Molecular Epidemiology of Methicillin-Resistant Staphylococcus hominis (MRSHo): Low Clonality and Reservoirs of SCCmec Structural Elements

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Abstract

Background: Methicillin resistant Staphylococcus hominis (MRSHo) are important human pathogens in immunocompromised patients. However, little is known regarding its population structure and staphylococcal chromosomal cassette mec (SCCmec) content.

Methodology/Principal Findings: To assess the population structure and the SCCmec content of S. hominis, 34 MRSHo and 11 methicillin-susceptible S. hominis (MSSHo) from neutropenic patients collected over a 3-year period were studied. The genetic backgrounds of S. hominis isolates were analyzed by pulsed-field gel electrophoresis (PFGE) and SCCmec types were determined by PCR. Cassette chromosome recombinases (ccr) were characterized by PCR and ccrB sequencing. The 34 S. hominis isolates were classified into as many as 28 types and 32 subtypes (SID = 99.82%); clonal dissemination was occasionally observed. The main SCCmec structures identified were SCCmec type VI (4B) (20%), SCCmec VIII (4A) (15%), and a new SCCmec composed of mec complex A in association with ccrAB1 (38%); 27% of the isolates harbored non-typeable SCCmec. Overall, a high prevalence of mec complex A (73.5%), ccrAB1 (50%) and ccrAB4 (44%) were found. Importantly, ccrB1 and ccrB4 from both MRSHo and MSSHo showed a high nucleotide sequence homology with those found in S. aureus SCCmec I, VI and VIII respectively (>95%).

Conclusions/Significance: The S. hominis population showed a limited clonality and a low genetic diversity in the allotypes of ccr and classes of mec complex. Moreover, our data suggest that S. hominis might have been a privileged source of mec complex A, ccrB1 and ccrB4, for the assembly of primordial SCCmec types.

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Introduction

Coagulase-negative staphylococci (CoNS) are the most frequently isolated bacteria from blood cultures of febrile neutropenic patients mostly in association with the use of intravenous catheters and are a predominant cause of nosocomial infections [1]. Methicillin-resistant S. epidermidis (MRSE), S. haemolyticus (MRSHae) and S. hominis (MRSHo) are all capable of causing infections and usually are more likely to be multiple resistant to antimicrobial agents than other CoNS. Among staphylococci, MRSHo is of increasing concern and today represents the third most common organism among clinical isolates of MRCoNS [2,3,4]. In spite of its clinical significance very little information on the epidemiology of MRCoNS has been published. In particular no information is available regarding S. hominis population structure, genetic diversity and capacity of dissemination. The whole genome sequencing of S. hominis was recently completed and was determined for two strains: S. hominis strain SK119 and S. hominis subsp. hominis C80 (http://www.ncbi.nlm.nih.gov/sites/entrez?db = genome&proj&term = S.hominis). Analysis of the nucleotide sequences available showed that both strains lack the determinant of methicillin resistance (mecA), but are multidrug resistant and exhibit resistance to several metal ions. Moreover, they contain a high number of transposases.

Methicillin resistance conferred by the presence of the mecA gene, encodes for an extra penicillin binding protein (PBP2A) with low affinity for all β-lactams [5]. The mecA gene is found inside a mobile genetic element designated staphylococcal cassette chromosome mec (SCCmec) [6], composed of two essential elements; the mec complex that contains mecA and intact or truncated forms its regulators (mecI and mecR1) and the ccr complex composed of cassette chromosome recombinases (ccr) that are involved in the integration and excision of the cassette [7]. Up until now, ten major types of SCCmec (type I to X) have been reported in S. aureus, that result from specific associations between a particular ccr gene allotype and class of mec complex (A–D) [8,9]. Several SCC-like elements that do not carry mecA but contain other characteristic genes (e.g., capsule gene cluster, fusidic acid...
resistance, or the mercury resistance operon) [8, 10, 11, 12] have also been described [13,14,15].

Although the majority of the work on the characterization of SCCmec has been carried out in MRSA, this element has been described as well in other CoNS namely in S. epidermidis, S. hominis, S. capitis, S. sciuri, S. warneri and S. saprophyticus [4].

Several recent reports suggest that in CoNS, SCCmec structures are highly diverse. In a large study of S. epidermidis it was observed that SCCmec IV and III were the most common, however, as much as 12% of isolates carried either new or non-typeable SCCmec types [16]. Data on SCCmec carried by S. hominis is scarce. In the few isolates characterized so far, SCCmec types Ib, III and new SCCmec types (1A, 1+4A, 5B) have been reported [7,17,18,19] as well as non-typeable SCCmec structures [17,4]. Besides carrying SCCmec and SCC-like cassettes, CoNS were suggested to be active players in the assembly of these mobile genetic elements. Previous studies have shown that a meca homologue, ubiquitous in Staphylococcus sciuri, may have been the evolutionary precursor of meca - the structural gene encoding PBP2a [20] and that the ccr and mec complexes from an unknown source were probably brought together in other CoNS [21], before the cassette was transferred into S. aureus [22]. Also, the recent finding of a 99% of nucleotide sequence homology between the SCCmec IV from S. epidermidis and S. aureus showed that SCCmec type IV in S. aureus was probably originated in S. epidermidis [23]. Similarly, the discovery of regions of high homology between a SCC non-mec containing ccrAB1 from S. hominis and SCCmec type I from S. aureus, lead to the suggestion that this SCC non-mec from S. hominis could have been the primordial form of SCCmec I [12]. Still several links are missing in the evolutionary history of SCCmec and the contribution of each CoNS species to SCCmec evolution is not known.

In this study, we provide what we believe are the first insights into the molecular epidemiology of S. hominis through the description of the diversity of clonal types in MRSHo and MSSHo and SCC elements in isolates from neutropenic patients collected at the bone marrow transplant centre of Tunisia. We also provide a detailed analysis on the most likely contribution of S. hominis to SCCmec evolution.

Materials and Methods

Ethics Statement

This study was performed with approval from the Local Medical Ethical Committee of Charles Nicolle Hospital, Tunis, Tunisia. Since the strains were de-identified and analyzed anonymously and the strains, not a human, were studied, this is exempt from human research committee approval according to the regulations of the Local Medical Ethical Committee of Charles Nicolle Hospital, Tunis, Tunisia and informed consent is not required according to the Ethical Committee.

Bacterial isolates

A total of 34 nosocomial MRSHo isolates consecutively sampled between 2002 and 2004 from neutropenic patients hospitalized at the bone marrow transplant centre of Tunisia, and recovered from blood cultures (29.4%), intravenous catheters (29.4%) and other specimens (41.2%), were analyzed. Isolates were hospital-acquired (specimens collected from patients hospitalized in the graft or hematological units (20), more than 48 h after admission, or from patients that had a history of hospitalization within 6 months prior the isolation date). The study included only one clinical isolate per patient. A group of 11 MSSHo collected over the same time period was included for molecular characterization of ccr genes.

Control isolates

S. aureus ATCC25923 [24] and S. epidermidis RP62A [25] were included for quality control of antimicrobial susceptibility patterns. The type strain of S. hominis ATCC27844 [1] was used as reference for ITS-PCR identification. S. aureus NCTC10442, N315, 85/2002, JCSC-14744, WIS and HDE288 [6,26–29] were included as controls for SCCmec type I, II, III, IV, V and VI, respectively. S. aureus strain COL [30] was used as a source for the meca probe and as positive control for ccrAB1 in hybridization assays; S. aureus WIS [29] was additionally used as a source for the ccrC probe; S. epidermidis RP62A was used as an internal control of PFGE and as a source of ccrAB2 probe; S. aureus 85/2002 was used as a positive control for ccrAB3 and ccrAB4 in hybridization assays; S. epidermidis ATCC12228 [31] was used as positive control for ccrAB2 and ccrAB4; and HDE288 [6] was used as a source of ccrAB4 probe.

Species identification

All isolates were tested by conventional identification phenotypic methods: mannitol fermentation, Gram staining, catalase, coagulase tests (BBL Coagulase Plasma Rabbit test, Becton Dickinson Microbiology systems, Cockeysville, USA) and DNAase activity. Isolates were characterized at the species level by API ID 32 STAPH system (BioMérieux, Marcy l’Étoile, France) according to manufacturer’s instructions. Species identification was confirmed by ITS-PCR, as described [32].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method on Muller-Hinton agar (Difco, Detroit, USA) according to the recommendation of the French Society of Microbiology «Comité de l’Antibiogramme de la Société Française de Microbiologie» (CA-SFM) [http://www.sfm.asso.fr]. Antimicrobial agents tested included penicillin G (6 µg, 10µ), oxacillin (5 µg), ceftoxitin (30 µg), cotrimoxazole (1,25/23,75 µg), streptomycin (10µ), amoxicillin (25 µg), amoxicillin-clavulanic-acid (20/10 µg), gentamicin (15 µg), kanamycin (30 µg), tobramycin (10 µg), erythromycin (15µ), pristinamycin (15 µg), lincomycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampicin (30 µg), ofloxacine (5 µg), ciprofloxacin (5 µg), vancomycin (30 µg), teicoplanin (30 µg), fosfomycin (30 µg), and fusidic acid (10 µg) (Sanofi Diagnostics Pasteur).

Methicillin resistance was confirmed by oxacillin (5 µg) and ceftoxitin (30 µg) disc diffusion tests after 24 h incubation at 37°C. The minimum inhibitory concentration (MIC) for oxacillin was determined by E-test (AB-biodisk, Dalvogen, Sweden) on Muller-Hinton agar (Difco, Detroit, USA) and interpreted as recommended by CA-SFM [http://www.sfm.asso.fr]. Multiresistance was defined as resistance to three or more antimicrobial classes.

DNA preparation

DNA for PFGE was prepared as described [33,34]. Genomic DNA for PCR was extracted as described before [32]. DNA probes for meca, ccrAB1, ccrAB2, ccrAB4 and ccrC were prepared using previously described primers [33,36] followed by purification by the Wizard PCR prep DNA Purification System (Promega, Madison, WI).

Detection of the meca gene

The presence of meca was determined by amplification by PCR [36] and confirmed for all isolates by hybridizing the XhoI (or ClaI) restriction band patterns with a DNA probe for meca.

PFGE typing

PFGE was performed as described [33,37] with the following modifications. XhoI (20 units/disk) was chosen as the restriction
enzyme for PFGE. The running conditions were the following: block1- pulse times 2 to 20 s, running time 11 h and block2- pulse times 2 to 7 s, running time 15 h; voltage 6V; angle 120° [30]. Low Range Lambda ladder DNA (New England BioLabs, Beverly, USA) was used as molecular weight PFGE marker. S. epidermidis RP62A was used to access inter-gel reproducibility. XhoI PFGE restriction band patterns were analyzed by visual inspection by counting the number of band differences; and automatically using BioNumerics Software (version 4.5) from Applied Maths (Sint-Martens-Latem, Belgium). Clusters (PFGE types) were defined using the Dice similarity coefficient and the unweighted pair group method with arithmetic means (UPGMA), with 1% of tolerance and 0.8% optimization, using a cutoff similarity value of 90%. PFGE types were identified by letters; and subtypes were identified by letters followed by a numeric subscript.

Southern blotting and DNA hybridization
XhoI DNA fragments in PFGE gels, were transferred by vacuum blotting as previously described [39] and hybridized with a DNA probe for meca using ECL direct Prime Labeling and detection systems (Amersham Biosciences, Buckinghamshire, United Kingdom), according to manufacturer’s instructions.

Analysis of SCCmec structure
The structures of ccr and mec complex were determined by conventional PCR reactions as described by Okuma et al. [35]. The cccAB1 was detected by PCR amplification using the primers described by Oliveira et al. [40]. The mec complex C1 was amplified by PCR using the primers defined by Katayama et al. [41]. As a first approach, SCCmec types in S. hominis were defined by the combination of the type of ccr complex and the class of mec complex [29,35] using the guidelines proposed for S. aureus by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) [8]. SCCmec was considered as non-typeable when ccr, the mec complex or both were non-typeable. The mec complex and ccr complex were considered non-typeable when no PCR amplification occurred for any of the primer pairs used.

ccrB typing
The ccrB typing was performed as previously described [42]. The ccrB nucleotide sequences were compared by the construction of an unrooted phylogenetic tree using the average distance clustering method and the default parameters set in the ccrBtyping tool (http://www.ccrbtyping.net). The measurement of statistical confidence of the clustering was performed by bootstrap resampling (1,000).

Genotypic diversity
Genotypic diversity was calculated by using Simpson’s index of diversity (SID) [43].

Results
Optimization of PFGE typing method for S. hominis
The enzyme SmaI generates a pattern of 4–5 fragments only, using the protocol defined for S. aureus [33,37]. For this reason we chose the enzyme XhoI and changed the running conditions. We obtained clear, reproducible and well separated banding profiles, containing 18–20 fragments ranging from approximately 6 to 165 Kb in size (Figure 1). The parameters that provided the best concordance between visual and automatic clustering classifications were 1% of tolerance and 0.8% optimization, using a cutoff similarity value of 90% that corresponded to up to 5 bands difference in macrorestriction band patterns, when analyzed visually. Isolates were considered to belong to the same PFGE type when they were put together in the same cluster, after automatic clustering analysis, using the parameters mentioned above. Unique band patterns within each PFGE type were considered as PFGE subtypes.

Genetic diversity and clonality of MRSHo
The molecular characterization of MRSHo isolates by PFGE classified the 34 isolates into 28 PFGE types (A–X), revealing an extremely high genetic diversity (SID = 90.75% for the PFGE type and SID = 99.92% for the PFGE subtype) (Figure 1). Six PFGE types (A, C, F, O, R, and X) contained two isolates each, and the remaining 22 isolates were included in unique types (Table 1). The combination of clinical and typing data revealed that MRSHo belonging to the same PFGE type were found with a 3-year interval in the same unit. This is the case, for example, of isolates of PFGE types A1 and A2 that were collected from two different patients in the hematological unit in 2002 and 2004, respectively. Moreover, cross dissemination of MRSHo among patients in the same ward and across different wards was also observed. This was illustrated clearly, by isolates of PFGE type R1 and R2 that were isolated from two patients both in the Graft Unit within a three year period; and by isolates of PFGE types X that were isolated within a five month interval from one patient in the Graft Unit and another in the Hematological Unit (Table 1).

In spite of the few examples of dissemination of S. hominis isolates, we could observe that, with the exception of two isolates (PFGE G), a unique PFGE subtype was associated to each patient. The results illustrate well the low clonality of the species.

Antibiotic susceptibility patterns
The antibiotic susceptibility to a panel of 22 antibiotics and the MIC for oxacillin was determined for the 34 meca-positive MRSHo. A total of 28 out of 34 isolates (80%) expressed phenotypic methicillin resistance (1.5 μg/ml). However, six isolates were susceptible to oxacillin in both disc diffusion and E-test (MICs 0.047–1.5 μg/ml), in spite of the fact they carried meca; this discrepancy was not investigated further, but might be due to the presence of point mutations in meca that can lead to the production of a less active PBP2a or two the existence of extremely heterogeneous oxacillin resistance profiles, that could not be detected by the methods used.

All MRSHo isolates were resistant to at least one of the non-β-lactam antibiotics tested, as follows: 29 (85%) were resistant to cotrimoxazole, 29 (83%) to fusidic acid, 25 (73%) to kanamycin, 23 (67%) to erythromycin, 21 (62%) to tetracycline, 15 (44%) to clindamycin, 18 (53%) to gentamicin, 18 (53%) to ofloxacin, 17 (50%) to ciprofloxacin, 16 (47%) to tetracycline, 15 (44%) to clindamycin, 6 (17%) to fosfomycin, 4 (12%) to rifampin and 3 (9%) to chloramphenicol. All the isolates were susceptible to pristinamycin, vancomycin and teicoplanin. As many as 94% of MRSHo isolates were multidrug-resistant.

No correlation was found between the PFGE type/subtype and the antibiogram profile. Although we found isolates with the same PFGE type (PFGE C) and the same highly resistant antibiotype, we also found cases where two isolates with exactly the same PFGE subtype (PFGE X) exhibited either resistance to seven different antimicrobial agents or susceptibility to all the antimicrobial agents tested (Table 1).

SCCmec distribution in S. hominis
The molecular characterization of the SCCmec carried by MRSHo showed that SCCmec VI (4B) was carried by 20% of the
isolates, and SCC\textit{mec} type VIII (4A) by 15%. A high proportion of MRSHo strains (38%, 13 out of 34) carried a unique new association between the \textit{mec} complex class and the \textit{ccr} complex, corresponding to \textit{ccrAB1} associated to \textit{mec} complex type A (1A). In addition, a high number of non-typeable SCC\textit{mec} structures (27%) were also found, that resulted from the finding of a single \textit{mec} complex and two different \textit{ccr} complex allotypes in the same isolate. They included: \textit{mec} complex class A, \textit{ccrAB1} and \textit{ccrAB4} (NT1) (two isolates); \textit{mec} complex class A, \textit{ccrAB1} and \textit{ccrC} (NT2) (two isolates); and class A, \textit{ccrAB4} and \textit{ccrC} (NT3) (one isolate). These results may correspond to the existence of a SCC\textit{mec} type with two \textit{ccr} complexes (like SCC\textit{mec} type III), or the existence of a SCC\textit{mec} in tandem with a SCC non-\textit{mec}. In addition, two isolates carried \textit{mec} complex type A but none of the \textit{ccr} genes described so far and in two isolates neither \textit{ccr} nor \textit{mec} complexes were typeable, although \textit{mecA} was present. This may correspond either to new SCC\textit{mec} type structures or to remnants of SCC\textit{mec} that have lost the \textit{ccr} complex.

Overall a high frequency of \textit{mec} complex class A (73.5%) and \textit{ccrAB1} (50%) and \textit{ccrAB4} (44%) was observed among MRSHo.

Regarding the distribution of SCC\textit{mec} among the different PFGE types, we found cases where isolates with the same PFGE type (PFGE types A, O and R) carried the same SCC\textit{mec} type (4B, 1A, and 4A, respectively). However, we also observed that the same SCC\textit{mec} could be carried by isolates of different PFGE types. For example, SCC\textit{mec} 1A was carried by as many as 12 different isolates.

**Figure 1. PFGE profiles after XhoI digestion of methicillin-resistant \textit{S. hominis} isolates.** The dendrogram showing the clustering of strains was performed by analysis of PFGE profiles with the Bionumerics software, with 1% tolerance and 0.8% optimization (this study). Clusters were defined at a cut-off value of 90%.

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Southern hybridization followed by ccr negative isolates analyzed, three harbored ccrC S. hominis ccrAB Identification of SCC PFGE types (Table 1), suggesting either multiple acquisitions of PFGE types and SCC mec two

table 1. Molecular and phenotypic characterization of methicillin-resistant S. hominis (MRSHo) isolates.

| Strain      | Date of isolation | Warda | Clinical product | MIC Ox (µg/ml) | mecA | SCCmec typingd | Resistance profilee | PFGE cluster |
|-------------|------------------|-------|------------------|----------------|------|----------------|---------------------|--------------|
| 6074ae      | 31-12-02         | HU Bl | 0.5              | +              | class B | type 4 | Vi            | P, Ox, E, SXT, Fu  | A1           |
| 4488        | 09-08-04         | HU Ca | 0.5              | +              | class B | type 4 | Vi            | P, S, K, E, RF, SXT, Fu | A2           |
| 2772        | 18-05-04         | HU Ca | 0.38             | +              | class B | type 4 | Vi            | P, Ox, Of, Cp, fo, Fu | B            |
| 5162a       | 09-11-02         | HU ISCa | 1.5 | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, L, Tc, SXT, Ox, Of, Cp, Fu | C1           |
| 3030a       | 08-07-03         | HU Ca | 3               | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, L, Tc, SXT, Ox, Of, Cp, Fu | C2           |
| 4244        | 24-09-03         | HU Ca | 0.19             | +              | NT      | NT      | NT            | P, K, E, Tc        | D            |
| 3140ae      | 18-07-03         | HU Bl | 8               | +              | class A | type 1 | new1 | P, Ox, Amx, Amc, S, G, K, T, E, Tc, Ch, SXT, Fu | E            |
| 2060        | 07-05-03         | GU Ca | >256             | +              | class A | NT      | NT            | P, Ox, Amx, Amc, G, K, T, E, Tc, SXT, Ox, Fu, Fo, Fu | F1           |
| 5305ae      | 02-10-04         | HU Bl | >256             | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, E, SXT, Ox, Cp, Fu | F2           |
| 4842        | 24-10-02         | GU ISCa | >256 | +              | class A | type 4 | VIII | P, Ox, Amx, G, K, T, E, Tc, SXT, Fo, Fu | G            |
| 4793a       | 21-10-02         | GU Bl | 0.047            | +              | class A | type 4 | 4+ccrC NT4   | L             | H            |
| 2916        | 30-06-03         | GU Ca | 1.5              | +              | class A | type 4 | VIII | P, Ox, S, G, K, T, E, Ch, SXT, Fu | I            |
| 3503an      | 21-06-04         | GU Bl | >256             | +              | class A | type 1 | new1 | P, Ox, G, K, K, T, E, SXT, Ox, Of, Cp, Fu | J            |
| 1781        | 22-04-03         | GU Ca | 0.064            | +              | class A | type 1 | new1 | P, Ox, G, K, T, E, SXT, Ox, Of, Cp, Fu | M            |
| 5486        | 26-11-02         | GU ISCa | 6  | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, E, Tc, SXT, Fu | O1           |
| 6624ae      | 06-12-04         | GU Bl | 6               | +              | class A | type 1 | new1 | P, Ox, G, K, K, T, E, Tc, SXT, Ox, Of, Cp, Fu | O2           |
| 5666        | 09-12-02         | GU ISCa | 4  | +              | class A | type 1 | new1 | P, Ox, T, E, SXT, L, LF, SXT, Ox, Of, Cp, Fu | P            |
| 2927b       | 01-07-03         | GU Ca | >256             | +              | class A | type 1 | new1 | P, Ox, Amx, Amc, S, G, K, T, L, SXT, Ox, Cp, Fu | Q            |
| 2730        | 27-06-02         | GU ISCa | 0.19 | +              | class A | type 4 | VIII | P, Ox, Amx, G, K, T, L, Tc, SXT, Fu | R1           |
| 4037b       | 20-09-02         | GU ISCa | 4  | +              | class A | type 4 | VIII | P, Ox, T, E, L, SXT, Ox, Of, Cp, Fu | R2           |
| 2762b       | 26-06-02         | GU ISCa | 24 | +              | class A | type 4 | VIII | P, Ox, Amx, G, K, T, L, Tc, SXT, Fu | S            |
| 312a        | 20-01-03         | GU Ca | 12              | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, SXT, Fu | T            |
| 1561        | 19-03-04         | GU Bl | 4               | +              | class A | type 1 | new1 | P, Ox, G, K, T, E, Tc, SXT, Ox, Of, Cp, Fu | U            |
| 4866b       | 21-10-03         | HU Pu | 1.5              | +              | class A | type 1 | 4+5T | P, Ox, G, K, T, E, L, Tc, SXT, Ox, Of, Cp, Fu | V            |
| 834b        | 11-02-04         | GU Ca | 4               | +              | class A | type 1 | new1 | P, Ox, Amx, K, T, TF, SXT | X1           |
| 4244        | 27-07-04         | HU Ca | 0.094            | +              | NT      | NT      | NT            | P, Ox, Amx, G, K, T, E, L, Tc, SXT, SXT, Ox, Fo, Fu | X2           |
| 5262b       | 13-11-02         | HU ISCa | >256 | +              | class A | NT      | NT            | P, Ox, Amx, G, K, T, E, Tc, SXT, Ox, Fo, Fu | Z            |
| 2320        | 26-04-04         | HU Bl | 0.25             | +              | class B | type 4 | VI            | P, Ox, Amx, Amc, G, K, E, L, RF, SXT, Ox, Of, Cp, Fu | Y            |
| 3070b       | 31-05-04         | HD Pu | 0.38             | +              | class B | type 4 | VI            | P, Ox, E, Tc, SXT, Ox, Of, Cp, Fu | W            |
| 4866an      | 04-09-04         | HU Bl | 8               | +              | class A | type 1 | new1 | P, Ox, Amx, G, K, T, Tc, Fu | AA           |

#H: hematological unit; HG: graft unit; HD: hospital day; 
#WB: blood; Ca: catheter; ISCa: insertion site of catheter; Pu: pus; 
#+: mecA gene present; 
#*: not typeable; 
#P: penicillin G; Ox: oxacillin; S: streptomycin; G: gentamicin; L: lincomycin; Amc: amoxicillin; Amc: amoxicillin-acid clavulanic; Tc: tetracycline; Ch: chloramphenicol; Cp: ciprofloxacin; Of: ofloxacin; SXT: cotrimoxazole; RF: rifampin; Fo: fosfomycin; Fu: fusidic acid.

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PFGE types and SCCmec type 4A was carried by four different PFGE types (Table 1), suggesting either multiple acquisitions of SCCmec or the existence of a highly adaptive and diverse clone.

Identification of ccrAB and ccrC genes in meca-negative S. hominis isolates

The presence of ccr genes among MSSHo isolates was tested by Southern hybridization followed by ccr typing. Among the 11 meca-negative isolates analyzed, three harbored ccrC, two carried ccrAB1, two ccrAB4, and one isolate carried ccrAB2. We also found one isolate that showed to carry the ccr genes by Southern hybridization but that was non-typeable by ccr typing by PCR. All these ccr genes found among MSSHo probably belong to SCC non-mec elements. Interestingly, all except three of the isolates carrying ccr genes showed resistance to three or more antimicrobial classes (Table 2). From the two MSSHo isolates recently sequenced, only one of the isolates (K119) carried a cassette chromosome recombinase with high homology with ccrC from the SCCmec V, which appears to be located far away from the orfX region (draft sequence data). Similarly to our findings, both strains
Nucleotide homology between \textit{ccr}B from \textit{S. hominis} and other \textit{Staphylococcus} species

In order to verify if \textit{ccrB4} and \textit{ccrB1} found in \textit{S. hominis} were similar to those previously described, \textit{ccrB} typing was performed for all MRSHo and MSSHo carrying a single \textit{ccrAB} allotype (28 MRSHo and 5 MSSHo). A high homology (97–99\%) was found between \textit{ccrB1} from \textit{S. hominis} isolates from our study and those of \textit{ccrB1} of other \textit{S. hominis} isolates in the database (SH13-27 and SH8-39). More interesting, a similar homology was found between \textit{ccrB1} from our collection and the \textit{ccrB1} from \textit{S. epidermidis} (SE6-42). Likewise, \textit{S. hominis ccrB4} showed a high nucleotide sequence homology (92 to 96.5\%) with \textit{ccrB4} from \textit{S. aureus} (HDE288) (Figure 2).

Thirteen out of the 33 isolates were non-typeable by \textit{ccrB} typing methodology, because of the presence of several superimposed peaks in sequence’s traces. As \textit{ccrB} typing makes use of degenerated primers, the existence of superimposed peaks may indicate the presence of multiple \textit{ccr} alleles of the same allotype, which the PCR multiplex methodology is unable to detect.

We also found four isolates for which PCR-based typing indicated the presence of \textit{ccrAB1}, but \textit{ccrB} sequencing indicated the presence of \textit{ccrAB4} (5162a, 3140ae´, 5486, and 1561). The results suggest that these isolates carry the two \textit{ccr} allotypes (\textit{ccrB1} and \textit{ccrB4}). However they could not be both detected by the same methodology most probably due to nucleotide differences in the primer regions used. Altogether, these apparently discrepant results, suggest a large pool of \textit{ccr} alleles in \textit{S. hominis}.

A total of four different \textit{ccrB1} and 10 different \textit{ccrB4} alleles were found in the 14 isolates from our collection, for which a \textit{ccrAB} allotype could be determined. The phylogenetic relationships among \textit{ccrB1} and \textit{ccrB4} alleles found in this study with those of control strains available in the database [44] are displayed in Figure 2. The \textit{ccrB4} alleles were clustered into three different branches: one containing the \textit{ccrB4} from the SCC\textit{php4} of \textit{S. epidermidis} strain ATCC12226, the other containing SCC\textit{mec} VI prototype \textit{S. aureus} strain (HDE288), SCC\textit{mec} VIII prototype \textit{S. aureus} strain (10682) and four isolates from our study (3162a, 3140ae´, 5486, and 1561); and the last one containing only four MRSHo (4793a, 2762b, 3070a, 4364a) and two MSSHo isolates (2573b, 2910) from our collection.

The \textit{ccrB1} allele was present in several species in the \textit{ccrB} database, being distributed in one major cluster that contained three prototype \textit{S. aureus} strains for \textit{ccrAB1} (COL, PER184, PL72), one \textit{S. epidermidis} isolate (SE 6–42), one \textit{S. hominis} (SH 13–27) isolate from the database and three \textit{S. hominis} isolates from our collection (two MRSHo and one MSSHo). The remaining five isolates were distributed by five different branches (\textit{S. flourenti} H65; \textit{S. aureus} MSSA4476, \textit{S. hominis} 2189 from our study, \textit{S. hominis SH8-39}, and \textit{S. haemolyticus} D64).

The phylogenetic analysis showed that \textit{ccrAB1} and \textit{ccrB4} from \textit{S. hominis} and those found in \textit{S. aureus} SCC\textit{mec} are highly similar and must have had a common origin.

**Discussion**

This study is the first to describe the molecular epidemiology of nosocomial \textit{Staphylococcus hominis} species. Our data showed that \textit{S. hominis} population was composed of a very high number of PFGE types and subtypes, suggesting that each \textit{S. hominis} clone as defined by PFGE can eventually be host specific and is only rarely disseminated. However, we also found \textit{S. hominis} isolates that were isolated with a 3-year interval in the same hospital. The results indicate that the great majority of \textit{S. hominis} infections most probably have originated from the endogenous flora of patients, rather than acquired from the environment or from cross transmission. However, only the study of isolates collected from healthy persons in the community with no recent hospital contact will enable to confirm this hypothesis.

The PFGE protocol optimized in this study illustrated well the genetic diversity of \textit{S. hominis} population, however it may have been too discriminatory, since it was not able to detect relatedness.

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**Table 2.** Molecular and phenotypic characterization of the 11 methicillin-susceptible \textit{S. hominis} (MSSHo) isolates.

| Strain | Date of isolation | Ward | Clinical Product | MIC Ox (µg/ml) | mecA | SCCmec typing | Resistance profile |
|--------|-------------------|------|-----------------|---------------|-----|---------------|-------------------|
| 2573b  | 17-06-02          | HU   | Ca              | 0.19          | -   | type 4        | NT P, E, Tc        |
| 2189   | 13-05-03          | HU   | BI              | 0.125         | -   | type 1        | NT P, Ox, Amx, S, G, K, T, E, Ch, Nf, SXT, Of, Cp, Fu |
| 3606a  | 21-08-03          | HU   | Th              | 0.125         | -   | type 2        | NT P, Ox, SXT, Fu |
| 2910   | 30-06-03          | GU   | BI              | 0.19          | -   | type 4        | NT P, Amx, SXT, Fu |
| 1342b  | 25-03-03          | GU   | Ca              | 0.19          | -   | type 2        | NT P, Ox, SXT, Of, Cp, Fu |
| 1756'  | 21-04-03          | DH   | BI              | 0.19          | -   | cccC          | NT P, Ox, E, L    |
| 1839a  | 25-04-03          | HU   | BI              | 0.25          | -   | NT            | NT P, S, K, E, L, Tc |
| 2347a  | 27-04-04          | HU   | Ca              | 0.064         | -   | cccC          | NT P               |
| 2654A  | 13-05-04          | GU   | BI              | 0.064         | -   | cccC          | NT -               |
| 5425   | 09-10-04          | HU   | Al              | 0.047         | -   | type 1        | ND                 |
| 3707b  | 01-07-04          | HU   | Bn              | 0.064         | -   | NT            | NT P, Tc, Fo      |

*aH: hematological unit; G: graft unit; DH: day hospital; 
bBI: blood; Ca: catheter; Th: throat; Al: alimentation parenteral; Bn: bone fragment; 
c-mec gene absent; 
dNT: not typeable; 
*ePenicillín G; Ox: oxacillin; S: streptomycin; Ga: gentamicin; K: kanamycin; T: tobramycin; E: erythromycin; L: lincomycin; Amx: amoxicillin; Tc: tetracycline; Ch: chloramphenicol; Cp: ciprofloxacin; Of: ofloxacin; SXT: cotrimoxazole; Rf: rifampin; Fu: fusidic acid.

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among the *S. hominis* isolates collected in this study. The genetic diversity observed may result from the existence of multiple transposases and integrases in the genome, as revealed by the analysis of the genome available for two *S. hominis* strains. To further clarify the origin of genetic diversity in this species it will be essential to develop a multilocus sequence typing scheme for *S. hominis*.

In spite of the low clonality of *S. hominis*, almost every isolate of this species was found to be multidrug resistant, accumulating resistance to virtually all classes of antimicrobial agents, particularly resistance to aminoglicosides, erythromycin, cotrimoxazole, and fosfomycin. The finding of multidrug resistance among *S. hominis* from this study that are part of an established resident flora may result from the long internment periods and aggressive and continuous antibiotic therapy that neutropenic patients are subjected to. This fact may turn this staphylococcal species into a particularly privileged reservoir and donor for other species sharing the same ecological niche, like *S. epidermidis*, *S. haemolyticus* and *S. aureus* [45].

Another interesting feature of *S. hominis* molecular epidemiology was the finding of a high frequency of SCCmec types containing ccrAB1, ccrAB4 and mec complex A in its composition, like VI (4B), SCCmec VIII (4A) and a new SCCmec type with mec complex class A associated to ccrAB1 (1A), and the complete absence of SCCmec structures containing ccrAB2, ccrAB3, ccrC and mec complex C.

Previous studies where SCCmec was characterized for only a few *S. hominis* isolates also showed that *S. hominis* predominantly carried, ccrAB1, ccrAB4 and mec complex A [18,46]. Most importantly, we found that ccrB1 and ccrB4 from both MRSHo and MSSHo analyzed here had a high homology with those ccrAB alleles of prototype strains of *S. aureus* carrying SCCmec type I and VI, respectively. Altogether, the results suggest that *S. hominis* is an important reservoir of ccrAB1, ccrAB4 and mec complex A and could have been the donor of these components to the assembly of SCCmec types I, VI, VIII, and the new type 1A. Another observation resulting from our study that sustains this hypothesis was the finding of ccrB internal regions of MRSHo (carrying SCCmec VI and the new SCCmec 1A) with 100% homology with those of MSSHo what suggests that already pre-existing SCC non-mec carrying these ccrAB alleles could have been the receptors of mec gene complex to give rise to SCCmec. Moreover, the finding in our study of a high number of ccrB1 and ccrB4 alleles among the small number of *S. hominis* analyzed, suggests that these ccr alleles have been in *S. hominis* long enough to be able to diversify and could have been the species where these ccr alleles were once originated.

In what respects to the origin of SCCmec type VI (4B) and VIII (4A), no previous studies documented their link to *S. hominis* or any other species. However, the involvement of *S. hominis* in the assembly of SCCmec I was previously suggested due to the finding...
of a SCC non-mec element in MSSHo with high homology with the SCCmec I from S. aureus [12]. In this study we identified a high number of S. hominis isolates carrying a new SCCmec type where ccrAB1 was associated to mec complex A, with intact mecI and mecR1. We believe that this new SCCmec type could have been the most direct precursor of SCCmec type I that emerged from the latter by mec and mecR1 IS1272-mediated deletion. However, most probably this deletion step did not occur in S. hominis since no IS1272 transposases were found in its genomes (http://www.ncbi.nlm.nih.gov/genome?Db=genome&Cmd=ShowDetailView&TermToSearch=7149; http://www.ncbi.nlm.nih.gov/genome?Db=genome&Cmd=ShowDetailView&TermToSearch=6409).

The methodology used in this study for the definition of SCCmec types in MRSHo was only based on PCR data for the two central elements of SCCmec – the mec complex and ccr complex. Although in S. aureus this is considered to be a valid approach, for CoNS species the SCCmec distribution and structure is still elusive and caution should be taken in interpretation of data. One limitation of the approach used is that it did not take into consideration the proximity of the two elements in the chromosome. Although the conclusions taken are the most parsimonious, the methodology used does not allow us to state that two elements found in the same isolate belong to the same SCC.

The prevalence of specific mec complex classes and ccr allotypes observed in this study was previously detected in other CoNS species like mec complex C in S. haemolyticus and mec complex B and ccrAB2 in S. epidermidis [46,47], suggesting that each CoNS might be a reservoir for specific and different components of the SCCmec types identified in S. aureus (Figure 3).

The study described here showed that S. hominis has a distinctive genetic diversity and has contributed in a specific mode to the assembly of SCCmec. The results emphasize the urgent need for studying each CoNS species as a separate entity with its own features and characteristics. Only by understanding the molecular epidemiology of the individual CoNS species it will be possible in the future to design effective infection control strategies against these microorganisms and understand their contribution for the evolution of the broad-spectrum beta-lactam determinant.

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Author Contributions
Conceived and designed the experiments: HdL MM ABH. Performed the experiments: OB MM ABH. Analyzed the data: OB MM ABH. Contributed reagents/materials/analysis tools: MM ABH. Wrote the paper: OB MM.

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