AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

EPIDEMIOLOGY

Next-Generation Sequence Analysis Reveals Transfer of Methicillin Resistance to a Methicillin-Susceptible *Staphylococcus aureus* Strain That Subsequently Caused a Methicillin-Resistant *Staphylococcus aureus* Outbreak: a Descriptive Study

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ABSTRACT Resistance to methicillin in *Staphylococcus aureus* is caused primarily by the *mecA* gene, which is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*). Horizontal transfer of this element is supposed to be an important factor in the emergence of new clones of methicillin-resistant *Staphylococcus aureus* (MRSA) but has been rarely observed in real time. In 2012, an outbreak occurred involving a health care worker (HCW) and three patients, all carrying a fusidic acid-resistant MRSA strain. The husband of the HCW was screened for MRSA carriage, but only a methicillin-susceptible *S. aureus* (MSSA) strain, which was also resistant to fusidic acid, was detected. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing showed that both the MSSA and MRSA isolates were MT4053-MC0005. This finding led to the hypothesis that the MSSA strain acquired the SCC*mec* and subsequently caused an outbreak. To support this hypothesis, next-generation sequencing of the MSSA and MRSA isolates was performed. This study showed that the MSSA isolate clustered closely with the outbreak isolates based on whole-genome multilocus sequence typing and single-nucleotide polymorphism (SNP) analysis, with a genetic distance of 17 genes and 44 SNPs, respectively. Remarkably, there were relatively large differences in the mobile genetic elements in strains within and between individuals. The limited genetic distance between the MSSA and MRSA isolates in combination with a clear epidemiologic link supports the hypothesis that the MSSA isolate acquired a SCC*mec* and that the resulting MRSA strain caused an outbreak.

KEYWORDS MRSA, MSSA, *Staphylococcus aureus*, *mecA*, methicillin resistance, next-generation sequencing, NGS, outbreak, staphylococcal cassette chromosome *mec*, SCC*mec*, wgMLST, within-host diversity

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community-acquired infections (1). The methicillin resistance gene *mecA* encodes an additional penicillin-binding protein (PBP), PBP2, which has a low affinity for all beta-lactam antibiotics and is not present in susceptible strains (2, 3). *mecA* is located on a mobile element called staphylococcal cassette chromosome *mec* (SCC*mec*). To date, 11 main types of SCC*mec* (types I to XI) have been defined, and they differ in both size and composition (http://www.sccmec.org/Pages/SCC_TypesEN.html). SCC*mec* also carries

Received 19 March 2017 **Returned for modification** 7 April 2017 **Accepted** 29 June 2017

Accepted manuscript posted online 5 July 2017

Citation Weterings V, Bosch T, Witteveen S, Landman F, Schouls L, Kluytmans J. 2017. Next-generation sequence analysis reveals transfer of methicillin resistance to a methicillin-susceptible *Staphylococcus aureus* strain that subsequently caused a methicillin-resistant *Staphylococcus aureus* outbreak: a descriptive study. J Clin Microbiol 55:2808–2816. https://doi.org/10.1128/JCM.00459-17.

Editor Karen C. Carroll, Johns Hopkins University School of Medicine

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genes that are responsible for the regulation of *mecA* transcription, i.e., those encoding the repressor MecI and the transmembrane beta-lactam-sensing signal transducer MecRI (4). For transfer, SCC*mec* carries specific genes called *ccr* genes, which encode recombinases of the invertase/resolvase family. Currently, three phylogenetically distinct *ccr* genes, *ccrA*, *ccrB*, and *ccrC*, have been reported (3).

The first MRSA strain emerged when a SCC*mec* with the *mecA* gene was integrated into the chromosome of a susceptible *S. aureus* strain. The mechanism responsible for the transfer of methicillin resistance has not been well understood. It has been suggested that SCC*mec* can be transferred between staphylococci and that *mecA*-positive coagulase-negative staphylococci may be a potential reservoir for these elements (3). This phenomenon has not been observed frequently, possibly due to the fact that clonal lineages have restriction barriers that control horizontal transfer between lineages (5, 6). However, some studies have reported the horizontal transfer of SCC*mec*. Berglund et al. described the transfer of SCC*mec* from a methicillin-resistant *Staphylococcus haemolyticus* strain to a methicillin-sensitive *S. aureus* (MSSA) strain, creating a new clone of MRSA that caused an outbreak on a neonatal ward (7). Bloemendaal et al. described a transfer of SCC*mec* from *Staphylococcus epidermidis* to a MSSA in an infant during antibiotic treatment (8).

This report describes a MRSA outbreak on the oncology ward in a Dutch hospital that was caused by a health care worker (HCW) carrying a methicillin-susceptible *S. aureus* strain, which acquired a SCC*mec* cassette and which was subsequently transmitted to patients.

RESULTS

Outbreak description. In September 2012, a patient who had been hospitalized for 5 weeks on the oncology ward developed a MRSA bacteremia. Contact tracing for MRSA revealed three other (asymptomatic) MRSA carriers: two patients and one HCW. No known risk factors for MRSA acquisition were present in the three patients or the HCW. The HCW was temporarily removed from patient care and started on decolonization treatment with mupirocin nasal ointment, chlorhexidine wash, oral rifampin, and co-trimoxazole. After initially successful decolonization, recolonization was documented after 1 month. A second decolonization treatment was prescribed but failed after 3 months. After the second failure episode, MRSA contact tracing was expanded to the husband, pets (dog and cat), three family members, and three close friends of the HCW, but all were found MRSA negative. In the end, decolonization of the HCW was achieved after four MRSA decolonization treatment courses. Remarkably, before final decolonization, the HCW tested MRSA positive exclusively in one culture site, the perineum. The index patient and second patient were not decolonized due to the medical condition of these two patients; the third patient lost colonization spontaneously.

Previous bacteriological findings in the HCW and the husband of the HCW. In March 2011 (18 months prior to the outbreak), a screening sample from the throat, nose, and perineum had been obtained from the involved HCW upon her new employment at the hospital, and a fusidic acid-resistant MSSA strain was detected. We hypothesized that the fusidic acid-resistant MSSA strain from the HCW acquired a SCC*mec* and subsequently caused an outbreak. Unfortunately, the fusidic acid-resistant MSSA strain was not available for further testing.

However, in September 2012, prior to the first decolonization attempt of the HCW, the husband of the HCW was tested for MRSA carriage, and a fusidic acid-resistant MSSA strain was detected in the nose, leading to the hypothesis that this MSSA isolate might be identical to the MRSA isolate from the HCW. To test our hypothesis, the MRSA outbreak isolates (from patients and the HCW) and the MSSA strain of the husband were further genotyped. The sequence of events is indicated in Fig. 1.

Microbiology and molecular typing. All isolates (MSSA and MRSA) were resistant to fusidic acid (MIC, $\geq$32 mg/liter). This is a relatively rare antibiogram in our setting; in the past 5 years, only 1% of all clinical isolates of *S. aureus* in our laboratory have been

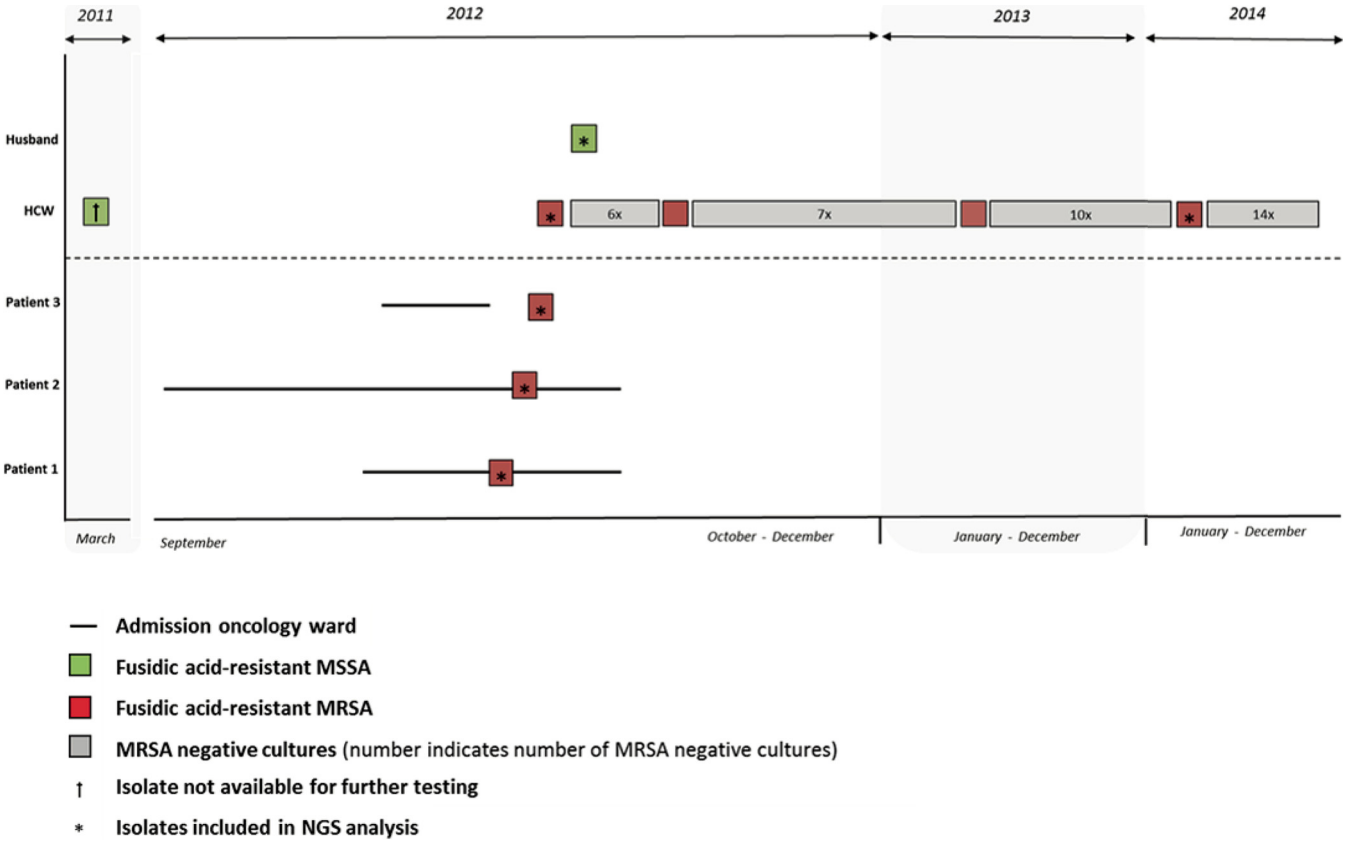

FIG 1 Timeline of the MRSA outbreak on the oncology ward and previous bacteriological findings in the HCW and the husband of the HCW. In September 2012, an outbreak occurred on the oncology ward involving an HCW and three patients, all carrying a fusidic acid-resistant MRSA strain. In March 2011, a screening sample had been obtained from the HCW, and a fusidic acid-resistant MSSA strain was detected. Unfortunately, this strain was not available for further testing. Prior to the first decolonization attempt of the HCW, the husband of the HCW was tested for MRSA carriage, and a fusidic acid-resistant MSSA strain was detected in the nose. Next-generation sequencing was performed on six isolates: the first MRSA isolate from patients 1, 2, and 3 and the HCW, the MSSA isolate from the husband, and one additional MRSA isolate from the HCW isolated in November 2014 after three MRSA treatment cycles.

found resistant to fusidic acid. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing showed that all the isolates involved were MT4053-MC0005, a type which had not been found before or since in the Netherlands.

Classical multilocus sequence typing (MLST) based on the next-generation sequencing (NGS) data revealed that all outbreak isolates yielded sequence type 5 (ST5), a sequence type belonging to clonal complex 5 (CC5). The 52 context isolates were also mainly ST5 ($n = 37$), but eight other STs were also found, albeit all belonging or closely related to CC5.

The minimum spanning tree based on all 1,861 genes of the whole-genome MLST (wgMLST) scheme showed that all outbreak isolates, the two MRSA isolates from the HCW, and the MSSA isolate from the partner of the HCW clustered closely together, with a maximum genetic distance of 17 alleles between two neighboring isolates (Fig. 2). The distance from one of the isolates in this cluster to the nearest unrelated context isolate was 184 alleles.

Single-nucleotide polymorphism (SNP) analysis showed that the outbreak isolates and the isolate from the HCW from 2012 had limited variation, with a maximum of 17 SNPs between the isolates. In comparison to the reference sequence, the MSSA isolate from the partner of the HCW had 44 SNPs while the isolate from the HCW obtained in 2014 had 52 SNPs. The genetic distance between the MSSA isolate and the MRSA isolate from the HCW in 2014 was 27 SNPs (Table 1).

Comparative genomics showed two deletions, of 87 bp and 818 bp, in the MSSA chromosome compared to the MRSA outbreak isolates and the MRSA isolate from the HCW in 2014. The 87-bp deletion sequence encodes the YbaK/EbsC protein, a tran-

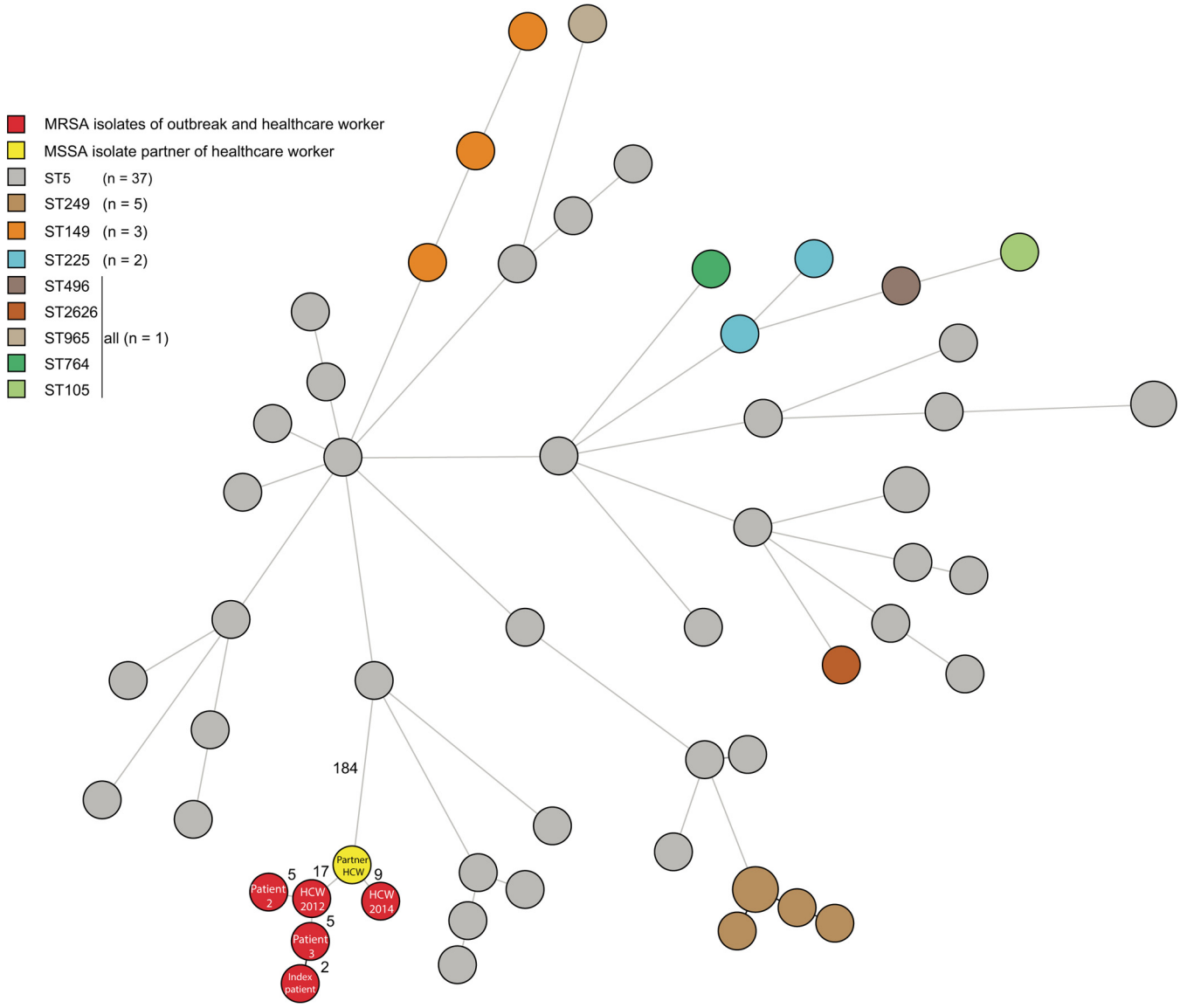

FIG 2 Minimum spanning tree based on next-generation sequencing of 58 MLVA complex 5 (MC5) *S. aureus* isolates. The minimum spanning tree was based on 1,861 genes. The size of each circle indicates the number of isolates. Colors represent MRSA isolates from the outbreak and the HCW (red) and the MSSA isolate from the partner of the HCW (yellow), while the other colors indicate the sequence types (STs) of the MC5 context isolates from the Dutch MRSA surveillance. The lengths of the lines between isolates represent the numbers of different alleles.

scriptional regulation protein. The 818-bp deletion sequence, located in two adjacent genes encoding serine proteases, caused an in-frame fusion of the two genes, creating a new serine protease-encoding open reading frame. No other deletions were found in the isolates.

SCC*mec* analysis showed that all four outbreak isolates carried a SCC*mec* type V (5C2&5C). However, compared to the SCC*mec* reference strain (AB512767), there was an 8.5-kb deletion in the region where the second *ccrC* gene was located in all outbreak isolates. In the MSSA isolate, the complete 38.8-kb SCC*mec* region was absent. Interestingly, the MRSA isolate from the HCW cultured in 2014 carried the same SCC*mec* type as the outbreak isolates but without the 8.5-kb deletion.

Analysis of the NGS data showed the presence of two plasmids with lengths of 4.3 kb (pRIVM6519-1, accession number CP015174) and 3.1 kb (pRIVM6519-2, accession number CP015175) in all four outbreak isolates. No plasmids were detected in the MSSA isolate, whereas the isolate from the HCW from 2014 contained only the pRIVM6519-2 plasmid.

TABLE 1 Microbiology and molecular data of the MSSA and MRSA isolates

Subject	Isolate	MRSA- or MSSA-positive sample sites	Isolation date	MLVA type	NGS data					
					MLST	No. of SNPs	Missing regions	SCC*mec* type	Plasmid(s)	Bacteriophage
Patient 1	MRSA	Nose, throat, blood culture[a]	24 June 2012	MT4053-MC0005	CC5	—[b]	—	V (5C2&5); *ccrC1* deletion	pRIVM6519-1 (4.3 kb); pRIVM6519-2 (3.1 kb)	Absent
Patient 2	MRSA	Nose, throat[a]	17 September 2012	MT4053-MC0005	CC5	17	None	V (5C2&5); *ccrC1* deletion	pRIVM6519-1; pRIVM6519-2	Absent
Patient 3	MRSA	Nose, throat[a], perineum	4 October 2012	MT4053-MC0005	CC5	5	None	V (5C2&5); *ccrC1* deletion	pRIVM6519-1; pRIVM6519-2	Absent
HCW	MRSA	Perineum[a]	21 September 2012	MT4053-MC0005	CC5	11	None	V (5C2&5); *ccrC1* deletion	pRIVM6519-1; pRIVM6519-2	Absent
	MRSA	Perineum[a]	19 November 2014	MT4053-MC0005	CC5	52	None	V (5C2&5)	Absent	Additional phage
Husband of HCW	MSSA	Throat[a]	25 September 2012	MT4053-MC0005	CC5	44	87 bp; 818 bp	Absent	pRIVM6519-2	Additional phage

[a]Isolate included in NGS analysis.
[b]—, reference genome.

Furthermore, an additional (identical) bacteriophage was present in the isolate from the HCW from 2014 and in the MSSA isolate from the husband of the HCW, but this bacteriophage was absent from the four outbreak isolates (Table 1).

DISCUSSION

We hypothesized that a fusidic acid-resistant MSSA strain acquired a SCC*mec* and subsequently caused an MRSA outbreak on the oncology ward in a Dutch hospital. The hypothesis was initially based on a rare antibiogram (*S. aureus* resistant to fusidic acid with *a priori* chance of 1% in our setting) and was further supported by the unique MLVA type MT4053-MC0005, a type which had not been found before or since in the Netherlands. NGS analysis together with a clear epidemiological link confirmed our hypothesis.

Whole-genome sequencing can provide improved resolution to define transmission pathways and characterize outbreaks (9). Other studies using next-generation sequencing of *S. aureus* isolates from outbreaks have been published, and most of these studies determined relatedness by the number of SNPs between genomes (10–13). In the present study, wgMLST typing was also applied. This gene-by-gene approach has the advantage that both SNPs and single recombination events are treated as one evolutionary event.

wgMLST showed that all outbreak isolates, the MSSA isolate, and the MRSA isolates of the HCW in 2014 clustered closely together in the minimum spanning tree with a large distance to the neighboring epidemiologically unrelated CC5 isolates. To date, for wgMLST of *S. aureus*, no clear minimum genetic diversity (in order to exclude a transmission event) has been established. Recently, this has been determined for Gram-negative bacteria in a large epidemiological study in hospitals (10), but for *S. aureus* this has not been done. However, considering the epidemiological linkage in a setting with hardly any circulation of MRSA (14) and in the context of epidemiologically unrelated CC5 strains, this study showed that the MRSA and MSSA isolates are almost certainly related to each other.

SNP analysis also showed that the outbreak strains were nearly identical. However, the numbers of SNPs found within the MSSA isolate and the MRSA isolate from the HCW in 2014 were higher than the distances observed with wgMLST and were above the threshold observed by Golubchik et al. of 40 SNPs or fewer to define recent transmission (9).

The observed differences between the methods can be explained by the fact that the SNP analysis also takes into account the variation between genes. Furthermore, more SNPs could have been identified, since we used one of the outbreak isolates as a reference chromosome for SNP analysis, whereas the wgMLST scheme has a fixed reference that is used for analysis of all *S. aureus* isolates. The higher number of SNPs present in the MSSA isolate could be explained by a different evolutionary path, since the time of transmission of the MSSA isolate between the husband of the HCW and the HCW is unknown.

The differences in number of SNPs between the MRSA isolates from the HCW in 2012 and 2014 might be explained by the fact that the HCW underwent multiple MRSA eradication therapies that could have caused the observed changes in the chromosome. When bacteria are exposed to environmental conditions (15), or when a mismatch repair system becomes disrupted (13), higher rates of point mutations can occur.

We found multiple variations in the compositions of the mobile genetic elements within (MRSA isolates from the HCW) and between (MSSA strain from the husband of the HCW versus the MRSA outbreak strain) individuals. A recent study suggested that the acquisition and loss of mobile genetic elements within nasal colonization populations are common phenomena (16). Also, individuals can harbor multiple variants of isolates, a phenomenon termed as within-host diversity (17). In the case of *S. aureus*, where long-term carriage is common, within-host diversity can be substantial. Longitudinal studies revealed that the size of population diversity can fluctuate over time, and relatively large differences have been detected within individuals (9, 18).

Our study has some limitations. First, only single colonies were sampled from each host, and therefore the diversity within the individuals was not taken into account. Second, the reference genome in this study was derived from a clinical sample (patient with bacteremia), in contrast to the other genomes, which were derived from asymptomatic carriers. Young et al. showed that *S. aureus* strains can acquire mutations during disease progression (18). However, except for the MSSA isolate, no deletions were found when the genomes of the outbreak isolates were compared to the reference genome.

Furthermore, in our study, a fusidic acid-resistant MSSA was detected first, followed by fusidic acid-resistant MRSA cultures from the HCW. This led to the hypothesis that the MSSA strain acquired the SCC*mec*. In contrast, some studies have reported (partial) excision of the SCC*mec* resulting in the conversion of MRSA to MSSA (19, 20). Chlebowicz et al. described an *in vivo* loss of type V (5C2&5)-like SCC*mec* in *S. aureus* ST398, due to recombination between two *ccrC* genes (19). The fact that the HCW was found MRSA positive at multiple times, over a long period of time, possibly indicates that the type V (5C2&5) SCC*mec* in the outbreak strain is relatively stable. Also, the SCC*mec* region in the MSSA isolate was entirely absent, which supports our hypothesis of acquisition rather than loss of the SCC*mec* cassette.

Lastly, this report describes a MRSA outbreak caused by an HCW. While screening of patients at risk of MRSA carriage is generally accepted, screening of HCWs remains controversial (21, 22). Several guidelines, mostly from regions with low MRSA prevalence, advocate screening of HCWs *inter alia* after exposure to MRSA-positive patients (23). On the other hand, the guidelines of several European countries and American professional societies advocate screening of HCWs only in selected situations, such as epidemiological outbreaks or when transmission continues despite implementation of basic control measures (24, 25). Furthermore, the Dutch national guideline recommends sampling all sites for patients but is inconclusive for perineal samples in HCWs (26). Here, the HCW tested MRSA positive exclusively in one culture site, the perineum. This extranasal carriage could be an explanation for the repeated treatment failure in the HCW, as perineal carriage is associated with treatment failure (27). This outbreak shows the potential role of HCWs as a cause of MRSA outbreaks. It underlines the importance of screening HCWs as part of a comprehensive infection control policy, and sampling all sites should be recommended for patients and HCWs.

In conclusion, the limited differences in the wgMLST and SNP analysis in combination with a clear epidemiologic link support the hypothesis that the MSSA isolate acquired a SCC*mec* and that the resulting MRSA strain caused an outbreak. Remarkably, relatively large differences in mobile genetic elements were found between the two MRSA isolates of the HCW. More research is needed to establish how much variation in genomes occurs during colonization in individuals, over long periods, and the variations in response to different hosts.

MATERIALS AND METHODS

Ethics statement. The data used in this study were part of routine practices in The Netherlands and did not require approval from an ethics committee. The data were anonymized and analyzed according to the local regulations and laws that apply to medical information.

Microbiology. For contact screening, swabs of the nose, throat, and perineum were collected using eSwab medium (Copan, Murrieta, CA, USA). The swabs were inoculated on chromID MRSA agar plates (bioMérieux, La Balme, France). The remaining eSwab fluid was transferred in Mueller-Hinton broth supplemented with 6.5% sodium chloride. The overnight Mueller-Hinton broth was subcultured onto both a chromID MRSA agar plate and sheep blood agar (26). Presumptive *S. aureus* colonies were confirmed with a latex agglutination test (Staphaurex Plus; Murex Diagnostics Ltd., Dartford, United Kingdom) and DNase (DNase agar; Oxoid Ltd., Basingstoke, United Kingdom). Antibiotic susceptibility testing was performed using an automated system (Vitek 2; bioMérieux, Marcy l'Etoile, France). Resistance to cefoxitin was measured using the cefoxitin disk diffusion method according to EUCAST standards (28).

Molecular typing. Confirmation of methicillin resistance and *S. aureus* species identification were performed by a PCR for detection of the staphylococcal protein A (*spa*) gene, the gene for methicillin resistance (*mecA*), and staphylococcal cassette chromosome *mec* (SCC*mec*) (GeneXpert SA nasal complete; Cepheid, CA, USA). Further genotyping was performed using MLVA for all isolates in this study (29).

Next-generation sequencing (NGS) was performed on six isolates, which comprised the first MRSA isolate from patients 1, 2, and 3 and the HCW, as well as the MSSA isolate from the husband of the HCW, and one additional MRSA isolate from the HCW isolated in November 2014 after three MRSA treatment cycles. To provide epidemiological and genetic context, 52 MRSA isolates yielding MC0005 were randomly selected from the national MRSA surveillance in 2016 and included in the analysis.

A complete reference genome of the isolate from the first patient (accession number CP015173) was obtained by a hybrid approach combining PacBio (Pacific Biosciences, Menlo Park, CA, USA) and Illumina HiSeq (Illumina, San Diego, CA, USA) data. For the five remaining isolates, NGS was performed using Illumina HiSeq. The NGS data were used for multilocus sequence typing (MLST) and whole-genome MLST (wgMLST) using the available wgMLST scheme in SeqSphere software version 2.3.0 (Ridom GmbH, Münster, Germany). Single-nucleotide polymorphism (SNP) analysis was performed by mapping the Illumina reads of the isolates against the consensus sequence of the reference genome obtained from the first patient. SNP identification was performed with CLC Genomics workbench version 9.5.3 (Qiagen, Hilden, Germany) using the criteria of a frequency of 75% and a forward/reverse read balance of 5%. In addition, the SCC*mec* types were identified using the consensus sequence of the reference genome in the CLC Genomics workbench version 9.5.3 (Qiagen, Hilden, Germany) and a blast search in the NCBI database. The plasmids and bacteriophages were identified by performing a blast search of the contigs of the DeNovo assembly of the unmapped reads of the reference sequence against the NCBI database. All resulting data were imported into BioNumerics version 7.6 for comparative analyses (Applied Maths, Sint-Martens-Latem, Belgium).

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