Virulence characteristics of community-associated *Staphylococcus aureus* and *in vitro* activities of moxifloxacin alone and in combination against community-associated and healthcare-associated meticillin-resistant and -susceptible *S. aureus*

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The increasing prevalence of community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) poses a challenge for antimicrobial therapy of skin and soft tissue infections (SSTIs). To determine whether another antimicrobial agent might enhance the activity of moxifloxacin against CA-MRSA, this study analysed its activity alone and in checkerboard combination with doxycycline, rifampicin, clindamycin, trimethoprim, sulfamethoxazole/trimethoprim (SXT) and vancomycin against recent SSTI clinical isolates, and also characterized the isolates for Panton–Valentine leukocidin (PVL), *agr* groups, staphylococcal cassette chromosome *mec* (SCC*mec*) types and δ -haemolysin production. For comparison, 25 strains of outpatient meticillin-susceptible *S. aureus* (MSSA), 24 strains of healthcare-associated (HA)-MRSA and six historical strains of vancomycin-intermediate *S. aureus* (VISA) were included. It was found that 21/25 CA-MRSA strains tested were PVL-positive, SCC*mec* type 4 and *agr* type 1, whilst 4/25 were PVL-negative, SCC*mec* type 2 and *agr* type 2. Two of the *agr* type 2 strains were negative for δ -haemolysin but all other strains were positive. Moxifloxacin MIC_{50/90} values ($\mu\text{g ml}^{-1}$) were 1/8 for CA-MRSA, 4/32 for HA-MRSA and $\leq 0.03/1$ for MSSA and MIC₅₀ of 2 for VISA. The D-test for inducible clindamycin resistance was positive for 3/27 CA-MRSA, 5/14 HA-MRSA and none of the MSSA isolates. In checkerboard studies, fractional inhibitory concentration indices (FICIs) showed that most interactions were additive or indifferent (FICI value >0.5 to ≤ 2) as follows: rifampicin 43/52 strains, clindamycin 44/44, SXT 44/47, trimethoprim 41/42 and vancomycin 37/43. The FICI values for doxycycline were 3–6 for 32/34 strains, indicating antagonism, suggesting that it should not be used in combination with moxifloxacin.

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INTRODUCTION

Community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) has become known as the ‘most common identifiable cause’ of skin and soft tissue infection (SSTI) across the USA (Moran *et al.*, 2006) and has altered

the clinician’s antimicrobial selection. As there are no reliable, predictable patient characteristics for CA-MRSA infection, combination therapy is initiated empirically. Moxifloxacin is approved by the Food and Drug Administration and recommended by the Infectious Diseases Society of America (Stevens *et al.*, 2005) for SSTIs; therefore, combined use of moxifloxacin with an anti-MRSA agent is a potential clinical consideration. In order to select the most appropriate agents, we evaluated the activity of six drugs combined with moxifloxacin against recent clinical isolates of CA-MRSA obtained from 33 patients with community-associated SSTIs. For comparison, we also studied 24 strains of healthcare-associated

Abbreviations: CA, community-associated; FICI, fractional inhibitory concentration index; HA, healthcare-associated; MRSA, meticillin-resistant *Staphylococcus aureus*; MSSA, meticillin-susceptible *Staphylococcus aureus*; PVL, Panton–Valentine leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*; SSTI, skin and soft tissue infection; SXT, sulfamethoxazole/trimethoprim; VISA, vancomycin-intermediate *Staphylococcus aureus*.

(HA)-MRSA, 25 strains of methicillin-susceptible *S. aureus* (MSSA) and six vancomycin-intermediate *S. aureus* (VISA) strains.

Jarraud *et al.* (2002) noted that CA-MRSA SSTIs are potentially more serious than SSTIs caused by other strains and that virulence factors such as Panton–Valentine leukocidin (PVL) may be a link between phylogenetics and virulence. In addition, Sakoulas *et al.* (2005) noted that there are ‘sparse data’ about associations of *agr* type II CA-MRSA with ‘infections with particular infectious syndromes’. Consequently, we selected the first 25 of our 33 CA-MRSA isolates, and determined the virulence genes and expression of PVL, *SSCmec* type, *agr* type and δ -haemolysis production.

METHODS

Test strains included 33 consecutive isolates of MRSA and 25 isolates of MSSA recovered from SSTIs in outpatients between 30 September and 17 October 2005; 24 strains of HA-MRSA from blood, respiratory samples and wounds collected between 20 October and 5 December 2005; and 6 historical strains of VISA obtained from Professor K. Hiramatsu (Juntendo University, Tokyo, Japan).

Determination of MICs and checkerboard synergy studies were carried out in cation-adjusted Mueller–Hinton broth with moxifloxacin alone and in combination with doxycycline, rifampicin, clindamycin, trimethoprim, sulfamethoxazole/trimethoprim (SXT) and vancomycin. MICs without synergy studies were determined for levofloxacin and erythromycin (CLSI, 2006). Moxifloxacin concentrations were 0.03–4 $\mu\text{g ml}^{-1}$ plus each of the other agents at four or five clinically appropriate concentrations. The fractional inhibitory concentration index (FICI) was calculated for all combinations (Krogstad & Moellering, 1986). Erythromycin-resistant, clindamycin-susceptible strains were tested for inducible resistance by the D-test. For quality control, we used *S. aureus* ATCC 29213 and ATCC 43300.

Staphylococcal cassette chromosome *mec* (*SCCmec*) type was determined by multiplex PCR methods as described previously by Zhang *et al.* (2005). The PVL genes, *lukS-PV* and *lukF-PV*, were detected by PCR using primers described by Lina *et al.* (1999). Identification of *agr* group was accomplished using specific primers. Clinical *S. aureus* isolates were studied using PCR and *agr* group-specific primers as follows: *agr* group I, 5′-CACTTATCATCAAA-GAGCC-3′ and 5′-CCACTAATTATAGCTGG-3; *agr* group II, 5′-GTAGAGCCGTATTGATTCC-3′ and 5′-TATTCATCTCTTTAA-GG-3; *agr* group III, *agrC* of class III: 5′-CTGCATTTATAGT-GGAATACG-3′ and 5′-GTTTCATTTCTTTAAGAG-3; *agr* group IV, 5′-GCCGCGAGCTTGGGAG-3′ and 5′-GAAGATACGTGGCAAAC-TGGT-3′. After amplification for 30 cycles (30 s of denaturation at 94 °C, 30 s of annealing at 55 °C and 1 min of extension at 72 °C), the PCR products were resolved by electrophoresis through 1.5% agarose gels followed by ethidium bromide staining and analysis. *S. aureus* ATCC 49775 was used as a positive control strain for all PCR runs. Expression of the *agr* gene cluster was determined by δ -haemolysin production using a strain of *S. aureus* (RN4220) that produces a large zone of β -haemolysis without interference by α - or δ -haemolysins. Briefly, RN4220 was streaked vertically and test strains were streaked perpendicular to it on sheep blood agar plates. *S. aureus* strains RN6607 and RN9120 were used as positive and negative controls, respectively. Evidence for enhanced haemolysin production was examined at the intersection of growth of the two organisms (Sakoulas *et al.*, 2003).

RESULTS

The determined MIC and FICI values are summarized in Table 1. The moxifloxacin MIC_{50/90} values ($\mu\text{g ml}^{-1}$) were 1/8 for CA-MRSA, 4/32 for HA-MRSA and $\leq 0.03/1$ for MSSA, and MIC₅₀ of 2 for VISA. The D-test was positive for 3/27 (11%) CA-MRSA strains that were resistant to erythromycin but appeared to be susceptible to clindamycin, 5/14 (36%) of the HA-MRSA strains and none of the MSSA strains.

In checkerboard studies, FICI values were calculated and most interactions were additive or indifferent (FICI >0.5 to ≤ 2); however, the FICI values for doxycycline were 3–6 for 32/34 strains, indicating antagonism. Approximately 10% of moxifloxacin-resistant strains (MIC $>0.5 \mu\text{g ml}^{-1}$) exhibited a reduction in their MIC to $\leq 0.5 \mu\text{g ml}^{-1}$ with the addition of clindamycin and SXT and ~25% with the addition of vancomycin, rifampicin and trimethoprim alone. There was no reduction with the addition of doxycycline.

Of the 25 CA-MRSA strains tested, 21 (84%) were PVL-positive, *SSCmec* type 4 and *agr* type 1, suggesting a dominant geographical clone. Four out of 25 (16%) were PVL-negative, *SSCmec* type 2 and *agr* type 2. Two of these four were negative for δ -haemolysin production, but all other strains were positive.

DISCUSSION

The association of virulence factors and clinical conditions has been studied infrequently (Sakoulas *et al.*, 2005). Jarraud *et al.* (2002) examined 198 strains, but only four of these were from patients with cellulitis and/or myositis, with one from *agr* group I and three from *agr* group III. Vandenesch *et al.* (2003) studied 117 CA-MRSA isolates from various countries worldwide by PFGE and multilocus sequence typing and noted ‘distinct genetic backgrounds associated with each geographic origin’ that were not related to the genetic background of the HA-MRSA strains. They found that *SSCmec* IV and PVL loci were common to all locations and that, although 97% were *agr* type 3, those from the USA were generally *agr* type 1 (USA 300), which is in accord with our findings. Diep *et al.* (2006) studied the evolution of CA-MRSA isolates from San Francisco over 8.5 years and found that the current epidemic phenotype strain was associated with the horizontal acquisition of *SSCmec* IV, PVL genes and also enterotoxin Q and K genes. Recently, *S. aureus* was isolated from 76% of patients (320/422) with SSTIs who presented to 11 university-affiliated emergency departments. MRSA accounted for 59% (range 15–74%) of these isolates with USA 300 accounting for 97% and *SSCmec* type IV and PVL being present in 98% of CA-MRSA isolates (Moran *et al.*, 2006). This was slightly higher than in our results, suggesting some diversity in SSTIs.

Our *in vitro* data showed that moxifloxacin was two to four times more active than levofloxacin on a weight basis,

Table 1. *In vitro* activity of moxifloxacin and other antimicrobial agents alone and in combination, and their FICI values against MRSA, MSSA and VISA strains

Antibiotic (susceptible breakpoint, µg ml ⁻¹)	MIC (µg ml ⁻¹)			Susceptible (%)	D-test positive*	FICI		Moxifloxacin-resistant strains with moxifloxacin MIC ≤0.5 in chequerboard (%)
	Range	MIC ₅₀	MIC ₉₀			Range (no.)	Median	
CA-MRSA (33 isolates)					3/27			
Moxifloxacin (≤0.5)	≤0.03–32	1	8	15.2				
Doxycycline (≤4)	0.125–4	0.125	0.25	100		2–5 (20)	3	0
Trimethoprim (≤8)	0.5–4	2	2	100		0.5–3 (23)	1	28.6
SXT (≤2/38)	0.06–0.25	0.06	0.125	100		0.5–3 (26)	1	10.3
Clindamycin (≤0.5)	0.125–>128	0.125	>128	93.9		0.5–2 (25)	2	10.7
Erythromycin (≤0.5)	0.5–>128	128	>128	9.1		NT		
Rifampicin (≤1)	0.008–0.015	0.008	0.008	100		0.75–3 (23)	2	25
Vancomycin (≤2)	0.5–1	1	1	100		0.56–3 (23)	2	23.8
Levofloxacin (≤1)	≤0.5–>16	4	>16	15.1		NT		
HA-MRSA (24 isolates)					5/14			
Moxifloxacin (≤0.5)	1–32	4	32	0				
Doxycycline (≤4)	0.06–2	0.125	0.25	100		3–6 (11)	5	0
Trimethoprim (≤8)	0.5–4	2	4	100		0.31–3 (16)	2	19
SXT (≤2/38)	0.06–0.25	0.125	0.125	100		0.27–2 (18)	1	22.2
Clindamycin (≤0.5)	≤0.06–>128	0.125	>128	62.5		0.13–2 (14)	0.51	50
Erythromycin (≤0.5)	0.5–>128	>128	>128	0		NT		
Rifampicin (≤1)	0.008–0.03	0.008	0.015	100		1–3 (18)	2	4.2
Vancomycin (≤2)	0.5–1	1	1	100		0.75–3 (16)	2	4.2
Levofloxacin (≤1)	4–>16	>16	>16	0		NT		
MSSA (25 isolates)					0/4			
Moxifloxacin (≤0.5)	≤0.03–4	≤0.03	1	88				
Doxycycline (≤4)	0.125–2	0.25	0.25	100		3–5 (3)	3	0
Trimethoprim (≤8)	2–4	2		100		1–2 (3)	1	33.3
SXT (≤2/38)	0.125–>8	0.125	0.25	92		0.38–2 (3)	1.5	0
Clindamycin (≤0.5)	≤0.06–>128	0.125	0.25	96		1–2 (5)	1	0
Erythromycin (≤0.5)	0.5–>128	0.5	>128	76		NT		
Rifampicin (≤1)	0.008–0.015	0.008	0.015	100		1–2 (12)	1.5	0
Vancomycin (≤2)	≤0.03–1	1	1	100		2–2	2	0
Levofloxacin (≤1)	≤0.5–>16	≤0.5	4	88		NT		
VISA (6 isolates)					–			
Moxifloxacin (≤0.5)	1–4	2	–	0				
Doxycycline (≤4)	4–8	4	–	83		–	–	
Trimethoprim (≤8)	2–>16	4	–	50		2–2 (4)	2	
SXT (≤2/38)	0.125–>8	0.125	–	50		2–2 (4)	2	
Clindamycin (≤0.5)	>128–>128	>128	–	0		NT		
Erythromycin (≤0.5)	>128–>128	>128	–	0		NT		
Rifampicin (≤1)	0.008–>8	2	–	33.3		2–3 (6)	–	
Vancomycin (≤2)	1–8	4	–	33.3		NT		
Levofloxacin (≤1)	4–16	8	–	0		NT		

NT, Not tested.

*D-test for inducible clindamycin resistance.

which might be the result of a single mutation in *grlA* or *gyrA*, as postulated by Noguchi *et al.* (2005). As the time above the MIC is a key parameter in the killing of *S. aureus*, the protein binding of moxifloxacin versus levofloxacin (30–50 % vs 24–38 %) and the time above the MIC (moxifloxacin has a half-life of 10–14 h vs 7 h for levofloxacin) would give advantage to moxifloxacin (although one should only

consider free drug concentrations). Clinically, several studies have suggested the utility of a fluoroquinolone plus rifampicin for the therapy of MSSA infections. Zimmerli *et al.* (1998) treated 33 patients with ciprofloxacin plus rifampicin for orthopaedic prosthesis infections and had a 100 % cure rate in the experimental group compared with 58 % in the control group (ciprofloxacin plus placebo)

($P < 0.02$). Schrenzel *et al.* (2004) treated 104 patients with deep-seated *S. aureus* infections, mostly MSSA, and found that a fluoroquinolone plus rifampicin was effective in 78 % of cases. Our data support these studies and suggest that moxifloxacin plus rifampicin would be effective for MSSA infections.

In our study, 15.2 % of CA-MRSA isolates were susceptible to ≤ 0.5 μg moxifloxacin ml^{-1} and 63.6 % to 1.0 μg moxifloxacin ml^{-1} . It is important to note that there is disparity between Food and Drug Administration and CLSI suggested breakpoints, but, despite *in vitro* activity, clinically moxifloxacin is not indicated for use in any MRSA infection, and prudent practice would suggest the co-administration of a second agent if MRSA were suspected. In our study, up to 29 % of moxifloxacin-resistant isolates decreased their moxifloxacin MICs to ≤ 0.5 μg ml^{-1} with the addition of a second agent, especially trimethoprim alone.

We found that 100 % of our CA-MRSA isolates were susceptible to doxycycline, SXT, trimethoprim, vancomycin and rifampicin, and 93.9 % to clindamycin, but only 9.1 % to erythromycin. This is similar to the results of Moran *et al.* (2006), who reported that 95 % of their MRSA isolates were susceptible to clindamycin, 6 % to erythromycin, 60 % to fluoroquinolones, 100 % to SXT and 92 % to tetracyclines. In contrast, Buck *et al.* (2005) noted that 201/517 SSTI CA-MRSA isolates (39 %) from patients in Minnesota were susceptible to erythromycin and 433/517 (84 %) to ciprofloxacin, whilst susceptibility to other agents was more typical (clindamycin 92 %, rifampicin 99 %, tetracycline 91 % and SXT 99 %). However, they found that, unlike CA-MRSA isolates, only 77 % (44/57) of invasive HA-MRSA isolates were susceptible to clindamycin.

Differences between the susceptibilities of MRSA and MSSA to moxifloxacin were noted by Metzler *et al.* (2004), who found that moxifloxacin was more potent (MIC_{90} 0.06 mg l^{-1}) than levofloxacin (MIC_{90} 0.25 mg l^{-1}) against MSSA isolates, and this was also true at mutation-prevention concentrations (MPCs) (moxifloxacin 0.25 mg l^{-1} vs levofloxacin 1 mg l^{-1}); however, MIC_{90} and MPC_{90} were higher for both drugs against the MRSA isolates. Metzler *et al.* (2004) did not look at the comparative resistance prevention of moxifloxacin versus levofloxacin in combination with SXT or rifampicin. In our study, moxifloxacin $\text{MIC}_{50/90}$ values were lowest for MSSA, intermediate for CA-MRSA and highest for HA-MRSA; except for levofloxacin, the activity of the other agents against HA-MRSA was similar to that for CA-MRSA. In contrast, moxifloxacin was active against 23/25 (92 %) of our MSSA isolates at < 1 $\mu\text{g ml}^{-1}$ (range ≤ 0.03 –4); clindamycin and erythromycin were active against 96 and 76 %, respectively.

For the six VISA strains that we tested, the moxifloxacin MICs were 1–4 $\mu\text{g ml}^{-1}$. SXT was active against 50 %, doxycycline against 83 % and rifampicin against 33.3 % of

strains. All were clindamycin- and erythromycin-resistant. This suggests that the VISA strains are multidrug-resistant and that selection of antimicrobial therapy must be based on individual susceptibility testing.

Our study shows that most interactions with moxifloxacin against MRSA were either additive or indifferent ($\text{FICI} > 0.5$ to ≤ 2) for rifampicin, clindamycin, SXT, trimethoprim and vancomycin. Of note is the extremely high activity of rifampicin alone against all MRSA and MSSA isolates; future studies to determine whether or not moxifloxacin prevents the emergence of rifampicin-resistant *S. aureus* would be of interest. The FICI values for doxycycline were 3–6 for 32/34 strains, indicating antagonism and suggesting that this combination should be avoided.

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