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Healthcare-associated outbreak of meticillin-resistant *Staphylococcus aureus* bacteraemia: role of a cryptic variant of an epidemic clone

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SUMMARY

Background: New strains of meticillin-resistant *Staphylococcus aureus* (MRSA) may be associated with changes in rates of disease or clinical presentation. Conventional typing techniques may not detect new clonal variants that underlie changes in epidemiology or clinical phenotype.

Aim: To investigate the role of clonal variants of MRSA in an outbreak of MRSA bacteraemia at a hospital in England.

Methods: Bacteraemia isolates of the major UK lineages (EMRSA-15 and -16) from before and after the outbreak were analysed by whole-genome sequencing in the context of epidemiological and clinical data. For comparison, EMRSA-15 and -16 isolates from another hospital in England were sequenced. A clonal variant of EMRSA-16 was identified at the outbreak hospital and a molecular signature test designed to distinguish variant isolates among further EMRSA-16 strains.

Findings: By whole-genome sequencing, EMRSA-16 isolates during the outbreak showed strikingly low genetic diversity ($P < 1 \times 10^{-6}$, Monte Carlo test), compared with EMRSA-15 and EMRSA-16 isolates from before the outbreak or the comparator hospital, demonstrating the emergence of a clonal variant. The variant was indistinguishable from the

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ancestral strain by conventional typing. This clonal variant accounted for 64/72 (89%) of EMRSA-16 bacteraemia isolates at the outbreak hospital from 2006.

Conclusions: Evolutionary changes in epidemic MRSA strains not detected by conventional typing may be associated with changes in disease epidemiology. Rapid and affordable technologies for whole-genome sequencing are becoming available with the potential to identify and track the emergence of variants of highly clonal organisms.

Introduction

Transmission of *Staphylococcus aureus* in hospitals, particularly of meticillin-resistant *S. aureus* (MRSA), is a global health challenge. Since its earliest detection, the epidemiology of MRSA has been characterized by the repeated emergence of new clones that expand and then decline.[1–3] In the UK, two clones designated epidemic MRSA (EMRSA)-15 [corresponding to clonal complex (CC)22 and multi-locus sequence type (ST)22] and EMRSA-16 (also known as USA200 and corresponding to CC30, ST36) became dominant in the 1990s, accounting for >95% of nosocomial MRSA infection.[4] The prevalence of both clones began declining in around 2004, EMRSA-16 at a faster rate.[5]

In 2004, a large general teaching hospital in southern England (Brighton) experienced a sudden increase in rates of MRSA bacteraemia that persisted until 2007. Increases in hospital infections are often attributed to poor infection control. However, outbreaks of MRSA infection may also be caused by the importation of new strains.[6–8] In the Brighton outbreak, conventional typing methods failed to detect novel strains and showed that the nationally prevalent EMRSA-15 and EMRSA-16 strains caused 92% of bacteraemias.[9] A curious finding was that although no new lineage appeared, EMRSA-16 dominated in Brighton at a time when EMRSA-16 rates were falling nationally in relation to EMRSA-15.[4] Although failure in infection control seemed possible, it was considered unlikely since it would have most probably resulted in an increase in all bacterial strains.

In theory, clonal variants arising within epidemic MRSA strains could, like new lineages, contribute to changes in infection rate or severity. This has not been demonstrated to date. Conventional approaches to typing of *S. aureus* [such as typing of the *spa* gene or multi-locus sequence typing (MLST)] are poorly discriminatory and would be unable to detect such variation within EMRSA clones. Whole-genome sequencing has the potential to detect subclones within dominant lineages of MRSA and to reveal the genetic basis for clonal expansion and virulence.[7,10–13]

We hypothesized that the emergence of a new clonal variant within EMRSA-16 might account for the outbreak in Brighton. Therefore the aim of this study was to investigate the outbreak using bacterial whole-genome sequencing, in comparison with control isolates from Oxford, where trends represented those seen in the rest of the UK.

Methods

Setting, microbiological sampling and selection of isolates for typing

Brighton and Sussex University Hospitals NHS Trust (BSUH) is an 890-bed general and teaching hospital on the south coast of England. Rates of MRSA bacteraemia reported to the Health Protection Agency mandatory reporting scheme demonstrate that the hospital experienced an outbreak of between 2004 and 2007 during which the peak rate of MRSA bacteraemia was markedly higher and later than that seen in comparable hospitals (Figure 1).

To test whether a clonal variant of EMRSA-15 or -16 might explain the outbreak, we analysed two sets of bacteraemia isolates collected at Brighton before (January 2000 to March 2001) and during (August 2006 to March 2007) the outbreak. Isolates underwent *spa*-typing and meticillin-resistant isolates were assigned as EMRSA-15 (*spa*-type t032) or EMRSA-16 (*spa*-types t018) respectively. These isolates then underwent whole-genome sequencing.[9] As a control population, we whole-genome-sequenced systematically collected bacteraemia and carriage MRSA isolates from similar time-periods from the Oxford University Hospital NHS Trust (OUH), a 1600-bed general and teaching hospital in southern England, at which MRSA bacteraemia rates were close to the national average across the study period (Figure 1).

Based on the results of the whole-genome sequencing we subsequently tested additional available Brighton isolates collected in 2004, early in 2006 and from April 2007 to March 2011 using a molecular signature test (described below). In total, 215 Brighton MRSA isolates from individual episodes of bacteraemia between 2000 and 2011 were *spa*-typed, of which 82 (38%) were EMRSA-15 and 93 (45%) EMRSA-16, from which 32 and 38 whole-genome sequences were obtained respectively (online-only Figure 1).

Whole-genome sequencing and bioinformatics

A full description of whole-genome sequencing and analysis may be found in the online-only methods. Paired-end, indexed libraries were produced for Illumina sequencing from 1.25 μg of sample DNA. Read data were assembled *de novo* using Velvet, and Staphylococcal Cassette Chromosome (SCC)*mec* types were identified by comparison of Velvet assemblies with a panel of type-representative SCC*mec* sequences.[14] Reads from EMRSA-16 and -15 isolates were mapped to lineage-specific reference genomes as previously described.[15–17] The evolutionary history of each lineage was reconstructed using ClonalFrame.[18] Lineage-specific genetic insertions were identified by mapping to a library of unique *S. aureus* sequences obtained from GenBank. Sequence read data have been deposited with the European Nucleotide Archive under accession ERP001489.

Molecular signature typing of EMRSA-16 isolates

Having identified the variant of EMRSA-16 in Brighton, a molecular signature test was designed to identify this variant

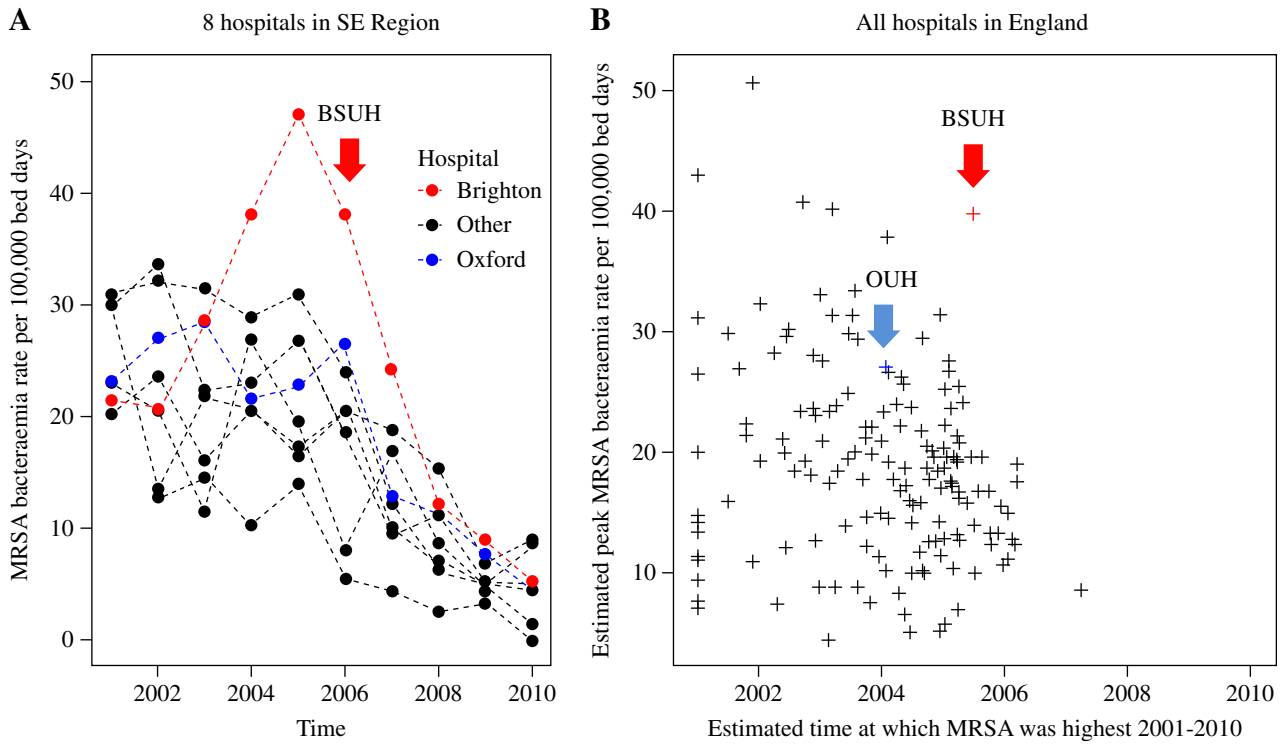

Figure 1. (A) Meticillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia at Brighton and Sussex University Hospitals NHS Trust (BSUH; red), Oxford University Hospitals (OUH; blue) and six other hospitals in South East England (SE; black) as reported to the Health Protection Agency Mandatory Reporting Scheme for England and Wales (www.hpa.org.uk). The rate at Brighton rose from 21.4 episodes per 100,000 bed days (national average of 17.1) in 2001 to a peak of 47.1 in 2005, taking the hospital from 41st to 1st highest out of 167 hospitals in England and Wales. (B) Estimated date and magnitude of MRSA bacteraemia rate for all hospitals in England and Wales between 2001 and 2011 derived from Mandatory Reporting Scheme data and bed occupancy (KH03 statistic) from NHS England. The peak MRSA bacteraemia rate at Brighton was higher and later than at almost all other hospitals.

EMRSA-16 strain in isolates that had not been whole-genome sequenced. The assay targeted five single nucleotide polymorphisms (SNPs) (S1–S5) on the three branches ancestral to the Brighton EMRSA-16 clonal variant (B1-B3 in Figure 2, Supplementary Table I). SNPs were [position in MRSA252 reference, alleles (REF/variant)]: S1, nt 1138701 (A/G); S2, nt 486162 (C/T); S3, nt 1497289 (A/T); S4, nt 542018 (C/T); S5: nt 770373 (G/A). The test was validated using five positive control isolates confirmed by whole-genome sequence data as clonal variant isolates and 10 non-clonal controls. All genotypes observed were either ancestral or the EMRSA-16 variant at all five SNPs.

Clinical profile of the outbreak

To assess the impact of the clonal variant on disease presentation, we compared age, sex, ward location and associated routine laboratory data (haemoglobin, neutrophil count, total white cell count, platelets, creatinine, urea, C-reactive protein and albumin) at the time of bacteraemia between the clonal variant and all other episodes of MRSA bacteraemia at Brighton during the study period. Differences were sought by *t*-test or Mann–Whitney *U*-test for parametrically and non-parametrically distributed variables respectively and Fisher's exact test for differences in proportions. $P \leq 0.05$ was considered significant.

Ethical approval

Isolate storage and data collection was approved in Brighton by the BSUH Research and Development office as a service evaluation, involving anonymized data from patient records and not requiring formal ethical review. Oxford 1997–1998 isolates were collected from the routine microbiology laboratory and stored without personally identifying information. Oxford 2003–2007 isolates were collected for epidemiological studies covered by Statutory Instrument Regulations 2002 No. 1438, section (iii) 'Communicable disease and other risks to public health (Health Service Control of Patient Information)' of Section 60 of the Health and Social Care Act and therefore did not require research ethics committee approval.[19]

Results

Use of whole-genome sequencing to define the diversity of dominant MRSA clones during the outbreak

For all MRSA isolates of *spa*-types t032 (CC22) and t018 (CC30), whole-genome sequencing confirmed membership of the respective EMRSA lineage: all CC22 isolates carried type IV SCC*mec* characteristic of EMRSA-15, and all but one CC30 isolate carried SCC*mec* II characteristic of EMRSA-16. The

Figure 2. Phylogenetic trees for epidemic meticillin-resistant *Staphylococcus aureus* (EMRSA)-15 and EMRSA-16 isolates analysed from Brighton (red) and Oxford (blue) during the study. The *x*-axis indicates date, where branch tips are the date of sampling and branches join at the estimated dates of their presumed common ancestors. The EMRSA-16 tree is annotated to show branches labelled B1—B3 which are ancestral to all outbreak isolates and include single nucleotide variants S1—S5 used in the molecular signature assay to identify clonal variants. In addition, a 5.3 kb recombinational replacement was mapped to branch B1 and the three separate insertions of 20 kb of highly similar non-reference sequences are indicated by BE.

remaining CC30 isolate was identified as a probable community-associated MRSA since it carried SCC*mec* V, was genetically distinct and was ciprofloxacin sensitive; it was excluded from further analysis.

Relationships within EMRSA-16 and EMRSA-15 groups were reconstructed (Figure 2), using sampling times to place dates on the phylogenetic tree (details in Supplementary methods). The most recent common ancestor of EMRSA-16 strains in our sample is estimated to have occurred in 1990 (95% credible interval March 1989–February 1991), coinciding closely with the reported 1991 emergence of EMRSA-16 in the UK.[20] There is strong clustering of isolates in the EMRSA-16 tree with respect to sampling time and location. The most recent common ancestor of EMRSA-15 strains in our sample is estimated to have occurred in 1994 (95% credible interval May 1993–October 1994) although EMRSA-15 was first described in English hospitals in 1991.[21,22]

EMRSA-15 showed no dominant local clones and few clusters with recent common ancestors (<3 years) (Figure 2). By contrast, 5/14 (36%) early versus 22/24 (92%) late Brighton EMRSA-16 genomes belonged to the largest recent genetic cluster for their time-period, compared with 11/23 (48%) versus 13/25 (52%) early versus late Oxford genomes respectively. The EMRSA-16 isolates from the Brighton 2006–2007 period showed strikingly low molecular and lineage diversity, with the most recent common ancestor of the 22 EMRSA-16 genomes forming a single tight genetic cluster (termed the EMRSA-16 'variant') estimated to have existed in 2004 (95% credible interval February 2004–January 2005), coinciding with the rise in MRSA bacteraemias in Brighton (Figure 1A). The genome of each Brighton EMRSA-16 clonal variant differed from its nearest neighbour by a median of 7 (range: 0–22) mutations. Such close genomic relationships are consistent with recent transmission and clonal expansion, i.e. rapid, recent emergence.[11] The formation of such a large cluster so quickly is highly unlikely under a standard coalescent model for the structure of an idealized population ($P < 1 \times 10^{-6}$, by simulation).[23]

Contribution of the EMRSA-16 variant to the outbreak

To explore the contribution of the EMRSA-16 variant to the outbreak, we tested all available EMRSA-16 isolates that had not been whole-genome-sequenced using the SNP-based molecular signature test described above. Among all 215 Brighton MRSA strains, 66 of the 93 EMRSA-16 isolates were identified as the clonal variant. Among the isolates from 2000 to 2001, 0/15 of EMRSA-16 strains were the variant, in 2004 2/6 (33%) were the variant, whereas after 2006, 64/72 EMRSA-16 strains (89%) represented the clonal variant (see online-only Figure 2).

MRSA bacteraemias caused by the EMRSA-16 variant occurred in patients of all ages and throughout the hospital. There was no difference in age or sex of cases. However, the proportion of MRSA bacteraemias caused by it differed between units with a greater proportion of clonal variant MRSA bacteraemias in intensive care (19/33 MRSA bacteraemias) and a lower proportion on the renal unit (7/46) ($P < 0.001$). After adjustment for patient location, MRSA bacteraemia episodes caused by the variant were associated with a significantly higher total white cell count [difference +4.0 ($1.7–6.3 \times 10^9$/L); $P < 0.001$] and neutrophil count [+3.9 ($1.7–6.0 \times 10^9$/L); $P < 0.001$] than episodes caused by non-variant strains (non-variant EMRSA-16, EMRSA-15 and other strains) with 40% of variant cases having neutrophil counts $>15 \times 10^9$/L versus only 14% of the other strains. Platelet count was also higher in episodes caused by the variant [difference +62 ($15–108 \times 10^9$/L); $P = 0.009$]. There was no significant difference in other laboratory values including C-reactive protein (see online-only tables).

Genomic innovations associated with the EMRSA-16 clonal variant

We sought genomic events (mutation, homologous recombination, gain or loss of gene content) on the branches of the phylogeny ancestral to the variant (B1, B2, B3 in Figure 2) that might explain its emergence via a selectively favoured change in phenotype. Twenty-five point mutations (21 non-synonymous, 11 non-synonymous substitutions on B3, the branch immediately ancestral to the clonal expansion) were identified across the three branches, including two non-synonymous changes in *ebh*, a gene encoding a very large cell wall-associated fibronectin-binding protein consistent with patterns recently reported among EMRSA-16 strains in which this gene was hypothesized to have an effect on virulence.[12,24] A 5.3 kb homologous recombination event was detected on B2 (Figure 2) which shows high similarity to regions of the (phi)Sa3 family phage in the MRSA252 upstream of the regions encoding the Panton–Valentine Leucocidin (PVL) genes. In addition 3.2 kb of unique sequences (termed 'BE' in Figure 2) were identified, variants of which had also inserted on other parts of the EMRSA-16 tree. BE comprises part of a larger (~20 kb) fragment of a phiNM4-like phage sequence whose position could not be uniquely determined using short-read data.

Discussion

Nosocomial infection rates, particularly those for MRSA bacteraemia, are regarded as key indicators of infection control effectiveness.[25] Not only do nosocomial infections have significant impact on patient outcomes, but high rates may also adversely affect reputation, leading to loss of revenue, and further declines in quality.[26] The emergence of novel pathogen strains may influence disease epidemiology.[6,7] One challenge with clonal organisms such as *S. aureus* is that conventional methods may fail to detect the emergence of novel variants within major lineages, although it is plausible that such variants could influence rates and severity of disease. Advances in microbial whole-genome sequencing make it possible for the first time to detect variants of epidemic MRSA strains and assess their impact.[7]

We applied whole-genome sequencing to investigate a three-year hospital outbreak of MRSA bacteraemia and demonstrated the emergence of a clonal variant of an epidemic MRSA lineage that was undetectable using conventional genotyping. The appearance of the variant coincided with the onset of the outbreak in 2004, and accounted for virtually all EMRSA-16 isolates by the peak of the outbreak. MRSA bacteraemia rates began to decline in 2007, possibly as a result of intensive infection control measures, although no specific additional intervention was made. The variant caused bacteraemias in patients of all ages and throughout the hospital. It was most prevalent in intensive care and gastroenterology units. Bacteraemias caused by the variant were associated with higher

neutrophil counts than other MRSA bacteraemias. There are several possible explanations for this since the neutrophil response to an infection depends on the immune status of the patient and the source of the infection.

One explanation for the emergence of the variant is that one or more genomic innovations may have conferred a selective advantage leading to the clonal expansion. Although our data do not definitively demonstrate that the clonal variant caused the outbreak, the counter explanation that it was a feature of the outbreak also indicates a transmission advantage of the variant within it. Relatively small fitness advantages may have a substantial impact on transmission, might be specific to a local environment and could relate to several aspects of bacterial ecology, for example, the reported interaction between microbial genetics and infection control practice in the selection of chlorhexidine resistance in MRSA.[27,28] Other studies have demonstrated the impact of new clones on the epidemiology of *S. aureus* infection within specific hospital units; our data indicate dissemination across different units within the hospital.[7,8] As whole-genome sequencing enters clinical practice we will come to understand how often new clonal variants emerge within healthcare settings and how often this is associated with clinically significant changes in disease phenotype or transmission. Our failure to identify a specific genetic explanation for the emergence of the variant is consistent with reports describing the difficulty of establishing a causal link retrospectively.[18,29] The potential relationship between mutations in *ebh* or the recombination events and higher neutrophil counts in bacteraemia caused by the clonal variant requires further investigation.

Another potential explanation for the appearance of the variant is a 'founder effect', in which a breakdown in infection control allowed the emergence of a randomly selected strain which was not typical of the ancestral population. However, it is unlikely that this would preferentially favour a single clone and could alone explain the outbreak. Furthermore, it seems unlikely that a single MRSA lineage would have expanded across the entire hospital by chance, surpassing other prevalent MRSA lineages. Similarly, a lapse in infection control specific to a single unit is not compatible with the presence of the variant across the hospital. Staff were not screened for MRSA during the period of the outbreak, so we cannot exclude the possible involvement of one or more healthcare workers in distributing the variant. Further studies of the role of healthcare workers in nosocomial transmission of *S. aureus* are warranted.

A limitation of this study is its dependence on archived isolates: as such the sampling time-frame is interrupted. Although this impedes precision in plotting the time-course of the outbreak, the use of time-scaled phylogenies means that it does not undermine the central observation.

Microbial whole-genome sequencing is providing insights into the national and international spread of MRSA lineages.[11,12,30] This technology is entering clinical practice and has the potential to be applied rapidly enough to influence patient care.[7,8] Our study demonstrates the emergence of a clonal variant within a nosocomial epidemic MRSA lineage associated with an outbreak. If, at the time of the outbreak, we had been using whole-genome sequencing for routine surveillance, the novel variant would have been recognized, allowing targeted investigation of transmission routes and sources, and the initiation of evidence-based control measures. This study demonstrates the potential for whole-genome sequencing to improve understanding of the epidemiology of MRSA within hospitals.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jhin.2013.11.007.

Conflict of interest statement
None declared.

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