al-Malat S, Christmann J, Schaumburg F, et al. (2011) Characteristics of hospital patients colonized with livestock-associated methicillin-resistant staphylococcus aureus (MRSA) CC398 versus other MRSA clones. The Journal of Hospital Infection 79: 292–296.
5. Jarrard S, Mougell C, Thiolouze J, Lina G, Meugnier H, et al. (2002) Relationships between staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. Infection and Immunity 70: 631–641.
6. Lindsay JA, Moore CE, Day NP, Peacock SJ, Witney AA, et al. (2006) Microarrays reveal that each of the ten dominant lineages of staphylococcus aureus has a unique combination of surface-associated and regulatory genes. Journal of Bacteriology 188: 669–676.
7. Moore PC, Lindsay JA. (2001) Genetic variation among hospital isolates of methicillin-sensitive staphylococcus aureus: Evidence for horizontal transfer of virulence genes. Journal of Clinical Microbiology 39: 2760–2767.
8. Sakwinska O, Kuhn G, Balmelli C, Francioli P, Giddey M, et al. (2009) Genetic diversity and ecological success of staphylococcus aureus strains colonizing humans. Applied and Environmental Microbiology 75: 173–183.
9. Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, et al. (2010) Examination of the MRSA population using transmission and intercontinental spread. Science (New York, N.Y.) 327: 469–474.
10. Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D, et al. (2010) Geographic distribution of staphylococcus aureus causing invasive infections in europe: A molecular-epidemiological analysis. PLoS Medicine 7; e1000921.
11. Koren L, Ramaswamy SV, Gravis EA, Naithi S, Musser JM, et al. (2004) Spa typing method for discriminating among staphylococcus aureus isolates: Implications for use of a single marker to detect genetic micro- and macroevolution. Journal of Clinical Microbiology 42: 792–799.
12. Strommenger B, Braulke C, Heuck D, Schmidt C, Pasemann B, et al. (2008) Spa typing of staphylococcus aureus as a frontline tool in epidemiological typing. Journal of Clinical Microbiology 46: 574–581.
13. Friedrich AW, Wite W, de Lencastre H, Heynowska W, Scheres J, et al. (2008) A european laboratory network for sequence-based typing of methicillin-resistant staphylococcus aureus (MRSA) as a communication platform between human and veterinary medicine: an update on SeqNet. Euro Surveillance : Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin 13: 10802.
14. Deurenberg RH, Rijnders MJ, Sebastian S, Welling MA, Beisser PS, et al. (2009) The staphylococcus aureus lineage-specific markers collagen adhesin and toxic shock syndrome toxin 1 distinguish multilocus sequence typing clonal complexes within the clonal complexes. Diagnostic Microbiology and Infectious Disease 65: 116–122.
15. Francois P, Huynhe A, Charbonnier Y, Bento M, Herzig S, et al. (2005) Use of an automated multiple-locus, variable-number tandem repeat-based method for rapid and high-throughput genotyping of staphylococcus aureus isolates. Journal of Clinical Microbiology 43: 3346–3355.
16. Holmes A, Edwards GF, Girvan EK, Hannant W, Daniel J, et al. (2010) Comparison of two multilocus variable-number tandem-repeat methods and pulsed-field gel electrophoresis for differentiating highly clonal methicillin-resistant staphylococcus aureus isolates. Journal of Clinical Microbiology 48: 3600–3607.
17. Schools LM, Spalding EC, van Luit M, Huisjendw XD, Phister GN, et al. (2009) Multiple-locus variable number tandem repeat analysis of staphylococcus aureus: Comparison with pulsed-field gel electrophoresis and spa-typing. PLoS One 4: e6302.
18. Koser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, et al. (2012) Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. The New England Journal of Medicine 366: 2267–2275.
19. Nubel U, Nitsche A, Layer F, Strommenger B, Witte W. (2012) Single-nucleotide polymorphism genotyping identifies a locally endemic clone of methicillin-resistant staphylococcus aureus. PLoS One 7; e52698.
42. Xue T, You Y, Hong D, Sun H, Sun B. (2011) The staphylococcus aureus KdpDE two-component system couples extracellular K+ sensing and agr signaling to infection programming. Infection and Immunity 79: 2154–2167.
43. Zhang M, Wang H, Tracey KJ. (2006) Regulation of macrophage activation and inflammation by spermine: A new chapter in an old story. Critical Care Medicine 28: N60–6.
44. Monecke S, Ehrlich R, Slickers P, Wiese N, Jonas D. (2009) Intra-strain variability of methicillin-resistant staphylococcus aureus strains ST22-MRSA-I and ST5-MRSA-II. European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology 28: 1383–1390.
45. Haenni M, Saras E, Chatre P, Medaille C, Bes M, et al. (2012) A USA300 variant and other human-related methicillin-resistant staphylococcus aureus strains infecting cats and dogs in france. The Journal of Antimicrobial Chemotherapy 67: 326–329.
46. Lamy B, Laurent F, Gallon O, Doucer-Populaire F, Etienne J, et al. (2012) Antibacterial resistance, genes encoding toxins and genetic background among staphylococcus aureus isolated from community-acquired skin and soft tissue infections in france: A national prospective survey. European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology 31: 1279–1284.
47. Horvath A, Dobay O, Kardon S, Ghidan A, Toth A, et al. (2012) Varying fitness cost associated with resistance to fluoroquinolones governs clonal dynamic of methicillin-resistant staphylococcus aureus. European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology 31: 2029–2036.
48. Kawaguchiya M, Urushihara N, Kuwahara O, Ito M, Mise K, et al. (2011) Molecular characteristics of community-acquired methicillin-resistant staphylococcus aureus in hokkaido, northern main island of japan: Identification of sequence types 6 and 59 panton-valentine leucocidin-positive community-acquired methicillin-resistant staphylococcus aureus. Microbial Drug Resistance (Larchmont, N.Y.) 17: 241–250.
49. Löffler B, Hussain M, Grundmeier M, Bruck M, Holzinger D, et al. (2010) Staphylococcus aureus panton-valentine leucocidin is a very potent cytotoxic factor for human neutrophils. PLoS Pathogens 6: e1000715.
50. Nienaber JJ, Sharma Kaünstel BK, Clarke-Pearson M, Lammertithon S, Park L, et al. (2011) Methicillin-susceptible staphylococcus aureus endocarditis isolates are associated with clonal complex 30 genotype and a distinct repertoire of enterotoxins and adhesins. The Journal of Infectious Diseases 204: 704–713.
51. Peacock S, Moore CE, Justice A, Kantzanou M, Story L, et al. (2002) Virulent combinations of adhesin and toxin genes in natural populations of staphylococcus aureus. Infection and Immunity 70: 4987–4996.
52. Young BC, Gehlchik T, Batty EM, Fung R, Larner-Svensson H, et al. (2012) Evolutionary dynamics of staphylococcus aureus during progression from carriage to disease. Proceedings of the National Academy of Sciences of the United States of America 109: 4550–4555.
53. Reiko RG. (2011) Telling the whole story in a 10,000-genome world. Biology Direct 6: 34.
54. Degnan JH, Rosenberg NA. (2006) Discordance of species trees with their most likely gene trees. PLoS Genetics 2: e68.
55. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis correctly for stratification in genome-wide association studies. Nature Genetics 38: 904–909.
56. Novembre J, Stephens M. (2008) Interpreting principal component analyses of spatial population genetic variation. Nature Genetics 40: 646–649.