Shift in dominant hospital-associated methicillin-resistant Staphylococcus aureus (HA-MRSA) clones over time

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Objectives: The majority of HA-MRSA infections are caused by endogenous infection and by only a small number of clones. The reasons for the success of some clones over others are unknown.

Methods: We investigated the evolution of an MRSA population from a large, acute-care teaching hospital in London, UK over a 10 year period. MRSA incidence and antibiotic prescribing were correlated with changes in resistance genes and prevalence of clonal groups.

Results: Three clones caused the majority of infections, CC30 SCCmecII (EMRSA-16), CC22 SCCmecIV (EMRSA-15) and ST239 SCCmecIII. Clones that were multidrug resistant were selected for, and CC22 became dominant once it acquired a wide range of extra resistance genes. CC22 MRSA was also the fittest clone in an independent growth assay and a competition assay, and had a greater ability to survive desiccation. No individual isolate was fully drug resistant, and there was evidence of substantial horizontal gene transfer (HGT) as well as resistance gene loss within the clonal groups. The exception was fluoroquinolone resistance, which was rarely lost by any of the dominant hospital clones, suggesting that this resistance contributes to selection and survival of HA-MRSA. In support of this, a decrease in hospital-wide ciprofloxacin (a fluoroquinolone) prescribing was strongly associated with an overall decrease in MRSA infection.

Conclusion: Our data suggest successful HA-MRSA clones such as CC22 SCCmecIV are resistant to fluoroquinolones as well as fitter and able to acquire, but not necessarily accumulate, resistance to a wide range of additional antibiotics.

Keywords: antibiotic resistance, fitness, fluoroquinolones

Introduction

Staphylococcus aureus is a commensal bacterium in 25% of humans and is found predominantly in the nose, throat, armpit and groin, where it causes no harm.1 This opportunistic pathogen is a common cause of hospital-acquired infection (HAI). The major reservoir of infecting isolates is the patient’s own flora.2,3 The population structure of S. aureus in humans consists of about ten independently evolving lineages,4 and virtually all are resistant to penicillin due to the carriage of the bla gene cassette encoding β-lactamase. Methicillin-resistant S. aureus (MRSA) are prevalent in hospitals and have acquired the mecA gene on genetic cassettes called SCCmec.5 This renders them resistant to nearly all β-lactamase-resistant β-lactams, including methicillin, fluoroxacillin, carbapenems and cephalosporins.

In some cases, different SCCmec elements have moved into the same lineage, hence we define an MRSA clone as a lineage with a conserved SCCmec type. Isolates from the same clone share the same SCCmec type, core variable genes4 and restriction-modification system,5 but carry different sets of mobile genetic elements (MGEs) and therefore different combinations of antibiotic resistances. Different hospital-associated (HA) MRSA clones are successful in different countries or geographical locations.7,8 For example, the major clones in the UK for the last 15 years have been CC22 SCCmecIV (also known as UK EMRSA-15), and CC30 SCCmecII (also known as ST36 SCCmecII and UK EMRSA-16).9,10 MRSA were first described in 1961, and only rose above 2% of global S. aureus infections after the 1980s.11,12 Thus, methicillin resistance alone is not sufficient for success in hospitals. In the UK,
MRSA rapidly expanded, via clones CC30 and CC22, in the 1990s, to become responsible for 40% of all hospital S. aureus infections. This expansion was in addition to existing levels of methicillin-susceptible S. aureus (MSSA) infections. However, the reasons for the success of these HA-MRSA clones instead of earlier clones or other introduced HA-MRSA is currently unknown. Recently the clonal structure of the population of MRSA within the UK has shifted, and CC22 has become the dominant clone instead of CC30.

In addition to being resistant to β-lactamase-resistant β-lactams, MRSA are frequently resistant to other antibiotics prescribed in hospitals. Resistance to all antibiotic classes has been described in S. aureus, although resistance to the glycopeptides (vancomycin for instance) is still rare. There are two main routes to resistance: either a gene is acquired upon an MGE (e.g. β-lactam, aminoglycoside, erythromycin, clindamycin, tetracycline and chloramphenicol resistance) or it is due to point mutations within chromosomal genes (e.g. fluoroquinolone and rifampicin resistance). Some resistances can be due to either mechanism (e.g. fusidic acid, trimethoprim, co-trimoxazole and mupirocin resistance). Horizontal gene transfer (HGT) of resistance (both mutations and MGE) is likely to be mediated by bacteriophage through generalized transduction. Gain and loss of MGE has been reported in individual patients during the course of colonization or infection.

Few studies of MRSA have sought to examine the incidence of MRSA clonal types and their resistance profiles in the context of hospital-wide antibiotic prescribing and infection control. In this multidisciplinary study, firstly we phenotypically and molecularly characterized MRSA isolates collected over a 10 year period in a UK hospital, in order to uncover the clonal dynamics and the links between antibiotic resistance and clonal success. Secondly, we characterized and compared the fitness of the most successful clones. Our third task was to determine whether factors such as antibiotic prescribing and infection control measures contribute to the selection and success of HA-MRSA clones. Our study revealed novel and surprising explanations for the success of some clones, and strategies that may contribute to the control or reduction of MRSA infections.

Methods

Bacterial isolates

A random selection of MRSA isolates was taken from wound swabs and blood culture specimens submitted to the Medical Microbiology department at St George’s Healthcare NHS Trust (London, UK). This is a large, acute teaching hospital serving south-west London. The isolates from 1999 have been previously reported. The remaining isolates were from: November 2002 – April 2003 (‘2003’), July 2006, November 2008 and April – July 2009. Sample size did not correlate with MRSA incidence. All isolates were frozen as 20% glycerol stocks and confirmed as S. aureus by femB PCR. For the fitness studies, we also included two strains that have been fully sequenced, MRSA5096 (CC22) and MRSA252 (CC30) (sequences accessible at http://www.sanger.ac.uk/resources/downloads/bacteria/staphylococcus-aureus.html).

Antimicrobial susceptibility testing

Antibiograms were determined using the BSAC guidelines for antimicrobial susceptibility testing (version 7).
**Fitness measures**

Growth rates were calculated for all strains between all 3–7 h timepoints from independent growth (2–7 h timepoints from mixed growth) using \( \frac{\log_2(x_2/x_1)}{(t_2-t_1)} \), where \( x \) is the OD\(_{600}\) reading or colony forming units count at time \( t \) minutes from inoculum. Only positive values were considered. From each of the six replicates, an average measure for the lineage was taken. Additional measures were also included.

**Independent growth**

The ratio of CC30 and ST239 OD\(_{600}\) values to those of CC22 were calculated for 7 h growth.\(^{25}\)

**Mixed growth**

\( n_i(t) \) represents the population density at time \( t \) (h) from inoculation for lineage \( i \) (CC30 or CC22). The average hourly growth rate was calculated as \( \ln(n_i(24))/\ln(n_i(0))/24;^{26}\) a similar comparison was calculated for bacterial count after 7 h.

**Survival**

The percentages of the inoculum surviving until the final timepoints (24 or 120 h) were considered,\(^{27,28}\) as was the average death rate per day, evaluated by \( K = 2.3 \times (B_0 - B_i)/t \) where \( B_x \) is the log\(_{10}\) transformed population density at \( x \) days from inoculum.\(^{29,30}\)

**Statistical measures**

Comparisons of growth measures were performed using either Student’s two-tailed \( t \)-test or a one-way ANOVA test (for more than two lineages), followed by Tukey’s post hoc analysis test for multiple comparison (\( \alpha = 0.05 \)). If the variance between two samples was found to be significant in an \( F \) test of equality of variances, then Welch’s correction was used with a Student’s \( t \)-test. Fisher’s exact test was used to test for significant differences in levels of resistance between groups. An \( F \) test was also used to test whether the slope of a linear regression between the average number of resistances held by an isolate and time was significantly non-zero (GraphPad Prism 5).

**Incidence of MSSA and MRSA infections**

The Medical Microbiology database was searched for the numbers of blood culture or wound specimens reporting positive for MRSA or MSSA.

**Antibiotic use**

Monthly in-patient antibiotic consumption data were obtained from the hospital pharmacy computer system and converted to number of defined daily doses (DDDs), as defined by the WHO Collaborating Center for Drug Statistics Methodology in its Anatomical Therapeutic Chemical (WHO/ATC) (version 2007; http://www.whocc.no/ddd). These figures were then divided by the number of occupied bed days (OBDs) and are reported as DDDs/1000 OBDs.\(^{31,32}\) As mupirocin is a topical antibiotic with no DDD, levels of mupirocin use were calculated from prescription levels of tubes of ointment (2% mupirocin), where 3 g tubes were prescribed for nasal decolonization, and 15 g tubes for wound treatment.

Pearson’s correlation was used to evaluate the relationship between all antibiotics (DDD/1000 OBDs 2004–09) and MRSA incidence (as in Cook et al.).\(^{23}\)

**Results**

The dominant MRSA clone changed to CC22 over time

CC30 and CC22 clones were the most prevalent, but the dominance reversed from CC30 to CC22 after 2003 (Figure 1). This is in agreement with the nationwide picture.\(^{10,14}\) In 2003, a new clone, ST239 SCCmecIII, was detected; this was thought to have originated in Asia and was also responsible for an outbreak in another London hospital,\(^{32}\) but had almost disappeared by 2006.

Antibiotic resistance in MRSA

Resistance to all classes of antibiotics (except glycopeptides) was found for our isolates, and the distribution of resistances was highly variable (Figure 2). Of isolates belonging to the three dominant HA-MRSA clones (CC22, CC30, ST239) 99% were resistant to ciprofloxacin, while only 40% of non-dominant MRSA (from clones CC5, CC8, CC45, ST59, CC1 and CC51, see Figure 1) were resistant. The difference in proportion resistant to ciprofloxacin between dominant and non-dominant clones was significant by Fisher’s exact test (\( P < 0.001 \)). MSSA isolates were usually susceptible (data not shown). Ciprofloxacin resistance was relatively constant.

All other resistances showed evidence of loss and acquisition by isolates, resulting in a range of new antibiograms emerging each year with different combinations of antibiotic resistances. New antibiograms were found each year, and no particular antibiogram profile was selected for and maintained (Figure 2). Antibiotic resistances did not accumulate within individual isolates; instead, the ability to acquire and lose resistances appears to have been selected for.
Figure 2. Antibiograms for MRSA clonal groups. Resistance to 18 antibiotics was used to generate each isolate’s antibiogram, and variation in the antibiograms of MRSA from each clonal group for 1999–2009 is shown. CC22 acquired multiple antibiotic resistances by 2006. Antibiotic resistance patterns in CC22 and CC30 were highly variable, resistances did not accumulate in individual isolates, and multidrug-resistant isolates were not selected for. Black indicates phenotypic resistance, white susceptibility.
In 1999 and 2003, CC22 was resistant to ciprofloxacin, and many isolates were also resistant to erythromycin. But by 2006, 58% of CC22 isolates were additionally resistant to combinations of aminoglycosides, tetracycline, fusidic acid, trimethoprim and mupirocin. By 2008 chloramphenicol and gentamicin resistance had also appeared in some CC22 MRSA isolates. The number of CC22 isolates holding six or more phenotypic resistances differed significantly prior to, and after, 2006 (Fisher’s exact test, $P=0.0002$). This coincided with the expansion and dominance of CC22 over other MRSA clones (Figure 1). As mentioned above, although new resistances were acquired by CC22 in 2006, resistances had not accumulated within isolates by 2009 (Figure 2). This was confirmed by the slope of the linear regression between time and average number of antibiotic resistances held by each isolate being not significantly different from zero for CC22 after 2006 ($F$ value $=0.0007$, $P=0.984$).

A detailed molecular analysis of 24 of the same CC22 isolates from 2009 using a 62 strain whole-genome microarray$^{19}$ showed an enormous range of MGE variation in this clone; several isolates encoded resistance genes. This suggests frequent HGT of MGE into this clone, as well as frequent loss of MGE. The changes were substantial and the MGEs appear to be ‘shuffling’ between isolates. This is reflected in the phenotypic resistance data (Figure 2) and statistical analyses reported here, and is consistent with previous studies reporting the movement of MGEs within patients during colonization and infection.$^{18-20}$

CC30 also showed evidence of frequent acquisition and loss of multiple resistances, but within this clone it was occurring from 1999. CC30 isolates generally carried more resistances than CC22 isolates (Figure 2), but again these resistances did not accumulate in a single successful isolate; an $F$ test showed that the slope of the linear regression between time and average number of resistances was not significantly different from zero for CC30 isolates from 1999 to 2009 ($F$ value $=0.613$, $P=0.491$). ST239 was uniformly resistant to ciprofloxacin, tetracycline and trimethoprim, and did not acquire or lose any additional resistances. Other non-dominant MRSA clones were detected, but incidence was low and no other introduced clone expanded.

**CC22 was fitter than CC30**

We used *in vitro* models to compare the ability of CC22 and CC30 to grow independently in rich broth, to compete with each other for nutrients within the same culture, and to survive stress and desiccation (Figure 3). Surprisingly, the results clearly show that in independent growth CC22 had a shorter lag phase (adapted to rich conditions faster) and a higher growth rate than both CC30 and ST239 (Figure 3a; Table S2, available as Supplementary data at JAC Online). CC22 reached a statistically significantly lower OD$_{600}$ level after 24 h, which could have been due to elevated bacterial clumping. Clumping was not seen at earlier timepoints.

In the co-culture experiments between CC22 and CC30, the earlier and higher exponential growth rate led CC22 to outcompete CC30. Eight replicates of each of the same two isolates show this large variation (Figure 3b). In six of the eight replicates, the CC22 isolate dominated, while in the remaining two replicates the CC30 isolate and CC22 isolate grew to similar densities. Overall, the CC22 isolate had a higher average viable count after 7 h and a higher average growth rate, both of which were statistically significant (Table S3; available as Supplementary data at JAC Online). CC22 also survived desiccation better, giving it an advantage in a model mimicking survival on hospital surfaces (Table S4; available as Supplementary data at JAC Online). While we have not exhausted all growth conditions encountered by MRSA in the hospital, the fact that CC22 was dominant in all of the assays, including suboptimal survival conditions, suggests it has a significant fitness advantage.

**A shift in clone dominance did not correlate with a change in MRSA incidence**

The overall incidence of MRSA isolated from blood and wound specimens sent to the diagnostic microbiology laboratory decreased substantially in the middle of 2007 (Figure 4a). This did not correlate with the shift in dominant clone to CC22, which occurred prior to July 2006 (Figure 1).

**Changes in antibiotic prescribing over time**

The total amount of antibiotics prescribed at St George’s Healthcare NHS Trust changed very little over time (Figure S1; available as Supplementary data at JAC Online). The only substantial hospital-wide change occurred in 2007, when a policy of reduced prescribing of ciprofloxacin and cephalosporins was introduced. The timing of this decrease, as measured by antibiotics dispensed by the pharmacy in DDDs/1000 OBs, coincided very closely with the decrease in MRSA incidence in mid-2007 (Figure 4a). There was little overall change in the prescribing of β-lactamase-resistant β-lactams in total (Figure 4a) or of any other antibiotic (Figure S1) apart from ciprofloxacin. Over the period January 2004–June 2009, the strongest correlation between antibiotic use and MRSA incidence was found for ciprofloxacin ($r=−0.817$, 95% CI $−0.7173$ to $−0.8845$, $P<0.01$). Use of cephalosporins was also strongly correlated with MRSA incidence ($r=−0.689$, 95% CI $−0.5360$ to $−0.7978$, $P<0.01$). However, the decline in prescribing of cephalosporins was compensated for by an increase in the prescribing of other β-lactamase resistant β-lactams, such as co-amoxiclav and piperacillin/taxobactam (Figure 4a). This resulted in an overall negative correlation between this class of antibiotics (to which all MRSA are resistant) and MRSA infection incidence ($r=−0.293$, 95% CI $−0.4993$ to $−0.0543$, $P=0.017$).

Hospital clones of MRSA were nearly universally resistant to ciprofloxacin (a fluoroquinolone) and this resistance was not lost. A possible explanation is that the hospital-wide prescribing of antibiotics to which MRSA is universally resistant, such as ciprofloxacin and β-lactamase-resistant β-lactams, selects for MRSA in colonized patients. This selection would lead to a higher likelihood of endogenous infection. If so, this could link the decrease in prescribing of ciprofloxacin with the decline in MRSA infection incidence.

**Changes in infection control and management policies over time**

During 2005–09 a range of additional infection control policies was introduced into the hospital to combat MRSA and other HAIs, particularly *Clostridium difficile*. Many of these were
Successful HA-MRSA are fit and resistant

Figure 3. Fitness differences between MRSA clones. (a) Mean and SEM of six replicates of independent growth of seven CC22 MRSA strains (shown with blue filled squares), 5 CC30 MRSA strains (red filled triangles) and 4 ST239 MRSA strains (black filled circles). CC22 isolates grew significantly faster than other lineages as measured by maximal growth rate and OD600 values at 7 h \( P < 0.01 (***) \); see Table S2 available as Supplementary data at JAC Online, but tended to clump by 24 h. No fitness cost was associated with antibiogram data. (b) Co-culture growth of a single representative CC30 isolate and a single representative CC22 isolate from an initial 1:1 inoculum, plotted with blue filled squares and red filled triangles, respectively \( \text{n = 8 each} \). Eight replicates of each of the same two isolates are presented, to show the large variation. In six out of eight experiments, the CC22 isolate dominated. The differences in bacterial count after 7 h and average hourly growth rate were significant \( P < 0.02 (**); \) see Table S3 at JAC Online. (c) CC22 could be recovered at significantly higher levels than CC30 after desiccation (mean of five experiments and SEM), plotted with blue filled squares and red filled triangles, respectively. CC22 showed superior survival at both 24 and 120 h \( P < 0.05 (*) \) and a lower average daily death rate \( P < 0.02; \) see Table S4 at JAC Online). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
non-pathogen-specific measures, such as hand washing, education, hospital cleaning, behavioural changes and improved line-care. Our analysis does not allow us to conclude whether these contributed to the shift in dominant clone. However, these measures did not appear to be responsible for the decrease in MRSA, because the MSSA infection incidence did not decrease over the same time period (Figure 4a).

Specific infection control strategies to target MRSA were also introduced, in particular screening of patients for MRSA carriage and decolonization of positive patients, with mupirocin ointment to the nose and minor wounds and chlorhexidine washes. This is unlikely to be associated with the shift in dominant clones because resistance to mupirocin in CC30 isolates (36.2%) was much more common than in CC22 isolates (3.1%), and this policy should have selected for CC30. The increase in prescriptions of mupirocin began in 2006, and it also did not correlate with the decrease in MRSA incidence (Figure 4b). A policy to shorten the length of patient stay in hospital in 2005 also did not correlate with MRSA infection incidence (data not shown). While we cannot rule out the possibility that other unknown factors played a role in the decrease in MRSA incidence, our data suggest ciprofloxacin prescribing contributed to the selection of MRSA in hospitals leading to infection, and that successful MRSA have an advantage if they are ciprofloxacin resistant.

**Discussion**

Our data show that prescribing of all antibiotics in the hospital had an impact on the selection and survival of MRSA, which is predominantly a commensal organism. In 2009, 1.8% of all patients entering our hospital were colonized with MRSA at admission, and six-monthly snapshots of antibiotic use showed 30% of inpatients were receiving a wide range of antibiotics at any one time (data not shown). Therefore, the selective pressure on commensal MRSA to evade multiple antibiotics while colonizing patients is substantial, and this pressure along with their ability to acquire resistances is likely to give them an advantage over other commensals.

CC22 has become the dominant HA-MRSA clone in the UK. Surprisingly, our data show this is not linked to either a change in antibiotic use or to a decline in the incidence of MRSA. Instead, its dominance coincided with the acquisition of a range of new resistances. Prior to 2006 CC22 MRSA were relatively antibiotic susceptible (Figure 2), but from 2006 onwards, when they became dominant (Figure 1), isolates from this clone had gained a wide range of additional resistances (Figure 2). CC22 was also the fittest clone, which is likely to have given it an advantage when competing with other MRSA. We hypothesize that this higher relative fitness allowed the relatively antibiotic-susceptible CC22 MRSA to survive prior to 2006, and then to
Successful HA-MRSA are fit and resistant

outcompete CC30 (which was already relatively antibiotic resistant) once it gained additional resistances.

The difference in fitness between MRSA clones was unexpected. The reason for this fitness difference is currently unknown, although we can speculate that the SCCmecIV element in CC22 is less burdensome than the larger SCCmecII element in CC30. This fitness difference could be behind the success of CC22 both in the UK and in other European countries, Australasia and Asia, where it is often a dominant clone. As CC22 has adapted to become multidrug resistant in UK hospitals, it may have spread or further adapted elsewhere. This flexibility, in addition to the fitness, could allow rapid adaptation to different antibiotic pressures in environments with different antibiotic usage patterns (with or without prescription). Knowledge of the dominating clone and its fitness and antibiotic resistance profiles would be valuable in developing control strategies in different settings.

Ciprofloxacin resistance was significantly associated with successful, dominant HA-MRSA in our hospital, and was not lost by, or shuffled between, these isolates in the same manner as other resistances. Previous studies have shown that ciprofloxacin resistance is due to mutations in gyrA and grlA genes, which are located in a region of the chromosome separate to SCCmec. Therefore the frequent co-occurrence of methicillin and fluoroquinolone resistances is not due to resistances ‘hitch-hiking’ on the same MGE. The fact that fluoroquinolone resistance is very occasionally lost by MRSA isolates indicates these resistances are not physically linked within the cell.

Ciprofloxacin was licensed in 1987, and at that time the MRSA clones circulating at low levels in UK hospitals were susceptible to this antibiotic. CC22 and CC30 were resistant to fluoroquinolones when they were first described in the early 1990s, and this resistance could have promoted their rapid expansion. These MRSA did not replace the resident MSSA population but their emergence resulted in an additional burden in the number of S. aureus infections, consistent with the explanation that dominant MRSA would have been selected because of resistance to fluoroquinolones and not just methicillin. Non-dominant MRSA were still found, but over half of the non-dominant MRSA were susceptible to fluoroquinolones. The inability of community-associated MRSA clones such as USA300 (CC8) to establish in susceptible to fluoroquinolones. The situation in the USA appears to be changing, with reports of increasing levels of fluoroquinolone-resistant USA300 isolates in the UK. The situation in the USA appears to be changing, with reports of increasing levels of fluoroquinolone-resistant USA300 isolates in the community-associated USA300 clone adapting to cause hospital-acquired infection.

A decrease in MRSA prevalence was associated with a decrease in ciprofloxacin prescribing in this study, as has also been suggested from data in several previous studies, collated in a recent systematic review. Also, a recent US study, where electronic medical records were introduced into a hospital, highlighted a decrease in MRSA incidence specifically associated with a decrease in ciprofloxacin prescribing. We cannot discount that a decline in prescribing of cephalosporins also contributed to a decrease in MRSA incidence; however, this would imply that cephalosporins select for MRSA more effectively than other β-lactamase-resistant β-lactams. This should be explored further.

There is plausibility and precedents for changes in the prevalence of resistance to an antibiotic when the usage of that antibiotic is altered. We show here that the combination of antibiotic prescribing data and incidence of resistant bacteria can suggest important new strategies to combat infection. We speculate that a hospital-wide decrease in prescribing of all β-lactamase-resistant β-lactams, not just cephalosporins, could also result in a drop in MRSA infection incidence. However, a decrease in the prescribing of antibiotics to which MRSA regularly loses resistance is unlikely to affect MRSA incidence.

In conclusion, our data suggest that in order to be successful in our hospital, an HA-MRSA clone must be fit, resistant to fluoroquinolones and able to acquire and lose multiple other resistances. MRSA may continue to evolve and adapt to the altering selective environment in our hospital over the next few years, but unless a fitter clone appears, the dominant clone is likely to remain CC22. In the meantime, MRSA appear to rely on ciprofloxacin for selection in hospitals, and this Achilles heel could be exploited to reduce the incidence of MRSA infection.

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Transparency declarations

None to declare.

Supplementary data

Tables S1–S4 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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