ORIGINAL ARTICLE

Rapid Whole-Genome Sequencing for Investigation of a Neonatal MRSA Outbreak

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ABSTRACT

BACKGROUND

Isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) belonging to a single lineage are often indistinguishable by means of current typing techniques. Whole-genome sequencing may provide improved resolution to define transmission pathways and characterize outbreaks.

METHODS

We investigated a putative MRSA outbreak in a neonatal intensive care unit. By using rapid high-throughput sequencing technology with a clinically relevant turnaround time, we retrospectively sequenced the DNA from seven isolates associated with the outbreak and another seven MRSA isolates associated with carriage of MRSA or bacteremia in the same hospital.

RESULTS

We constructed a phylogenetic tree by comparing single-nucleotide polymorphisms (SNPs) in the core genome to a reference genome (an epidemic MRSA clone, EMRSA-15 [sequence type 22]). This revealed a distinct cluster of outbreak isolates and clear separation between these and the nonoutbreak isolates. A previously missed transmission event was detected between two patients with bacteremia who were not part of the outbreak. We created an artificial "resistome" of antibiotic-resistance genes and demonstrated concordance between it and the results of phenotypic susceptibility testing; we also created a "toxome" consisting of toxin genes. One outbreak isolate had a hypermutator phenotype with a higher number of SNPs than the other outbreak isolates, highlighting the difficulty of imposing a simple threshold for the number of SNPs between isolates to decide whether they are part of a recent transmission chain.

CONCLUSIONS

Whole-genome sequencing can provide clinically relevant data within a time frame that can influence patient care. The need for automated data interpretation and the provision of clinically meaningful reports represent hurdles to clinical implementation. (Funded by the U.K. Clinical Research Collaboration Translational Infection Research Initiative and others.)

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N Engl J Med 2012;366:2267-75.

Microbial whole-genome sequencing is poised to enhance diagnostic and public health microbiology.[1-3] Its discriminatory power has already been shown in a number of recent outbreaks, including cholera,[4] tuberculosis,[5] and *Escherichia coli* O104:H4.[6,7] The next step is to translate this technology from a research tool into one with clinical utility in the routine diagnostic setting. A compelling target is invasive methicillin-resistant *Staphylococcus aureus* (MRSA) infection, a major public health problem related primarily to health care.[8] In 2005, an estimated 94,360 invasive MRSA infections (including 18,900 hospital-acquired cases of bacteremia) occurred in the United States, associated with 18,650 deaths.[9] On average, hospital stays for MRSA infections cost $14,000, as compared with $7,600 for all other stays, with twice the length of hospitalization: 10.0 days for MRSA infections versus 4.6 days for all other stays.[10]

Efforts to reduce MRSA infection largely revolve around the prevention of person-to-person transmission, the investigation of transmission events, and control of outbreaks. Detecting transmission events in health care settings is central to effective infection control yet is based on imperfect lines of evidence. These include the probability of contact with a known MRSA carrier and the antimicrobial susceptibility pattern ("antibiogram") of the isolates in question — which is useful in only a minority of cases when transmission involves an isolate with a distinct antibiogram. Bacterial genotyping can improve the evidence but to a limited degree only, because in any given institution, most infections are caused by strains belonging to a very restricted number of bacterial genotypes[11] that cannot always be differentiated further by means of current genotyping methods.

We have shown previously that whole-genome sequencing can be used to describe the intercontinental and local transmission of MRSA,[12] but in the past an obstacle to its application in the clinical setting has been the inability to provide sequence data within a clinically relevant time frame. Here, we attempted to overcome this temporal barrier in a retrospective investigation of a MRSA outbreak in a neonatal intensive care unit (NICU) by using a rapid-sequencing platform.

Methods

Bacterial Isolation, Identification, and Drug-Susceptibility Testing

MRSA was detected in blood cultures and swabs used to screen for MRSA colonization with the use of an automated system and plating onto selective medium, respectively. Bacterial identification was achieved by means of a commercial latex agglutination kit (Pastorex Staph-Plus, Bio-Rad Laboratories) and the coagulase test. Antimicrobial-susceptibility testing was performed with the use of a disk-diffusion method[13] for the following drugs: cefoxitin, erythromycin, ciprofloxacin, gentamicin, tetracycline, rifampin, fusidic acid, and mupirocin. Additional antimicrobial susceptibilities to clindamycin, kanamycin, tobramycin, trimethoprim, and linezolid were determined by means of an automated system (see the Supplementary Appendix, available with the full text of this article at NEJM.org).

DNA Sequencing and Analysis

DNA extracted from each MRSA isolate (50-ng samples) was prepared for sequencing with the use of a kit (Nextera DNA Sample Prep Kit, Epicenter). Samples were pooled together and then run on a sequencer (Illumina MiSeq) for paired-end 150-bp sequencing. The sequence data were then aligned to a reference isolate (HO 5096 0412) to identify single-nucleotide polymorphisms (SNPs) as well as regions with insertions or deletions (indels). This reference isolate is defined, by means of multilocus sequence typing, as sequence type 22, the most common hospital-associated MRSA clone in the United Kingdom. We used SNPs in the core genome for the phylogenetic analysis, in this way excluding variation in the accessory genome that may have arisen through horizontal gene transfer from unrelated lineages (see the Supplementary Appendix).

Sample preparation and DNA sequencing were performed by Illumina in advance of commercial release of the MiSeq system, and the entire data set for each sample was provided by them for analysis.

RESULTS

DESCRIPTION OF OUTBREAK

Cambridge University Hospitals National Health Service (NHS) Foundation Trust has 1170 beds and 340,000 occupied-bed-days per year. The Rosie Hospital, the mother and baby hospital of the Trust, contains a 17-bassinet NICU and an adjacent 10-bassinet Special Care Baby Unit (SCBU). All infants admitted to these units are screened for MRSA carriage on admission and then weekly thereafter.

An infant (Patient 1, the index case) who was transferred from a district general hospital to the NICU for specialist care in 2009 was colonized with MRSA at the time of admission (Fig. 1). Each isolate studied is identified with the number of the patient from whom it was isolated, followed by a B if the patient had bacteremia or a C if the patient had carriage of MRSA. The antibiogram of the MRSA isolate revealed differences from the prevalent MRSA clone in our hospital and across the United Kingdom[11] (the epidemic MRSA clone EMRSA-15, sequence type 22, typically resistant to cefoxitin, erythromycin, and ciprofloxacin[14]): the isolate from Patient 1 was also resistant to gentamicin and mupirocin. Infection-control measures were initiated, and a decolonization protocol undertaken, but this failed to eradicate MRSA colonization. Patient 2 was admitted 1 week after Patient 1, with a negative result on MRSA screening, but MRSA colonization was found on repeat screening 37 days later. This isolate from Patient 2 had the same unusual antibiogram as the isolate from Patient 1, suggesting a transmission event.

MRSA bacteremia developed in Patient 1 at 77 days after admission (outbreak isolate 1B). This triggered an investigation by the infection-control team and a program of targeted measures, including emphasis on hand washing and use of personal protective equipment (gloves and aprons) along with a rolling audit of compliance; restricted movement of staff, infants, and equipment within the unit; and a program of deep cleaning. Parents were informed and asked to comply with infection-control procedures. All infants in the NICU and SCBU were screened for MRSA carriage, as were the 181 staff who came into contact with the infants. New admissions were screened, and all patients were screened weekly thereafter.

The first screening in the outbreak investigation identified four more infants colonized with MRSA. Isolates from three of the infants (Patients 5, 6, and 8) had the same antibiogram as the isolate from the index case, whereas the isolate from the fourth infant (Patient 13) had a different antibiogram (showing resistance only to cefoxitin, erythromycin, and ciprofloxacin) and was not considered to be part of the outbreak. Within 13 days after the index case of bacteremia, seven other infants (Patients 3, 4, 7, and 9 through 12) acquired MRSA with the same antibiogram as the outbreak isolate. Infants positive for MRSA carriage were placed in the same bay as other MRSA-colonized infants and underwent a program of decolonization. Three staff members tested positive for MRSA that had a different antibiogram than the outbreak isolate and received decolonization therapy from the Occupational Health Department according to hospital policy. Both the NICU and SCBU were closed for 9 days (except for emergencies in the Cambridge University Hospitals NHS Foundation Trust), refusing admission to 24 infants who therefore had to be admitted by other hospitals in the regional neonatal network.

GENOMIC INVESTIGATION

We sequenced the MRSA bacteremia isolate from Patient 1 (isolate 1B) as well as isolates from six patients with MRSA carriage (isolates 6C, 7C, 8C, 10C, 11C, and 12C) that were believed to be part of the NICU outbreak. We also sequenced MRSA isolates from seven patients not thought to be associated with the outbreak: Patients 14 and 15 with MRSA carriage (isolates 14C and 15C) from infants in the NICU before admission of the index patient, and five isolates associated with MRSA bacteremia in Patients 16 through 20 (isolates 16B through 20B) on other wards during the NICU outbreak (Fig. 1).

We initially interrogated the whole-genome sequence data to define the sequence type of the 14 MRSA isolates (Fig. 2A). All seven isolates associated with the NICU outbreak were sequence type 22. The two MRSA carriage isolates cultured from infants in the NICU before the outbreak (isolates 14C and 15C) were sequence types 5 and 22, re-

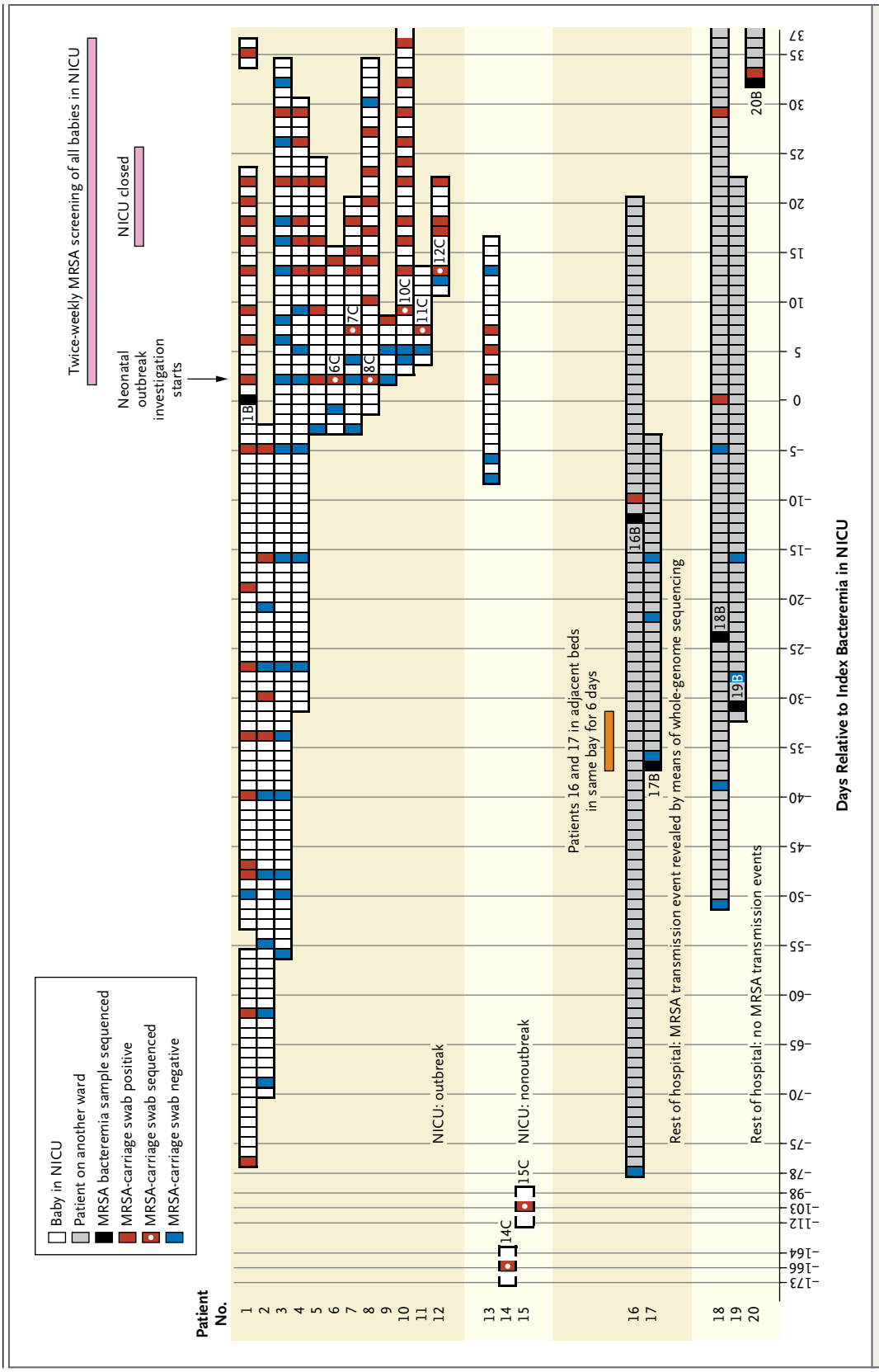


Figure 1. Timeline of the Neonatal Intensive Care Unit (NICU) Outbreak and Methicillin-Resistant *Staphylococcus aureus* (MRSA) Bacteremia Cases on Other Wards.

The index patient (Patient 1) and 11 other infants (Patients 2 through 12) were involved in a MRSA outbreak that was characterized by a distinct antibiogram (resistance to cefoxitin, erythromycin, ciprofloxacin, gentamicin, and mupirocin). Patient 13 was colonized with an isolate with a different antibiogram (resistance to only cefoxitin, erythromycin, and ciprofloxacin), and Patients 14 and 15 had been in the NICU before the outbreak; none of these three were considered to be part of the outbreak. Five other patients (Patients 16 through 20) with bacteremia that developed during the time of the outbreak are shown, in addition to key events throughout this period. Cases of MRSA bacteremia (B) and MRSA carriage (C) are indicated along with the number of the affected patient (e.g., Patient 14's MRSA carriage is indicated as "14C").

spectively. Of the five MRSA bacteremia isolates from other wards, two (isolates 19B and 20B) were sequence type 22, two (isolates 16B and 17B) were sequence type 1, and one (isolate 18B) was sequence type 36.

We mapped the genome sequences of the 10 isolates of sequence type 22 against the reference sequence type 22 and identified 449 variable sites in the core genome (see Fig. S1 in the Supplementary Appendix). Phylogenetic analysis revealed two distinct groups separated by 102 SNPs (Fig. 3). One group contained all the isolates associated with the NICU outbreak, and the other group contained the reference genome together with the three isolates not considered to be part of the outbreak. The outbreak isolates were more tightly clustered than the group of nonoutbreak isolates. In addition, the nonoutbreak isolates of sequence type 22 differed from each other by at least 136 SNPs, suggesting that no transmission events had occurred between the patients carrying these isolates.[12] These observations were not discernible from the antibiograms, which were identical or nearly identical (isolates 15C, 19B, and 20B) (Fig. 2A).

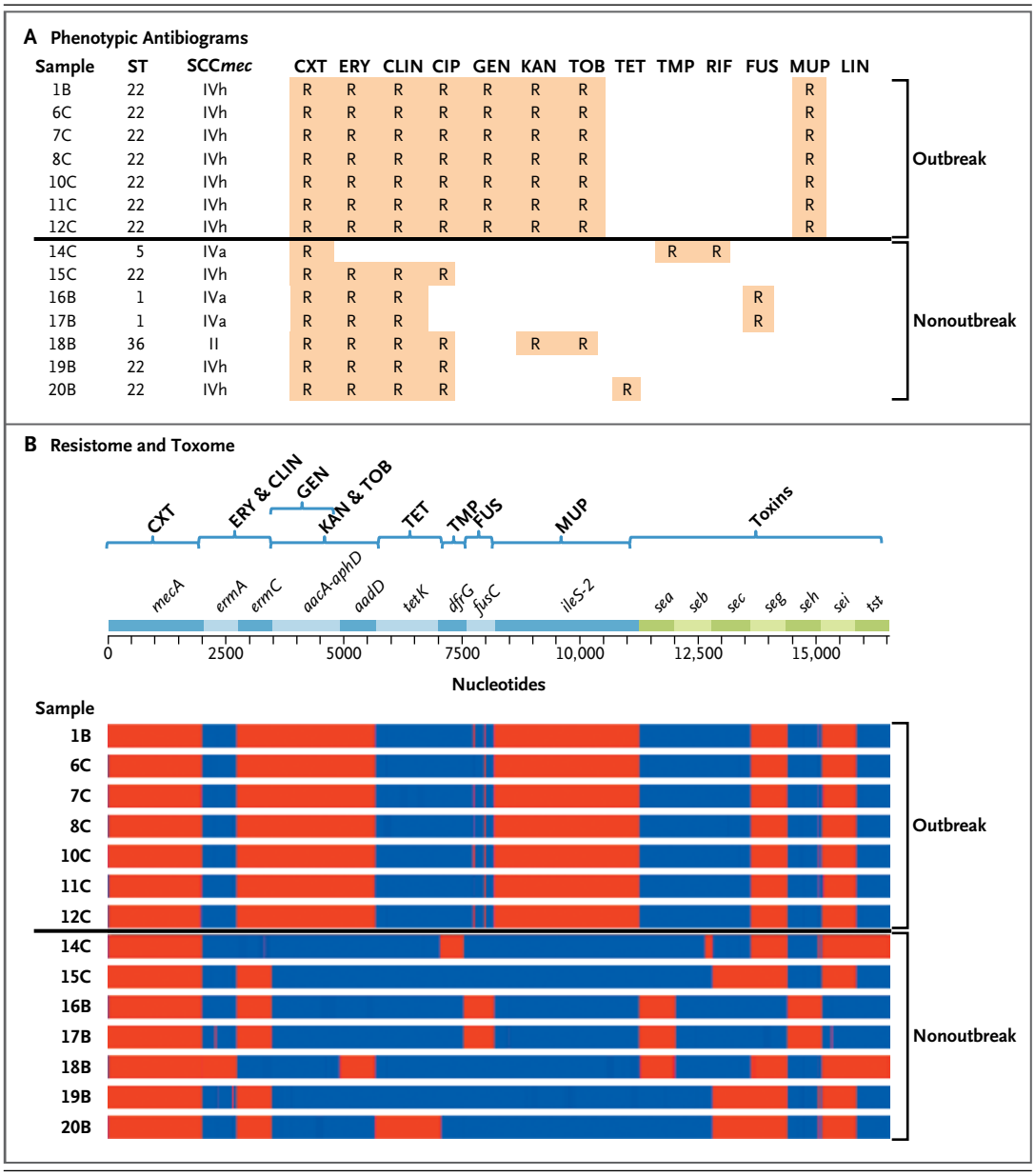
Isolate 6C was genetically divergent as compared with the other outbreak isolates (Fig. 3) and contained more unique SNPs than the remaining six outbreak isolates together (51 vs. 41 SNPs; Fig. S1 in the Supplementary Appendix). Isolate 6C also took significantly longer to grow on solid medium than other outbreak isolates, irrespective of the culture medium used. We suspected that this isolate might be a hypermutator strain with one or more defects in DNA repair pathways. This proved to be the case, with a mutation (Trp291Stop) observed in the *mutS* gene, part of the DNA proofreading mismatch repair pathway, that truncated two thirds of the protein. We confirmed that this led to an elevated mutation frequency by using a standard assay that determined the rate of spontaneous resistance to rifampin (see the Supplementary Appendix). The mutation frequency of isolate 6C ($3.28\pm0.78\times10^{-6}$) was 6.9 times that of isolate 7C ($4.77\pm0.80\times10^{-7}$), its closest genetic relative. This was consistent with the increase in mutation frequency by a factor of 7.1 of a *mutS*-knockout mutant ($1.59\pm0.57\times10^{-6}$) relative to that of *S. aureus* RN4220, its isogenic parent ($2.24\pm0.35\times10^{-7}$). Of the 51 SNPs found only in isolate 6C, several were found in genes associated with central metabolism (Table S1 in the Supplementary Appendix), which may account for the observation that the growth of 6C was slower than the growth of the other outbreak isolates.

Isolate 19B was noted to be a small-colony variant, and we used the whole-genome sequence data to investigate the basis for this. This isolate had 75 unique SNPs and 14 unique indels. A single adenine-nucleotide insertion at nucleotide position 1091 generated a frameshift mutation of *hemG* (the protoporphyrinogen oxidase gene) resulting in the translation of a truncated HemG (366 amino acids vs. the full-length 466). This gene is part of the heme biosynthetic pathway that, if perturbed, is known to be responsible for small-colony variants.[18,19]

Two of the nonoutbreak bacteremia isolates (16B and 17B) were sequence type 1, a community-associated MRSA clone that has been isolated previously from injection-drug users admitted to our hospital.[15] These two isolates were mapped to a sequence-type-1 reference chromosome (MSSA476),[20] resulting in the identification of 280 and 281 SNPs in the core genome for isolates 16B and 17B, respectively. Only a single SNP distinguished the two isolates from each other, leading us to suspect a missed transmission event. Patient 17 had presented at our hospital from the community with a groin abscess associated with MRSA bacteremia and was placed in a six-bed side room with other MRSA-positive patients. Patient 16 had a history of MRSA colonization and occupied a bed adjacent to Patient 17 for 6 days (Fig. 1). We propose that transmission occurred from Patient 17 to Patient 16, supported by the finding that the historical MRSA isolate from Patient 16 had an antibiogram (resistance to cefoxitin, erythromycin, and ciprofloxacin) different from that of the subsequent bacteremia isolate (16B) (Fig. 2A).

RESISTOME AND TOXOME EXPERIMENTS

We examined whether we could determine the antimicrobial-resistance profile for the agents tested in this study on the basis of whole-genome sequencing data (Fig. 2A). Resistance mechanisms arise by means of either the acquisition of a specific gene or mutations in the existing genome. Genes encoding potential antimicrobial resistance that may be acquired by *S. aureus* were assembled to form a resistome for comparison against the sequenced genomes (Fig. 2B). Point mutations associated with resistance to ciprofloxacin and rifampin were identified by searching for SNPs that



have previously been reported to determine resistance to these agents.[16,17] We found complete concordance between the genotypic evidence for resistance and the phenotypically derived antibiogram. We also assembled a toxome, by using all the toxin genes tested for at reference laboratories in the United Kingdom to identify those present in at least one isolate (Fig. 2B).

DISCUSSION

The primary objective of this study was to determine whether whole-genome sequencing could distinguish between MRSA isolates associated and not associated with a putative outbreak, with the use of a sequencing platform with a clinically relevant turnaround time. This was achieved by reconstructing a phylogenetic tree that showed a clear distinction between isolates in the outbreak and nonoutbreak groups. This finding alone is valuable; we also showed that the data enabled additional analyses of immediate clinical value for no additional cost.

We were able to assemble a resistome of genes coding for antibiotic resistance and compare this with the resistance pattern defined by standard

Figure 2 (facing page). Phenotypic Antibiograms, Resistome, and Toxome.

Panel A shows the antibiogram, or antimicrobial susceptibility pattern, for each of the 14 isolates sequenced, and Panel B shows the resistome and toxome. The samples are identified with the patient number followed by a B for bacteremia or a C for carriage of MRSA, along with the sequence type (ST). For each antimicrobial drug, resistance is indicated with an R; its absence indicates susceptibility. The staphylococcal cassette chromosome (SCC) *mec* types are also shown. In Panel B, at the top are genes responsible for drug resistance (the resistome) and toxin genes (the toxome). The toxin genes considered were limited to those that are tested for in the United Kingdom (staphylococcal enterotoxins [*sea* and *sej*], exfoliative toxins [*eta* and *etd*], toxic shock syndrome toxin [*tst*], and Panton–Valentine leukocidin [PVL] [*lukS-PV* and *lukF-PV*]). Only those genes present in at least 1 isolate are shown. Underneath these is a "heat map" showing the presence (red) or absence (blue) of the relevant genes for each of the samples (sequences used in the resistome and toxome are listed in Table S2 in the Supplementary Appendix). Antimicrobial resistance in MRSA can arise by way of either gene acquisition or chromosomal mutations. Gene acquisition accounted for resistance to 10 antimicrobial drugs in the 14 isolates studied. Consistent with a prior study in our hospital of sequence type 1 community-associated MRSA,[15] both transmission isolates 16B and 17B were PVL-negative and carried *sea* and *seh*. Not shown are the chromosomal mutations detected that accounted for resistance to rifampin (RIF) or ciprofloxacin (CIP): mutations in *rpoB* (Leu466Ser and His481Asn) accounted for RIF resistance in isolate 14C[16] and two mutations (Ser80Phe mutation in *grlA* and Ser84Leu in *gyrA*) were responsible for CIP resistance in all ST 22 and ST 36 isolates,[17] and no mutations responsible for linezolid (LIN) resistance were found. CLIN denotes clindamycin, CXT cefoxitin, ERY erythromycin, FUS fusidic acid, GEN gentamicin, KAN kanamycin, MUP mupirocin, TET tetracycline, TMP trimethoprim, and TOB tobramycin.

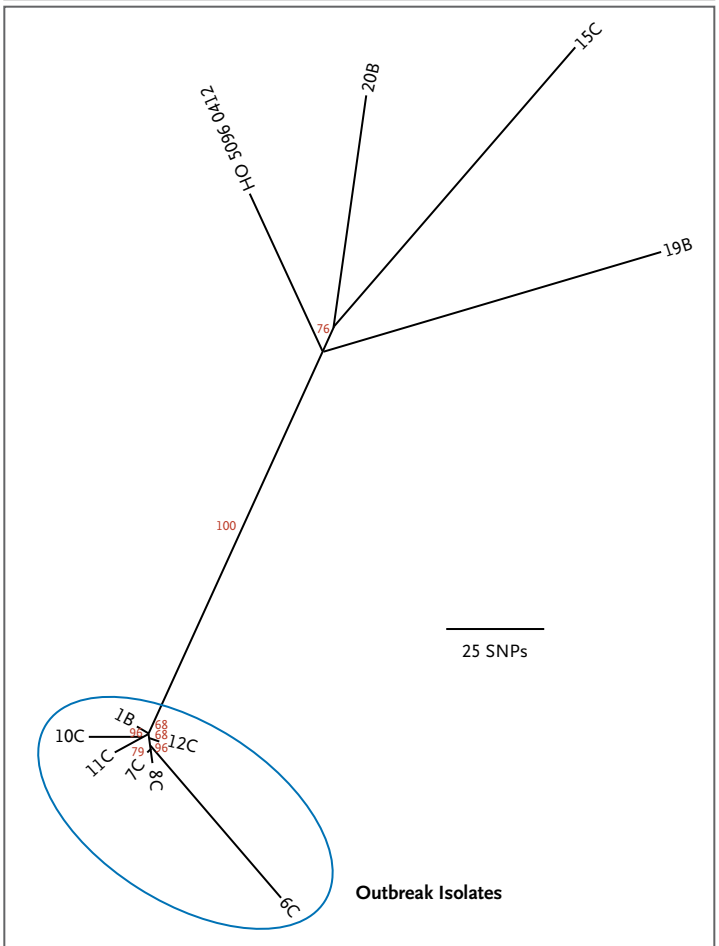


Figure 3. Results of Phylogenetic Analysis of the 10 MRSA Isolates of Sequence Type 22.

An unrooted maximum likelihood tree of the 10 sequence type 22 MRSA isolates identified, together with a reference sequence type 22 isolate (HO 5096 0412), is shown. The isolates are identified with the patient number followed by a B for bacteremia or a C for carriage of MRSA. Bootstrap values are shown in red. Outbreak isolates are circled in blue. Six of the seven outbreak isolates clustered closely together. The seventh isolate (6C) had an extended branch length that could be explained by the fact that it had a hypermutator phenotype. The remaining sequence type 22 isolates associated with carriage by an infant in the NICU (15C) or bacteremia in patients on other wards of the same hospital (19B, 20B) were distantly related to the outbreak isolates and to each other. SNP denotes single-nucleotide polymorphism.

antimicrobial-susceptibility testing. Antimicrobial phenotypes and genotypes showed concordance, providing proof of principle that whole-genome sequencing could be used (after a more extensive evaluation of larger, more diverse strain collections) to guide therapy and represents a powerful tool for the discovery of new drug-resistance mechanisms.[21] We also created a toxome allowing for immediate identification of the toxin genes present in the study isolates that currently have to be determined with the use of multiple polymerase-chain-reaction (PCR) reactions at reference laboratories.[22] One of the isolates in the study was a small-colony variant, which are known to be associated with persistence and treatment failure.[23] The genetic defect for this phenotype was rapidly identified, indicating that it is possible to construct large genetic data sets to better understand and clinically study important microbial variants. Finally, the isolation of a hypermutator strain highlights that it is not possible to use a simple cutoff of SNPs between isolates to decide whether or not they are part of a recent transmission chain; this information must be determined from the topologic characteristics of the phylogenetic tree.

Whole-genome sequencing showed that the infection-control team had correctly identified an outbreak and associated transmission events, aided by an unusual antibiogram for our setting. However, antibiograms are typically less informative for outbreak investigations of sequence type 22, as was evident in our study because two isolates (15C and 19B) are clearly unrelated genotypically despite sharing an identical antibiogram. The ability to distinguish between isolates in this way has important practical infection-control management implications, in that it is often as important to identify isolates that are not part of an outbreak as those that are. Prompt measures are essential to control a suspected outbreak, but unnecessary ward closure and other disruptive control measures can be avoided if unrelated isolates can be identified rapidly. Confirmation of transmission provides the opportunity to target infection-control measures and reduce the risk of further transmission events occurring. Whole-genome sequencing revealed that we had missed a transmission event of a community-associated MRSA isolate infecting a patient who had been placed in a side room containing patients with MRSA on the basis of a history of MRSA carriage. This provides evidence to suggest that putting MRSA-infected patients together on a ward could be a driver for the replacement of hospital-associated MRSA by community-associated MRSA and its dissemination in health care settings and the community.

The findings of our study indicate that there could be value in performing whole-genome sequencing in real time as an integral part of the control of MRSA in the hospital setting. We envisage its initial introduction into regional sequencing facilities that maintain close contact and communication with local heath care institutions. The necessary features for service delivery of this technology include a rapid turnaround time, affordability, and the provision of clinically relevant information to health care personnel that can be interpreted without specialist knowledge of whole-genome sequencing. The recent availability of new sequencing systems[4,6,7] constitutes an important step toward this goal. After extracting DNA from an overnight culture, it took us approximately 1.5 days to prepare the DNA libraries and sequence the isolates, although for fewer samples and shorter sequence-read lengths, faster protocols could be used that would reduce the time period to under a day. The approximate cost of all the materials for whole-genome sequencing is $150 per isolate, including sample preparation, library quality control (quantification and size assessment), and sequencing, which is roughly equivalent to the cost of two PCR tests (e.g., Cepheid Xpert) used to screen for MRSA carriage.[24] Given the competition between current and emerging sequencing platforms,[4,6,7,25] the price and turnaround time will most likely fall, as shown over the past decade.[26] Once data interpretation is fully automated, we predict that whole-genome sequencing will become a standard tool for infection control and will provide the capability to monitor the spread and evolution of major pathogens both within and outside of hospitals in real time.

Supported by grants from the U.K. Clinical Research Collaboration Translational Infection Research Initiative; the Medical Research Council (G1000803), with contributions from the Biotechnology and Biological Sciences Research Council, the National Institute for Health Research (NIHR) on behalf of the U.K. Department of Health, and the Chief Scientist Office of the Scottish Government Health Directorate (to Dr. Peacock); the Health Protection Agency (to Dr. Peacock); the NIHR Cambridge Biomedical Research Centre (to Drs. Cartwright and Peacock); the U.K. Department of Health (to Dr. Enright); and the Wellcome Trust (to Ms. Chewapreecha, Mr. Croucher, Drs. Harris, Holden, Hsu, Parkhill, and Peacock, and Ms. Sanders).

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Mrs. Marie Blackman Northwood, Ms. Aurelie Breton, Mrs. Tracy Brooke, Mrs. Rachael Doughton, Ms. Claire Jenkins, and Dr. Claudia Lopes for their help with this project; and the Sanger Institute Pathogen Production Group and Core Informatics Group for the sequencing of HO 5096 0412.

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