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Recombinations in Staphylococcal Cassette Chromosome \textit{mec} Elements Compromise the Molecular Detection of Methicillin Resistance in \textit{Staphylococcus aureus}

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Abstract

Clinical laboratories are increasingly using molecular tests for methicillin-resistant \textit{Staphylococcus aureus} (MRSA) screening. However, primers have to be targeted to a variable chromosomal region, the staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}). We initially screened 726 MRSA isolates from a single UK hospital trust by recombinase polymerase amplification (RPA), a novel, isothermal alternative to PCR. Undetected isolates were further characterised using multilocus sequence, \textit{spa} typing and whole genome sequencing. 96\% of our tested phenotypically MRSA isolates contained one of the six \textit{orfX}-SCC\textit{mec} junctions our RPA test and commercially available molecular tests target. However 30 isolates could not be detected. Sequencing of 24 of these isolates demonstrated recombinations within the SCC\textit{mec} element with novel insertions that interfered with the RPA, preventing identification as MRSA. This result suggests that clinical laboratories cannot rely solely upon molecular assays to reliably detect all methicillin-resistance. The presence of significant recombinations in the SCC\textit{mec} element, where the majority of assays target their primers, suggests that there will continue to be isolates that escape identification. We caution that dependence on amplification-based molecular assays will continue to result in failure to diagnose a small proportion (~4\%) of MRSA isolates, unless the true level of SCC\textit{mec} natural diversity is determined by whole genome sequencing of a large collection of MRSA isolates.

Introduction

\textit{Staphylococcus aureus} is an important human pathogen and is responsible for healthcare-, community- and livestock-associated colonisation and infection [1]. Infections are especially problematic if the bacteria are methicillin-resistant \textit{S. aureus} (MRSA), which also exhibit phenotypic resistance to related \textit{\beta}-lactam antibiotics including flucloxacillin, cefoxitin and oxacillin. As such there is a great demand for rapid tests to detect MRSA.

Methicillin-sensitive \textit{S. aureus} (MSSA) becomes MRSA when it acquires the SCC\textit{mec} (staphylococcal cassette chromosome \textit{mec}) genetic element. This element contains the \textit{mec} gene that encodes the protein PBP2A (penicillin binding protein 2A). Co-colonising coagulase-negative staphylococci (CNS) are thought to act as the reservoir for the \textit{mec} gene with \textit{S. hominis} suggested to be the origin [2]. CNS species are commensal human skin organisms but have been found to act as a reservoir for entire SCC\textit{mec} elements [3]. Co-colonisation of CNS carrying \textit{mec} elements and MSSA means that nucleic acid tests (NATs) for \textit{mec} and an MSSA-specific gene can give false positive results. NATs for MRSA therefore need to specifically detect the insertion of the SCC\textit{mec} element into the \textit{orfX}
gene of S. aureus. This testing is performed by amplifying with a primer in the SCC\textit{mec} element and a primer in the \textit{orfX} gene of the \textit{S. aureus} chromosome [4]. Clinical microbiology laboratories are increasingly turning towards such molecular testing for the first-line identification of MRSA carriage. However, a recent systematic review concluded that there is insufficient evidence on the clinical effectiveness of PCR over other hospital MRSA screening methods [5]. In particular, a growing number of \textit{S. aureus} isolates are phenotypically resistant but produce false negative results using currently marketed real-time PCR assays [6–9]. Sensitivities for currently marketed PCR assays are as low as 69\% compared to culture [10], resulting in MRSA isolates being misidentified as MSSA [11,12]. Some of the false negatives are likely due to the diversity of the \textit{orfX}-SCC\textit{mec} junctions, as to date at least twenty types have been identified worldwide [13]. This variability means that a unique primer must be developed for each novel \textit{orfX}-SCC\textit{mec} sequence if it is to be detected. The prevalence of different junction types is not well known, but it is suggested that types i, ii, iii, iv, v and vii, account for over 90\% of worldwide strains tested [4]. We decided to investigate if this was true at a large UK hospital by screening several hundred MRSA isolates using a Recombinase Polymerase Amplification (RPA) multiplex assay.

RPA is a novel, isothermal nucleic acid amplification chemistry [14]. The need for complex thermal cycling instruments for PCR is replaced by three core enzymes that operate optimally at 37–40 °C. The first enzyme, a recombinase, binds to primers, forming filaments that can then recombine with homologous DNA. The second enzyme, a single-stranded DNA binding protein, binds to the strand of DNA that is displaced by the primer, preventing the dissociation of the primer. The final core enzyme is a strand-displacing polymerase that copies the DNA, adding bases onto the 3’ end of the primer, forcing open the DNA double helix as it progresses. When opposing primers are used, exponential amplification occurs, with reactions typically running to completion in 5–20 minutes depending upon amplicon size and starting template copy number. Real-time fluorescent detection of RPA reactions is achieved with TwistAmp exo probes. These feature an internal fluorophore and a quencher a few bases apart, with an intervening abasic site (tetrahydrofuran, THF). If the TwistAmp exo probe binds to a complementary sequence then this THF becomes a substrate for exonuclease III and is cleaved, separating fluorophore and quencher. As amplicon is generated, increasing numbers of probes are cleaved, typically giving a detectable signal in 5–10 minutes. RPA reactions are provided as stable, hophilised pellets that contain all of the necessary enzymes and reagents (www.twistdx.co.uk).

As the price of whole-genome sequencing fell significantly after our initial study, it became possible to expand the scope of our investigation and use whole genome sequencing to further identify the precise reasons why our RPA multiplex failed to detect some of the isolates as MRSA.

Failing to identify carriage of MRSA in hospital inpatients will have significant consequences for the individual patient and for general infection control. With the high variability in both \textit{orfX}-SCC\textit{mec} junctions and the sequences of SCC\textit{mec} elements circulating in hospitals it remains to be seen if one assay will be effective as a screening tool. Rapid whole-genome sequencing has revolutionised the investigation of MRSA outbreaks and transmission [15,16] but it can also aid us in identifying isolates that cannot be detected with current molecular assays and enable researchers to alter their tests to detect them.

**Methods**

**Strains**

Central Manchester University Hospitals NHS Foundation Trust (CMFT) is a large academic trust comprising of specialist hospitals for children, dentistry and ophthalmology, together with a large teaching hospital and community services. MRSA isolates were obtained from clinical samples collected via standard hospital screening procedures from the Trust. A total of 726 isolates were collected from 726 patients at CMFT between December 2008 and June 2009 (n = 580) and between July 2009 and November 2009 (n = 146).

Isolates were first screened using a multiplex RPA test. The CMFT standard screening procedure was to collect nasal and groin swabs and combine to inoculate 15 ml nutrient broth (Oxoid, CM1) containing 7.5\% NaCl. Broths were incubated for a minimum of 18 hrs at 35°C in air and then subcultured on MRSA Select agar plates (BD Diagnostics). In addition to usual diagnostics, a single, pink, colony was picked from each positive plate after 24 hours incubation, streaked onto an anonymised blood agar plate and incubated for a further 24 hours. A lack of suitable biocontainment facilities at TwistDx meant that plates were scraped and bacteria resuspended and boiled for 20 minutes. Lysed bacteria were then diluted 1:1000 in sterile distilled water for use in RPA reactions.

**Recombinase polymerase amplification**

RPA primers differ from PCR only in length, with 30–38 bases being optimal for efficient recombinase filament formation. TwistAmp exo probes are typically 46–52 bases long, with a THF ≥30 bases from the 5’ end and ≥15 bases from the 3’ end. A fluorophore and a quencher are positioned either side of the THF such that cleavage by exonuclease III separates the two and fluorescence increases. TwistAmp exo probes have a C3-spacer or similar block at their 3’ end to prevent them amplifying DNA unless cleaved. Numerous overlapping primers were tested empirically with 25 copies of PCR product for each \textit{orfX}-SCC\textit{mec} junction type to determine the best combinations for multiplexing. Potential confounding SNPs were identified by BLAST searches and oligonucleotides targeted to the most conserved regions of each junction. \textit{orfX} was compared to CNS using BLAST to identify the most divergent sequences from \textit{S. aureus} to minimise the risk of false positives caused by amplification of methicillin resistant CNS. To determine the range of SCC\textit{mec} element types that TwistAmp MRSA was able to detect, 15 prototypic strains (Table S1) for SCC\textit{mec} types I–XI were tested with the assay [17]. TwistAmp MRSA was able to detect 11, representing SCC\textit{mec} types I–IV and VI–VIII. The SCC\textit{mec} type V prototype strain (WIS) was not detected by TwistAmp MRSA and was later identified as containing a type of junction, xii, not covered by the assay. Performing a BLAST alignment of all SCC\textit{mec} type V entries in GenBank other than WIS (both types 5C2 and 5C2\&5 accession numbers AB305629, AM999092, GG902038, FJ830660, AB475780, AB512767, AB462393 and CP003166) revealed that they all contained type iii junctions, suggesting that they would be successfully detected by the multiplex.

The selected oligonucleotides were tested for specificity with 10^6 copies of genomic DNA from \textit{S. saprophyticus} (ATCC 43867), \textit{S. epidermidis} (ATCC 35983) and \textit{S. hominis} (ATCC 51624) and gave no signal. We developed a multiplex RPA reaction that detected, but did not differentiate, junctions i, ii, iii, iv and vii, and vii. Isolates that were positive by this test were typed by uniplex assays for junction ii, i, iii, iv, and vii. 30 μl RPA reactions pellets that included the primers and probes were freeze-dried by TwistDx Ltd, Babraham,
Cambridge. For the multiple reaction a internal control sequence was also included to confirm that the reactions had worked. This internal control DNA was designed with the junction i and iii primers at opposite ends with the sequence detected by the control probe in between (Table 1).

RPA reactions were performed by adding 49 μl MRSA resuspension buffer and 1 μl of the boiled, diluted MRSA isolate. Once a strip of 8 reactions had been resuspended, lids were placed on the 0.2 ml PCR tubes and the strip vortexed and pulse-spun. Reactions were run for 20 minutes in Twista portable real-time fluorometers pre-heated to 39°C (Figure S1). Strips were removed, vortexed and pulse-spun and replaced after 4 and 6 minutes to agitate the reactions.

Isolates that were positive by the RPA orfX-SCCmec junction multiplex were then tested using multiplex RPA reactions to individual orfX-SCCmec junction targets. Isolates that were negative by the RPA orfX-SCCmec junction multiplex were sent for MLST, spa typing and high-throughput sequencing.

Further molecular characterisation

We used the MLST scheme previously developed [18] to characterise the isolates on the basis of the sequences of seven housekeeping genes (arc, arcD, gldE, synK, pta, spa and yqiL). The sequence type (ST) of each isolate was determined using the online MLST database [http://saureus.mlst.net/]. eBURST [http://eburst.mlst.net/] was used to determine founding genotypes. The spa type of each isolate was determined using methods described previously [19].

Review of MRSA SCCmec sequences available on GenBank

57 high quality annotated sequences of MRSA SCCmec regions were found by searching the GenBank Nucleotide database (February 2013). Features were extracted and manually curated into a protein fasta file and analysed using OrthoMCL v2.0. Maximal discrimination between similar proteins was achieved by using an inflation parameter (I) of 13. OrthoMCL could not distinguish between different Ccr and Mec allotypes due to their high amino acid similarity.

High-throughput sequencing and assembly

Extracted DNA from isolates undetectable by RPA to the orfX-SCCmec junction were run on an E-Gel 2% gel System (Invitrogen) and examined for quality of DNA and degree of degradation. DNA from 24 (80%) of the 30 isolates was judged to be of sufficient quantity and quality for library making and sequencing. Unfortunately the other six isolate cultures were unrecoverable and so further DNA could not be sourced. The isolates were sequenced on an Illumina Genome Analyzer II, with 101 base pair reads and a paired-end insert size of 400 bp to an average coverage of 100-fold. It is regrettable that we were not able to sequence all 30 undetected isolates and that cultivable isolates are not available for them. When this study was started the cost of whole-genome sequencing was prohibitive and it was only the subsequent precipitous drop in cost that allowed us to re-evaluate what it was possible to do with the samples that we had collected. The sequence reads were trimmed and corrected by Phred+33 quality values using Quake [20]. De novo sequence assembly was carried out using Velvet [21] with the optimal kmer size ranging from 33 to 75. Sequencing contigs and scaffolds were first automatically rearranged and ordered using ABACAS [22] and then homology with existing SCCmec types in GenBank determined using BLASTN on a custom database. Manual reordering was performed by visualisation of BLAST results in ACT [23]. Where the orfX-SCCmec junction was not fully assembled 20–30 iterations of IMAGE [22] were performed. SCCmec types were annotated using RATT [22] before undergoing manual annotation using Artemis [24]. All annotated SCCmec sequences have been uploaded to GenBank [accession numbers HF569093–HF569116]. Unfortunately frozen stocks of these bacteria were no longer available at CMFT by this time. With the sequence information that we have published however, researchers will be able to order synthetic versions of novel junction regions with which to test any new assays that they may wish to develop.

Results

Recombinase polymerase amplification

Of the 726 MRSA isolates tested, 696 (96%) could be detected by orfX-SCCmec junction multiplex RPA. orfX-SCCmec junction uniplex-RPA testing showed that most (653) of these 696 isolates were orfX-SCCmec junction-i, with the other orfX-SCCmec junction-i–iii primers at opposite ends with the sequence detected by the control probe in between (Table 1).

| Oligonucleotide      | Nucleotide sequence 5’-3’ | Reference sequence | Nucleotides (5’-3’) |
|----------------------|--------------------------|--------------------|--------------------|
| mrej-i               | CTGCAGGCTATGCAAAAATCATGAAATTTCTCAT | ABO33763.2        | 38813..38880       |
| mrej-ii              | ACTGACATCCTCAAAAACCTCATCATGCTCT | BA000018.3        | 34244..34215       |
| mrej-iii             | ATGTAATCTTCCTCATTATATAAATTTTTAAAT | ABO37671.1        | 67719..67753       |
| mrej-iv              | TCCATCTCTACCTTATGTTTTCCTCAATAATT | AY2667374.1       | 539..507           |
| mrej-v               | AACTCTGCTTTATATATTATAATTTAATTAAAAATCTGCTGAGAAA | AY2667381.1 | 489..466 |
| mrej-vii             | TTCACTTTTTATCTCATTAGTTTGGATTGCTAATT | AY2667375.1 | 531..497 |
| orfX                 | CAACGCTAATGCGTATACATATTAGTTTGAAGAAT | AY2667375.1 | 346..380 |
| orfX                 | CAACGCTAATGCGTATACATATTAGTTTGAAGAAT | BA000018.3 | 34046..34080 |
| orfX-probe           | CCGTTCATCTGATCGAATCGTTATGATGCAAATGCGT | BA000018.3 | 34083..34128 |
| internal control-probe | CGATCATGCCATCGAGTTATGATGCAAATGCGT | N/A | N/A |

IUPAC ambiguity codes are used where necessary. Non-standard bases are as follows: 1 = dT FAM; 2 = tetrahydrofuran; 3 = dT Black Hole Quencher (BHQ) 1; 4 = dT TAMRA; 5 = dT BHQ2. BHQ available from Biosearch Technologies, Novato, CA. doi:10.1371/journal.pone.0101419.s001

Table 1. Primers and TwistAmp exo probes used in multiplex and singleplex RPA reactions to identify orfX-SCCmec junction types in MRSA isolates.
types tested only representing a minority of cases (i and iii, 1.8%; iv, 0.3%; v, 0.1%; vii, 1.9%).

Comparison of MRSA SCCmec sequences in GenBank
To understand better the conservation of proteins in MRSA, the proteins from the 57 MRSA SCCmec sequences available in GenBank were compared using OrthoMCL (Figure S2). A core group of proteins appear in most SCCmec elements with the most frequent occurring proteins being mec (100% strains), an uncharacterised protein (OG63_2, 89.5%), IS431 transposase (89.5%), ughy (87.7%), coxR (84.2%), ccrB (82.5%) and maacC (82.5%). Strains clustered based on the SCCmec types proposed by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements [IWG-SCC] [25].

Further molecular characterisation
DNA from the original extract was available for 28 of the 30 arfx-SCCmec isolates for which the junction was undetectable by RPA and these underwent further analysis using MLST and spa typing. Only 24 of the isolates were recoverable for further DNA extraction for second-generation sequencing. Unfortunately, because isolates had been anonymised and the cultures taken from them boiled, it was not possible to return to CMFT and regrow any strains that had poor quality DNA. Table 2 shows the results of the distribution of ST, spa and SCCmec types (where available) within these 28 isolates. Eight STs in total were found, none of which were closely related except for ST30, which is a single locus variant (SLV) of ST36. 18 spa types were found among the 28 isolates tested. Sequencing of the SCCmec elements demonstrates conserved homology for many of the isolates but with one cassette displaying no homology and variable levels among the others (Figure 1). The elements identified by sequencing were reviewed by the IWG-SCC. As the elements are conjugates the SCC and SCCmec element subtypes have been assigned. When a novel conjugate element was described the first isolate that was found in our set becomes the archetype and elements with the same structure are called CMFTx-like for simplicity in the text.

Changes in SCCmec type II in ST36 isolates
The major MLST groups were 22 (9 isolates) and 36 (10 isolates). ST36 belongs to clonal complex 30 (CC30). All, but one of the isolates were spa type t018. One isolate was characterised as ST30, an SLV of ST36. Sequencing of the ten ST36 isolates demonstrated that their SCCmec elements were all type II as expected within this group, but with significant changes that prevented the RPA assay from working (Table 2). Seven of the isolates contain the same conjugate element, of which CMFT2 is an example (Figure 2a). This cassette has near identical gene order to the type II reference genome MRSA252 [26], with a class 2 ccr complex and class A mec complex, PUB110 and Tn554 (Figures 2a and 8a). As well as the SCCmec element there is an additional SCC carrying type I ccr genes close to the 5’ end of the element (Figures 2a and 8b). No such composite elements with this pattern are present in GenBank. Two isolates have a variant of this element, which have been labelled IIa-CMFT492-like. This has the same structure but lacks the pUB110 plasmid vector and Tn554 transposon (Figure 2a).

Changes in SCCmec type IV in ST22 isolates
ST22-MRSA-IV is the pandemic strain known as UK-EMRSA-15. In the UK ST22-MRSA-IV has become increasingly common, at the expense of ST36-MRSA-II [27], the other predominant group among the arfx-SCCmec junction-undetectable isolates. It is now responsible for 85% of MRSA bacteraemia cases in UK hospitals. Nine isolates belong to CC22, a common and widespread group that carries SCCmec type IV. Eight ST22-MRSA-IV strains were sequenced and four of them had elements that have a different structure to previously published type IV cassettes. CMFT201 shows the characteristic type IV features of a class 2 ccr gene complex and class B mec gene complex. However, similar to the variant type II elements discussed above, there is a further class I (A1B1) ccr complex situated at the 5’ end of the cassette (Figure 2b). Comparison to all SCCmec elements available in GenBank demonstrates remarkable homology to an unpublished strain 45394F (GU122149) (Figures 2b and S4a). The key differences in homology are at the 5’ end (Figure S4b) with the CMFT201 strain lacking genes hsdR and hsdM. As type I restriction and modification systems are encoded by all three genes; hsdR, hsdM, and hsdS, this may indicate an inability to synthesise R2M2S1 that usually modifies hemimethylated DNA and restricts unmethylated DNA [28].

An additional novel variant is only demonstrated by CMFT3119. The sequenced SCCmec element shares partial homology with two existing strains; ZH47 [29], and strain M1 (Figures 2c and S5) [30]. Similar to ZH47 it contains an additional ccrC along with the normal class 2 ccr gene complex. The larger SCCmec element in CMFT3119 is due to the addition of an arfx gene cluster. Therefore overall CMFT3119 also bears similarity to isolate M1, which also has a class B mec complex, class 2 ccr complex and part of the arginine catabolic mobile element (ACME) often found in S. epidermidis and S. saprophyticus. This may be a subtype of IVh as the J1 region bears homology to ST22-MRSA-IVh, and some isolates belonging to this genotype can contain a truncated ACME element [31].

The three further isolates sequenced from this clonal complex had smaller changes but of a great enough magnitude to still prevent molecular detection with RPA (Table 2).

SCCmec IVk also seen in ST149 isolates
The three isolates belonging to ST149, part of CC5, also have a type IVk SCCmec element. ST149 has previously been described in Malta where it appears to be common [32] and in a Libyan patient in Switzerland [33]. This clonal complex has previously been characterised as carrying multiple composite SCCmec elements [29]. As with the ST22-MRSA-IVk these isolates bore significant homology to 4539F but with deletion of the hsd complex.

Small numbers of ST59, ST130 and ST772 also found
The two ST59 isolates belong to the major community-associated CC59 lineage, a clonal group that has become widespread in the Asia-Pacific region. CC59 strains have been described in several countries including the UK [34–36]. Enough DNA was available for one of the two isolates to be sequenced with a type IVE element evident. However multiple RPA primers bind due to similarities to both arfx-SCCmec junction types iv and v, leading to no clear amplified fragment generation.

CMFT540 belongs to the clonal lineage CC130 with spa type t843, previously reported in bovine and more recently in humans in the UK, Denmark, Ireland and Germany [37–39]. Sequencing confirms that this also has a type XI element with very close homology to that of LGA251 [37].
Table 2. Further characterisation of isolates undetectable by recombinase polymerase amplification: MLST and spa typing for the 28 isolates and sequencing data for the 24 isolates for which DNA could be recovered.

| Isolate number | ST<sup>a</sup> | Clonal complex (CC)<sup>b</sup> | spa type | SCCmec<sup>c</sup> type | Homology to SCCmec (accession no.) | Main changes | Reason for lack of RPA result |
|----------------|-------------|-------------------------------|---------|-----------------------|---------------------------------|-------------|-----------------------------|
| CMFT109        | 15          | 15                            | t084    | *                     | *                               | *           | *                           |
| CMFT3119       | 22          | 22                            | t025    | IVh-CMFT3119-like     | ZH47 (AM292304) M1 (HM030720)    | + arc complex Substitution ccr 4 for 5 | Multiple sites for primer binding |
| CMFT36         | 22          | 22                            | t032    | IVj                   | JSC5670 (AB425824)              | + hsd complex | Fragment too large           |
| CMFT246        | 22          | 22                            | t223    | IVA (-ACME)           | USA300 (NC_007793)             | − ACME      | Too many primer mismatches  |
| CMFT503        | 22          | 22                            | t309    | IVA (-IS431)          | JKD6159 (KP0021144)            | + CDS at IS431 | Too many primer mismatches |
| CMFT201        | 22          | 22                            | t906    | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT211        | 22          | 22                            | t906    | *                     | *                               | *           | *                           |
| CMFT303        | 22          |                                | t906    | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT306        | 22          |                                | t6421   | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT353        | 22          |                                | t6421   | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT432        | 30          | 30                            | t017    | *                     | *                               | *           | *                           |
| CMFT2          | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT120        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT151        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT283        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT463        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT489        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT532        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + class 1 ccr | Too many primer mismatches  |
| CMFT492        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + ccr 1/−  pub1110/−  Tn554 | Too many primer mismatches |
| CMFT352        | 36          | 30                            | t018    | Ila-CMFT2-like        | MRS252 (NC002952)              | + ccr 1/−  pub1110/−  Tn554 | Too many primer mismatches |
| CMFT33         | 36          | 30                            | t021    | IL5                   | MRS252 (NC002952)              | pUB1110 inverted | Two fragments produced    |
| CMFT454        | 59          | 59                            | t216    | IVc                   | AR43/3330.1 (AJ810121)          | Minor J1 changes | Multiple sites for primer binding |
| CMFT374        | 59          | 59                            | t6419   | *                     | *                               | *           | *                           |
| CMFT340        | 130         | 130                           | t043    | XI                    | LG5251 (FR821779)              | No changes | Too many primer mismatches  |
| CMFT106        | 149         | 5                             | t526    | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT181        | 149         | 5                             | t5181   | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT3002       | 149         | 5                             | t5289   | IVk                   | 45394F (GU122149)              | − hsd complex | Fragment too large           |
| CMFT1723       | 772         | 1                             | t657    | V                     | W5 (AB121219)                 | + pepF (SAR1397) | Too many primer mismatches |

<sup>a</sup>Lack of good quality DNA for sequencing.

<sup>b</sup>Sequence type.

<sup>c</sup>Staphylococcal cassette chromosome mec.

Reasons for the negative RPA result for the sequenced isolates are given.

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element identified by sequencing was very similar to that of WIS (AB121219) except for an additional pepF, more commonly found in type II cassettes.

Discussion

Molecular methods for the detection of MRSA that are currently on the market make an attractive alternative to the slower methods of using chromogenic selective MRSA agar or by enrichment in a 7.5% NaCl nutrient broth [41–45]. However, the inherent weakness of all molecular tests is that they can only amplify sequences that they have been designed to detect. For many pathogens it is simple to identify conserved, signature sequences to target. However, in order to be confident that all MRSA cases are detected, a high sensitivity for all SCCmec element types is required. MRSA SCCmec elements display a high level of protein diversity resulting from significant nucleotide diversity within shared proteins. Despite this we have shown that an RPA assay can be used to assess the prevalence of the orfX-SCCmec junction types described by Huletsky et al, in a large UK teaching hospital [4]. 96% of bacteriologically confirmed MRSA isolates were detectable with the RPA assay. However, this does mean that, if used as a diagnostic assay, 30 MRSA isolates would have been false negatives. This is similar to the sensitivity rates seen for other molecular assays [46].
Figure 2. Variant SCCmec elements. a) SCCmec for archetypal type II, MRSA252 (NC_002952), compared to isolates CMFT2 and CMFT492. The cassette of CMFT2 shows the typical features of a type II SCCmec; a class A mec complex and a class 2 ccr complex. However, there is an additional SCC carrying type I ccr genes situated at the 5’ end of the element. CMFT492 is superficially similar and contains the same additional class 1 ccr complex. However, it lacks two of the major features of most type II SCCmec elements; the plasmid vector pUB110 and transposon Tn554. b) SCCmec type IVk: SCCmec for CMFT201 compared to CA05 (AB063172, type IV(2B)) and 45394F (GU122149). The cassette of CMFT201 shows the typical features of a type IV SCCmec (CA05); a class B mec complex and a class 2 ccr complex. However, there are additional SCC elements with an SCC carrying type I ccr genes situated at the 5’ end of the cassette. This is a similar structure to that shown by strain 45394F (unpublished). c) Type IVh variant SCC-SCCmec element for CMFT3119 compared to strains ZH47 (AM292304) and M1 (HM030720). The cassette of CMFT3119 shows the typical features of a type IV SCCmec; a class B mec complex and a class 2 ccr complex. However CMFT3119 contains an additional SCC carrying a ccrC gene upstream of the mec complex, similar to the recombination seen in ZH47. In contrast to ZH47 there is not a Tn4001 but instead part of the arginine catabolic mobile element (ACME), seen in S. epidermidis, S. haemolyticus and USA300, has been inserted. This arc gene cluster is very similar to that seen in the recently identified strain M1.

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A previous study at a UK hospital found the majority of molecularly characterised MRSA isolates carry a type IV SCCmec element [47] but there is little information available for cassette distributions for the whole country. In CMFT 90% of all isolates were found to be oβX-SCCmec junction type ii. Although oβX-SCCmec junction types and SCCmec types do not easily correlate, oβX-SCCmec junction type ii usually corresponds to SCCmec element types I, II or IVd [4].

With the increased interest in molecular-only MRSA screening, missing 30% resistant isolates during screening has significant health risks. It is only in recent years that we are starting to appreciate fully the array of SCCmec elements that may occur. PCR and RPA primers are therefore being developed for a genetically highly variable location. It has also been difficult to identify composite SCC-SCCmec elements as they would either produce a large number of PCR fragments that were difficult to interpret or have a very unusual hybridisation pattern [35]. Whole-genome sequencing followed by re-mapping does not work for genome regions displaying high levels of variation. No assembled reference sequences containing all SCCmec elements exists which leads to very low mapping coverage (Figures S6a and b). We have demonstrated that only de novo assembly allows the cassette to be fully characterised (Figure S6c).

The SCCmec element sequences give clues as to why the RPA assay failed to detect them. For the type IVk the insertion of the additional class 1 ccr complex leads to a 950 bp fragment being produced by the RPA primers; too large for an RPA assay optimised for amplicons of less than 300 bp. Whilst it is possible to optimise RPA to amplify longer fragments, amplification time is a risk. It is only in recent years that we are starting to appreciate fully the array of SCCmec elements that may occur. PCR and RPA primers are therefore being developed for a genetically highly variable location. It has also been difficult to identify composite SCC-SCCmec elements as they would either produce a large number of PCR fragments that were difficult to interpret or have a very unusual hybridisation pattern [35]. Whole-genome sequencing followed by re-mapping does not work for genome regions displaying high levels of variation. No assembled reference sequences containing all SCCmec elements exists which leads to very low mapping coverage (Figures S6a and b). We have demonstrated that only de novo assembly allows the cassette to be fully characterised (Figure S6c).

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Although previously recognised for its predilection for recombination, we have shown that the acquisition of composite SCC-SCCmec elements is not unique to ST149. Both of the most common MLST groups in UK hospitals, 36 and 22, were present amongst the RPA-undetectable isolates. Many of the ST36 isolates had a Ha cassette that had acquired an additional class 1 ccr complex from an SCC. In addition, two isolates had lost the puB110 and Tn554 seen in MRSA252. Although phenotype data was not available it is likely that the loss of the bleomycin- and kanamycin-resistance genes, and erythromycin- and spectinomycin-resistance genes, from the puB110 and Tn554 respectively, will have led to an MRSA strain susceptible to most non-methicillin antibiotics.

The composite SCC-SCCmec element of CMFT3119 showed a different arrangement to all of the other isolates. The variant IVh cassette was similar to that of ZH47 by having an additional class 5 ccr complex but it also contained an arc cluster like strain M1 [30]. arc gene complexes, or ACMEs, are common amongst ST9-MRSA IVa (USA300) isolates but not often seen outside of this clonal group for MRSA. However, they are seen more commonly in coagulase negative staphylococci. arc and opf genes are homologs of genes that are recognised bacterial virulence factors and encode an arginine deaminase pathway and ABC transporter systems respectively. The speG gene of ACME has also been shown to be a potential virulence factor by allowing the bacterium to circumvent polyamine hypersensitivity [49]. A native arc cluster can be found on the chromosome of S. aureus but the ACME-arc cluster inserts as an SCC-like element adjacent to the SCCmec in some strains. Animal models have suggested that the presence of ACME clusters in ST8-MRSA-IVa strains leads to an improved fitness and ability to colonise the skin and mucous membranes [50,51].

The remaining six isolates that were sequenced all contained relatively minor changes but each change was sufficient to interrupt RPA primer binding or lead to excessively large fragments (Table 2). The numbers of RPA undetectable isolates in this study are consistent with those seen in previous studies on the PCR assays available on the market, and suggests that there will always be a few escapee isolates. However the amount of diversity seen here in just one hospital is likely to only be the tip of an iceberg and hence much larger studies of isolates from hospitals in different geographical areas are needed to understand the true levels of natural diversity amongst the populations of MRSA prevailing in our hospitals. The latest whole-genome sequencing technologies make this possible by multiplexing up to 192 samples in a single pool and we have shown that a de novo assembly approach is a reliable method to identify novel SCCmec element sequences.

To effectively reduce post-operative infection rates all surgical patients are screened for MRSA colonisation. In addition all medical admissions are also screened in most hospitals to reduce overall levels of colonisation and the risk of bacteraemia. Effective isolation and treatment of patients with MRSA colonisation requires adequate identification of resistant S. aureus and the speedier time to results that the molecular assays provide has to be balanced against their reduced sensitivity. However, clinical laboratories need to be cautious in adopting fully molecular assays at present – it is likely that 4–5% of MRSA will be missed. We suggest that further uptake occurs only in the knowledge that a phenotypic assay is used to confirm negative isolates. We await larger sequencing studies to provide more information on the ultra-conserved areas of the cassette that can be used for primer design.

Supporting Information

Figure S1 Typical fluorescence (arbitrary units) curves for RPA oβX-SCCmec junction multiplex reactions. Fluorescence is generated by the cleavage of TwistAmp exo probes that have hybridised to amplicon produced by opposing primers. Primers are designed to amplify junction types i, ii, iii, iv, v and vii. a) Signal from the orfX probe (FAM) indicating the presence of the junction sequence. b) Signal from the internal control probe (TAMRA) showing that the reactions have worked. Two negative, no template, controls (NTC) were run. Reactions were run at 39°C for 20 minutes in Twista portable real-time fluorometers (www.twistdx.co.uk). The strips of 8×0.2 ml tubes were removed from Twista, agitated and replaced after 4 and 6 minutes – these are visible as spikes in fluorescence. Because RPA reactions are viscous and run at relatively low temperatures, agitation is necessary to disperse amplicons if there are not many starting template molecules.

(TIF)

Figure S2 Binary heatmap showing presence or absence of proteins within MRSA SCCmec elements. Presence or absence of proteins in SCCmec published sequences on GenBank using OrthoMCL to determine homology. Conventional gene names are shown for each orthologue group with uncharacterised (hypothetical) proteins listed with a relevant reference strain and locus tag in table S2.

(TIF)

Figure S3 Type II variant: homology between strains MRSA252 (NC_002952) and CMFT strain 2. a) Comparison between strain with novel SCCmec from CMFT and SCCmec type II reference strain shown on ACT (26) demonstrating very similar homology for the majority of the 3’ end of the cassette. b) Close-
up view of 5’ end of SCCmec demonstrating main divergence with additional class 1 cer complex inserted in CMFT2. (TIF)

Figure S4 Type IVk: homology between strains CMFT201 and 45394F (GU122149). a) Comparison between strain with novel SCCmec from CMFT and SCCmec type IVk strain on GenBank focusing on the 3’ end, which demonstrates considerable homology. b) Close-up view of 5’ end of SCCmec demonstrating the only area of significantly reduced homology; with absence of hsdR and hsdM in CMFT201. (TIF)

Figure S5 Type Ivh variant SCCmec for CMFT3119 compared to strains ZH47 (AM292304) and M1 (HM030720). The cassette of CMFT3119 shows significant homology to ZH47 with the addition of an arc gene cluster similar to that found in M1. (TIF)

Figure S6 Importance of assembly for examining SCCmec types in MRSA. a) VCF view on Artemis (27) for all 24 MRSA isolates sequenced (rows) with variants compared to MRSA252 shown as coloured vertical lines. Area shaded in pink is for SCCmec region. There appears to be little variation but b) is the same region in pink shown with a variation of varB (50) that displays areas of zero coverage as grey. The low variation seen in a) is due to a significant reduction in mapping depth over this region. c) BAM view of same reads of CMFT3540 piled onto completed assembly of CMFT340 (only SCCmec is annotated). (TIF)

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TABLE S1 SCCmec element prototype strains used.

| Reference          | Type   | Prototype Strain | Source |
|--------------------|--------|------------------|--------|
| 1. Lowry FD (1998) | Type IVk| SCCmec IVk | N Engl J Med 339: 520–532. |
| 2. Tsuhabakita S, Kucikahara-Arai K, Sasaki T, Hiranuma K (2010) | Type IVk| SCCmec IVk | Antonie van Leeuwenhoek 94: 351–355. |
| 3. Barbari F, Kuppi E, Hernandez D, Lebeaux D, Francois P, et al. (2010) | Type IVk| SCCmec IVk | J Infect Dis 202: 270–281. |
| 4. Huletsky A, Giroux R, Rossbach V, Gagnon M, Vaillancourt M, et al. (2004) | Type IVk| SCCmec IVk | J Clin Microbiol 42: 2281–2283. |

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Author Contributions
Conceived and designed the experiments: GAH-C OP MSF LOH AP. Performed the experiments: GAH-C LOH MFAEG MSF AD MSF. Analyzed the data: GAH-C LOH MSF. Contributed reagents/materials/analysis tools: OP MSF AD TGC AP. Contributed to the writing of the manuscript: GAH-C LOH MSF AD OP MFAEG MG TGC AP. Collected the nasal and groin swabs and carried out routine MRSA screening: AD. Developed the recombinase polymerase amplification assay, designed the primer sequences and isolated DNA from the MRSA isolates: MSF OP. Tested the RPA assay and performed spa typing and MLST: LOH. Purified the DNA and prepared whole-genome sequencing libraries: MFAEG MN. Assembled the genomes and performed the bioinformatics analysis: GAH-C. Wrote the first draft of the manuscript: GAH-C LOH MSF. Designed the original project to design and test an RPA assay for MRSA: MSF OP LOH AD. Designed the WGS project: GAH-C TGC AP MSF. Contributed to editing the manuscript: GAH-C LOH MFAEG OP MG AD TGC AP.

TABLE S2 Locus tags for uncharacterised protein clusters in Figure S2.

| Reference          | Type       | Prototype Strain | Source |
|--------------------|------------|------------------|--------|
| 1. Lowry FD (1998) | Type IVk   | SCCmec IVk       | N Engl J Med 339: 520–532. |
| 2. Tsuhabakita S, Kucikahara-Arai K, Sasaki T, Hiranuma K (2010) | Type IVk| SCCmec IVk | Antonie van Leeuwenhoek 94: 351–355. |
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