Comparison of antibiotic resistance, virulence gene profiles, and pathogenicity of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* using a *Caenorhabditis elegans* infection model

Terissa Thompson, Paul D. Brown

Department of Basic Medical Sciences, University of the West Indies, Kingston, Jamaica

Objectives: This study compared the presence of 35 virulence genes, resistance phenotypes to 11 anti-staphylococcal antibiotics, and pathogenicity in methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA).

Methods: Multiplex PCR analysis was used to differentiate *Staphylococcus aureus* isolates ($n=102$) based on characterization of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Singleplex and multiplex PCR assays targeting 35 virulence determinants were used to analyze the virulence repertoire of *S. aureus*. *In vitro* activities of the antibiotics were determined by the disk-diffusion method. The pathogenicity of representative isolates was assessed using *Caenorhabditis elegans* survival assays. Significance in virulence distribution and antibiotic resistance phenotypes was assessed using the Chi-squared tests. Kaplan–Meier survival estimates were used to analyze nematode survival and significance of survival rates evaluated using the log-rank test.

Results: Except for *sei* (staphylococcal enterotoxin I) ($P = 0.027$), all other virulence genes were not significantly associated with MRSA. Resistance to clindamycin ($P = 0.03$), tetracycline ($P = 0.048$), trimethoprim/sulfamethoxazole ($P = 0.038$), and oxacillin ($P = 0.004$) was significantly associated with MRSA. Survival assay showed MSSA having a lower median lifespan of 3 days than MRSA that had a median lifespan of 6 days. The difference in the killing time of MRSA and MSSA was significant ($P < 0.001$).

Conclusion: While antibiotic resistance was significantly associated with MRSA, there was no preferential distribution of the virulence genes. The quicker killing potential of MSSA compared to MRSA suggests that carriage of virulence determinants *per se* does not determine pathogenicity in *S. aureus*. Pathogenicity is impacted by other factors, possibly antibiotic resistance.

Keywords: MRSA, MSSA, Antimicrobial resistance, Virulence, *Caenorhabditis elegans*

Introduction

Staphylococcus aureus is a commensal pathogen that has managed to retain its pathogenic significance worldwide as a major etiological agent of nosocomial and community acquired infections.¹ Its pathogenesis is multifactorial and includes a comprehensive suite of virulence determinants that includes enterotoxins, quorum sensing mediators, cell lysis mediators, immune system evasion factors, as well as an array of toxins that facilitate tissue destruction.²

Chemotherapeutic therapy using antibiotics is the current form of treatment for staphylococcal infections;

however, multi-drug-resistant strains that acquire resistance quickly upon exposure to antibiotics have been known to complicate *S. aureus* treatment.³ The introduction of methicillin as well as other beta-lactamase stable antibiotics into clinical practice in 1960 resulted in the isolation of the first methicillin resistant *S. aureus* strain in 1961.⁴ This resistance resulted from the acquisition of the *mecA* gene carried on a genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*) by a methicillin-susceptible strain (methicillin-susceptible *S. aureus*, MSSA). *mecA* encodes a modified penicillin-binding protein (PBP2a) that retains transpeptidase activity in the presence of concentrations of methicillin that inhibits the native penicillin binding proteins.⁵

Correspondence to: P. D. Brown, Department of Basic Medical Sciences, University of the West Indies, Mona, Kingston 7, Jamaica. Email: paul.brown@uwimona.edu.jm

While methicillin-resistant *S. aureus* (MRSA) is the typical etiological agent isolated from moderate to severe *S. aureus* infections, MSSA has also been isolated with increasing frequency.⁶ In the face of the debate concerning the role pathogenesis plays in MRSA infections, the isolation of MSSA from patients with moderate to severe *S. aureus* infections has added complexity and a new paradigm. It is therefore worthwhile examining the virulence and antimicrobial resistance potential of MRSA and MSSA populations since establishment of any difference between MRSA and MSSA will aid patient treatment outcome.

The *Caenorhabditis elegans* invertebrate model has been exploited for over four decades to investigate and model different biological processes, including host–pathogen relationships of *S. aureus*.⁷ These initial successes have led to more recent use of *C. elegans* for the discovery of potential anti-infectives against *S. aureus*.^{8,9} The objectives of this study were to compare the carriage of virulence determinants and antibiotic resistance phenotypes between MRSA and MSSA isolates and examine their virulence potential using the nematode, *C. elegans*.

Methods

Bacterial strains, culture conditions, DNA extraction, and species identification

A total of 102 non-duplicate *S. aureus* isolates obtained from hospitalized patients with skin and soft tissue infections in Jamaica were used in the study. Isolates were confirmed as *S. aureus* based on Gram and catalase positivity, DNase tests and the presence of the *nuc* gene. Isolates were inoculated in 1 ml of tryptic soy broth (EMD Millipore, Darmstadt Germany) and incubated overnight with agitation at 35°C. Overnight (O/N) grown culture (500 ml) was used to prepare glycerol stocks using the protocol proposed by Sambrook and Russell¹⁰ with modifications where 500 µl of sterile 30% glycerol was added to an equivalent amount of O/N bacterial culture. The mixture was vortexed and stored at –80°C. Genomic DNA was extracted from the remaining 500 µl of O/N culture using the Wizard Genomic DNA Kit (Promega, Madison, WI, USA) with modification. The cultures were incubated at 80°C for 30 minutes for the lysis step.

SCCmec fingerprinting

The SCCmec genomic element was characterized by a multiplex PCR strategy proposed by Milheirico, Oliveira and de Lencastre.¹¹ PCR reactions (50 µl) contained Gotaq Green Master mix (Promega) (consisting of 200 µM of each dNTP, 3 mM MgCl₂, 1 U Taq polymerase; pH 8.5) and 2 µl of genomic DNA as template. Amplifications were performed in a GeneAmp PCR System 9700 thermal cycler under the following PCR conditions: 94°C for 4 minutes, 30

cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute and a post-extension step of 72°C for 4 minutes. PCR products were resolved on ethidium bromide-stained 2.5% agarose gels.

Assessment of virulence profiles of *S. aureus* strains

Amplification of accessory gene regulator (*agr*) alleles 1–4 was conducted using a multiplex PCR strategy previously described by Lina *et al.*¹² The capsular polysaccharide types 5 and 8 (*cap5* and *cap8*) genes were amplified from genomic DNA preparations according to Verdier *et al.*¹³

S. aureus microbial surface components adhesive matrix molecules (MSCRAMMs) were identified as previously described by Tristan *et al.*,¹⁴ using genomic DNA as the template in two separate multiplex PCR reactions. PCR1 amplified *bbp*, *cam*, *ebpS*, and *eno*, while PCR2 amplified *fnbA*, *fnbB*, *fib*, *clfA*, and *clfB*.

The presence of toxin genes *sea-sei*, *seg-sek*, *seq*, *tst*, *eta*, *etb*, *luks-PV-lukF-PV*, *lukE-lukD*, *hly*, *hly2*, and *edin* encoding staphylococcal enterotoxins A–I, G–K, and K, toxic shock syndrome toxin 1, exfoliative toxins A and B, PVL components S and F, LUKE and LUKD, beta, gamma and gamma variant hemolysin, and EDIN, respectively, was analyzed by PCR as previously described by Lina *et al.*,¹⁵ with slight modifications. Each toxin gene was detected for in a singleplex PCR reaction. PCR products were assessed on ethidium bromide-stained agarose gels.

Antibiotic susceptibility tests

Antibiotic susceptibility tests were conducted using the disc diffusion method by the guidelines and breakpoints established by the Clinical and Laboratory Standards Institute (CLSI).¹⁶ Eleven anti-staphylococcal antibiotics were used: oxacillin (1 µg), clindamycin (2 µg), chloramphenicol (30 µg), teicoplanin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), erythromycin (15 µg), quinupristin/dalfopristin (15 µg), tetracycline (30 µg), mupirocin (200 µg), gentamicin (10 µg), and ciprofloxacin (5 µg).

C. elegans pathogenicity assay

C. elegans strain AU37 were grown on *Escherichia coli* OP50 and maintained at 15°C. *S. aureus* test isolates were selected based on a stratified random sampling method and grown at 35°C overnight. A 1 : 20 dilution of the standard culture was made using Tryptic Soy Broth. L4 Stage nematodes were prepared using the hypochlorite method as described by Kurz and Eubank.¹⁷ Triplicates of NGM agar plates were inoculated with 10 µl diluted culture, incubated for 6 hours, and then allowed to equilibrate to room temperature for 60 minutes before being seeded with 20–30 L4 stage nematodes. Seeded

Figure 1 Frequency and distribution of *agr* alleles between MSSA and MRSA.

plates were incubated at 25°C and scored for live and dead worms every 24 hours for 10 days.

Statistical analysis

Statistical analyses of the examined parameters were conducted using the Statistical Package for Social Science version 14. Significance was assessed using Chi-squared analysis where $P \leq 0.05$ was described as significant. Nematode survival assays was assessed using the Kaplan–Meier method and survival differences were tested using the log-rank test.

Results

Virulence genotyping

Chi-squared analysis of the presence of the four *agr* alleles (*agr1*, *agr2*, *agr3*, and *agr4*), two capsule polysaccharides (*cap5* and *cap8*) and the nine MSCRAMMs (*bbp*, *can*, *eno*, *ebps*, *fib*, *fnbB*, *fnbA*, *clfA*, and *clfB*), and five toxins (*tsst*, *lukED*, beta-hemolysin, gamma-hemolysin, and gamma-hemolysin variants) showed no preferential distribution to MRSA isolates (Figs. 1–4). Twenty-eight (28%) of the isolates were PCR-negative for the *agr* locus, while 2% was positive for multiple alleles. No isolate

was positive for *agr* II. There was no significant difference in the carriage of virulence determinants between isolates that were *agr*-positive and those that were PCR-negative for the *agr* locus.

Of the eleven enterotoxins assessed (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *she*, *sei*, *sej*, *sek*, *seq*), only *sei* was found to be significantly associated with MRSA isolates ($P = 0.027$) (Fig. 5). PVL components S and F, exfoliative toxins A and B, and EDIN were not found in this study.

Figure 6 shows a comparison of the activity of 13 antistaphylococcal antibiotics against MRSA and MSSA. For every antibiotic tested, except for erythromycin, frequencies of resistance were higher for MRSA than MSSA. Tetracycline (48%), oxacillin (39%), and ciprofloxacin (33%) had the highest resistance frequencies, while teicoplanin (4%) and chloramphenicol (15%) were the lowest. Antibiotic resistance was found to be significantly associated with MRSA with oxacillin ($P = 0.004$), trimethoprim/sulfamethoxazole ($P = 0.038$), tetracycline ($P = 0.048$), and clindamycin ($P = 0.03$).

C. elegans infectivity and lifespan assays (Fig. 7) showed $q = 0.9$ cumulative survival to be 2 days for

Figure 2 Frequency and distribution of capsular polysaccharide types 5 and 8 between MRSA and MSSA isolates.

both MRSA and MSSA. Median lifetime ($q = 0.5$) for MRSA was 6 days compared to 3 days for MSSA isolates indicating that MSSA isolates had a faster killing rate than MRSA. Log-rank tests corroborated the difference in median lifetime, showing a significant difference in the survival curves for MRSA and MSSA with $P < 0.001$.

Discussion

Infections caused by *S. aureus* (including MRSA and MSSA) have been problematic to treat taking into consideration the virulence capacity of *S. aureus* and its ability to acquire antibiotic resistance genes from its environment. While MRSA infections have been associated with increased morbidity and mortality,¹⁸ it is important to identify the genetic factors, if any that contributes to MSSA being implicated in moderate to severe *S. aureus* infections.

Selection and adaptation have over time exploited the genetic diversity within the bacteria populations to yield pathogenic strains that are host-specific and adapted to their environment. In the case of MSSA, it is yet unknown whether the acquisition of genes for

virulence, the loss of genetic determinants favoring commensalisms, or host/environmental factors have caused them to be pivotal in disease states normally associated with MRSA.

Given that *S. aureus*'s pathogenesis is a direct consequence of the complement of the virulence-associated genes that the organism possesses, we sought to assess similarities in the virulence repertoire of MRSA and MSSA isolates, 35 putative virulence genes were assessed by PCR, and their presence or absence was noted. There was no significant difference in the presence of any of the alleles with MRSA or MSSA. However, 28% of the isolates were PCR-negative for the *agr* locus, while 2% was positive for multiple alleles. No isolate was positive for *agr2*. The two clinically important *S. aureus* capsular polysaccharides type 5 and 8 were assessed in the current study. While there was no preferential association of the capsule polysaccharide with either MSSA or MRSA, 49% of the isolates were *cap5*-positive and 20.6% positive for *cap8*. Twenty-seven percent (27%) of the isolates were PCR-negative for both genes. There was no assessment carried out to identify any

Figure 3 Frequency and distribution of *S. aureus* MSCRAMMs between MRSA and MSSA isolates. bbp: bone sialoprotein-binding protein; can: collagen-binding protein; eno: laminin-binding protein; ebps: encoding elastin-binding protein; fnaA: fibronectin-binding protein A; fnaB: fibronectin-binding protein B; fib: fibrinogen-binding protein; clfA: clumping factor A; clfB: clumping factor B.

of the other 11 capsule types that had been previously described for *S. aureus* though not predominant among clinical isolates¹⁹ or surface antigen 336, a surface polysaccharide found to be present in most *cap5/cap8*-negative *S. aureus* isolates.^{13,20} It is therefore possible that those isolates that were PCR-negative for the *cap5* and *cap8* genes could be using any of the other eleven capsule types or antigen 336 to escape phagocytosis by cells of the innate immune system.

Enterotoxin gene *sei* was the only virulence gene that was found to be significantly associated with MRSA ($P = 0.027$). A study conducted by Sila *et al.*²¹ that compared the prevalence of 13 virulence genes in MRSA and MSSA in a Czech hospital also found *sei* to be significantly associated with MRSA along with *seg*. However, the significant association of *sei* with MRSA in the present study does not represent an accumulation of virulence genes in MRSA that would result in an increased virulence phenotype. The association of *sei* with MRSA in this

and other studies^{22,23,24} that showed high prevalence of *sei* and other genes from the enterotoxin gene cluster (*egc*) including *seg* warrants further investigation to elucidate the reason enterotoxins associated with the *egc* cluster have high prevalence, given that they are not food-borne isolates.

The findings suggest that in the case of the 35 virulence genes examined in the isolates, the complement of virulence genes in MRSA and MSSA is similar. The absence of a significant difference in the presence of virulence genes between MRSA and MSSA has been noted previously by Kocsis *et al.*²⁵ and and Rozgonyi *et al.*²⁶ The high level of clonality within the *S. aureus* population that reduces genetic exchange between lineages and the similarity of the background genetic information of MRSA, favors similarity of virulence genes composition between the two groups. Also, given that virulence genes found on accessory genetic elements have been found to be stably integrated into the core genome of *S. aureus*

Figure 4 Frequency and distribution of staphylococcal enterotoxins between MRSA and MSSA isolates. SE: staphylococcal enterotoxin A, B, C, D, E, G, H, I, J, K, and Q.

Figure 5 Frequency and distribution of *S. aureus* toxins between MRSA and MSSA isolates. tsst: toxic shock syndrome toxin; β-haemo: β-hemolysin; γ-haemo: γ-hemolysin; γ-var: γ-hemolysin variant.

Figure 6 Comparison of resistance rates of MRSA and MSSA to 13 antimicrobial agents. CIP: ciprofloxacin; GENT: gentamicin; MUP: mupirocin; TET: tetracycline; QUIN/DALFO: quinupristin/dalfopristin; ERYTH: erythromycin; SXT: trimethoprim/sulfamethoxazole; TEIC: teicoplanin; CLIND: clindamycin; CHLOR: chloramphenicol; OXA: oxacillin.

from all clonal lineages and geographical area, there is support for the results of this study that found that the 35 virulence-associated genes examined are not preferentially distributed within the *S. aureus* population. The lack of any assessment of host factors or strain origin (except basic information that isolates were from hospitalized patients with SSTIs) imposed some limitation on the study since such an assessment could have helped in explicating the presence of the virulence determinants.

Contrastingly, lifespan assays using *C. elegans* showed a significant difference ($p < 0.001$) in the killing potential of MSSA when compared to MRSA, with MSSA associated with a lower median lifespan of 3 days compared to 6 days for MRSA. Observational studies in support of the idea that patients with MRSA infections have worse clinical outcomes than those with MSSA^{27,28} have been published, which would suggest that in the present study, MRSA should be associated with a lower median lifespan than MSSA. It must be noted, however, that there are a myriad of factors, including differences in antibiotic treatment regimes, length of time before effective antibiotic treatment begins, as well as the relative fitness of the infective strain, which contribute to patient outcome.

Bacterial fitness is critical to any strain's survival in the environment. While the acquisition of resistance genes adds fitness and improves the bacteria's selective advantage in the presence of antibiotics, a fitness cost may be incurred in the absence of an antibiotic.^{29,30} Since this and other studies have shown that MRSA are usually multi-drug-resistant and are also significantly associated with antibiotic resistance, the carriage of antibiotic resistance genes in the absence of antibiotic pressure could account for the lowered pathogenicity seen in MRSA. Massey *et al.*³¹ showed that gentamicin resistant small colony variants had a lower fitness compared to the gentamicin-sensitive parental phenotype. It is therefore likely that antibiotic resistance lowered fitness and impacted the pathogenicity of MRSA.

Additionally, the effects of antibiotics on virulence expression may also impact MRSA's pathogenicity. With a low occurrence of correct first-time treatment, MRSA strains are more likely to be exposed to multiple antibiotics before successful treatment. While exposure to subcurative concentrations of certain antibiotics (including fluoroquinolones and beta-lactams) may increase the expression of certain virulence factors, others (for example, glycopeptides, clindamycin, and aminoglycosides) have been shown

Figure 7 Kaplan-Meier survival curves of *C. elegans* fed MRSA (green), MSSA (red), and *E. coli* OP50 strains.

to cause lowered induction or inhibition of several virulence genes.^{32,33} Further, Rudkin *et al.*³⁴ found that changes in the bacterial cell wall as a result of methicillin resistance led to an interference of the *agr*-mediated quorum sensing system that dictated lowered expression of toxin genes and decreased virulence in a murine model. Methicillin resistance could also alter pathogenicity *in vivo*. These examples of reduced virulence in conjunction with the associated reduced fitness caused by the carriage of antibiotic resistance genes are likely to play a part in the difference in pathogenicity between MRSA and MSSA when the lack of difference between the 35 virulence genes examined is taken into account.

Taken together, the lack of difference in the carriage of the 35 virulence genes examined in this study between MRSA and MSSA suggests that the virulence of MRSA is not responsible for the increased cost associated with higher mortality, longer hospital stays, and length and severity of infection as suggested by some authors.^{35,36} That, coupled with the lowered pathogenicity of MRSA in comparison to MSSA, suggests that other factors, possibly antibiotic resistance, are affecting MRSA pathogenicity.

Disclaimer Statements

Contributors PB conceived of and designed the study. TT carried out experimental work and spearheaded the data collection and analysis. Both contributed to manuscript preparation, final review and approved the final version.

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Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval Ethical approval was not required for this study.

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