J Antimicrob Chemother 2013; **68**: 2766–2772
doi:10.1093/jac/dkt286 Advance Access publication 8 August 2013

Journal of Antimicrobial Chemotherapy

Association of *norB* overexpression and fluoroquinolone resistance in clinical isolates of *Staphylococcus aureus* from Korea

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Received 4 February 2013; returned 27 March 2013; revised 4 May 2013; accepted 15 June 2013

Objectives: Although the prevalence of fluoroquinolone resistance among methicillin-resistant *Staphylococcus aureus* (MRSA) is known to be higher than in methicillin-susceptible *S. aureus* (MSSA), the reasons have never been identified.

Methods: We randomly selected 115 isolates of *S. aureus* collected from 10 different hospitals in Korea between June 2009 and May 2011. To investigate the difference in fluoroquinolone resistance mechanisms between MRSA and MSSA, we evaluated *gyrA* and *parC* mutations and the relative expression of the multidrug efflux pump genes *norA*, *norB* and *norC*.

Results: All 62 ciprofloxacin-resistant *S. aureus* had either *gyrA* or *parC* mutations. The S84L mutation of *gyrA* (59/62, 95.2%) and the S80F mutation of *parC* (61/62, 98.4%) were the most common. Fifty-eight (93.6%) strains had both the S84L mutation of *gyrA* and the S80F mutation of *parC*. Among the 115 isolates, *norB* overexpression was the most common, occurring in 49 (42.6%) strains. There were only two (1.7%) strains with *norA* overexpression and none with *norC* overexpression. Strains overexpressing *norB* were more common among ciprofloxacin-resistant *S. aureus* (33/62, 53.2%) than ciprofloxacin-susceptible *S. aureus* (16/53, 30.2%) ($P=0.013$). When we analysed 62 ciprofloxacin-resistant *S. aureus* strains, those overexpressing *norB* were more common in ciprofloxacin-resistant MRSA (28/46, 60.9%) than in ciprofloxacin-resistant MSSA (5/16, 31.3%) ($P=0.041$).

Conclusions: Increased expression of *norB* can be a factor that contributes to ciprofloxacin resistance in MRSA strains.

Keywords: efflux pumps, ciprofloxacin, *S. aureus*

Introduction

Fluoroquinolone resistance in *Staphylococcus aureus* has mainly been attributed to mutations occurring in the quinolone-resistance determining region (QRDR) of *parC*, encoding topoisomerase IV, and *gyrA*, encoding DNA gyrase A.[1] However, fluoroquinolone resistance can also be mediated by the chromosomally encoded multidrug resistance (MDR) efflux pumps NorA, NorB and NorC, which are widely present in different strains and are identified based on their ability to confer resistance to quinolones.[2–4] The contributions of active efflux mechanisms to resistance is less well defined, but can be additive with target alterations. Costa *et al.*[5] demonstrated that mutations in the QRDR confer resistance up to a certain level, above which resistance is mainly driven by efflux.

Ciprofloxacin resistance in methicillin-resistant *S. aureus* (MRSA) is common worldwide, but resistance in methicillin-susceptible *S. aureus* (MSSA) is substantially less common.[6–8] According to US data from 2010, the rate of ciprofloxacin resistance was only 14.3% among MSSA isolates, but >70% among MRSA isolates, with the overall prevalence of MRSA being 50.8%.[9] This phenomenon is similar in Korea. The rate of ciprofloxacin resistance among *S. aureus* was only 3.3% among MSSA, but 85.1% among MRSA.[10] The mechanistic reasons for this difference have not yet been elucidated.[1,6,11]

Although MRSA has been regarded as a nosocomial pathogen, new strains of MRSA causing serious infections have emerged in the community in recent years.[12] These community-associated (CA) MRSA isolates are more likely to be susceptible to antimicrobial agents than healthcare-associated (HA) MRSA, but differences in the mechanisms of resistance to fluoroquinolones have never been identified.[13]

This study therefore aimed to assess and characterize the patterns of fluoroquinolone resistance mechanisms in MRSA versus MSSA strains and CA versus HA strains among clinical isolates.

Materials and methods

Bacterial isolates and data collection

We randomly selected 115 non-duplicate clinical isolates of *S. aureus* collected from 10 different hospitals in Korea (Korean Invasive *Staphylococcus aureus* Infection Study Group; KOISA) between June 2009 and May 2011. Among them, 109 (94.8%) strains were isolated from blood, 4 (3.5%) from CSF and 2 (1.7%) from pleural fluid. Methicillin or oxacillin susceptibility data and clinical information were also collected. Seventy-one strains (61.7%) were MRSA and 44 (38.3%) were MSSA. The primary sites of infection were central venous catheters (22, 19.1%), skin and soft tissue (16, 13.9%), pneumonia (16, 13.9%), bone and joint (14, 12.2%), surgical site (6, 5.2%), urinary tract (4, 3.5%), intra-abdominal (2, 1.7%), CNS (2, 1.7%) and others (2, 1.7%). In 31 (27%) isolates the primary site of infection was not identified.

CA isolates were defined as those recovered from clinical cultures within 48 h of admission in patients lacking risk factors for MRSA acquisition.[12] Established risk factors included: (i) a history of hospitalization, surgery, dialysis or residence in a long-term care facility in the year before the date of culture; (ii) the presence of a permanent indwelling catheter or percutaneous medical device at the time of culture; or (iii) isolation of MRSA in the previous 6 months. The remaining isolates were deemed to be HA. According to this definition, 41 isolates (35.7%) were CA and 74 isolates (64.3%) were HA.

Antimicrobial susceptibility tests

MICs of ciprofloxacin (Sigma-Aldrich, St Louis, MO, USA) and moxifloxacin (LKT Laboratories, Minneapolis, MN, USA) were determined using the agar dilution method in accordance with CLSI guidelines with doubling drug dilutions ranging from 128 to 0.03 mg/L.[14] The breakpoints for resistance were those recommended by the CLSI guidelines. A ciprofloxacin MIC of 2 mg/L and a moxifloxacin MIC of 1 mg/L were regarded as intermediate, while a ciprofloxacin MIC of ≥4 mg/L and a moxifloxacin MIC of ≥2 mg/L were regarded as resistant. Intermediate strains were regarded as resistant.

Screening for mutations in gyrA and parC

For ciprofloxacin-resistant strains, PCR amplification of the QRDRs of *gyrA* and *parC* was carried out using the primers shown in Table 1.[15,16] All primers used in this study were synthesized at the Massachusetts General Hospital DNA Core Facility, Cambridge, MA, USA. Colonies were suspended in 50 μL of water in a microcentrifuge tube and boiled to prepare DNA templates for PCR. The PCRs were conducted by a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA). The amplification conditions were the same as previously described.[15] PCR-amplified DNA was purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced (Tufts University Core Facility, Boston, MA, USA). QRDR DNA sequences were compared with the sequences of *S. aureus* NCTC 8325 (GenBank accession numbers were: CP000253 for the complete genome; ABD29197.1 for GyrA; and ABD30448.1 for ParC).

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Bacteria were grown in Luria–Bertani broth to an optical density at 600 nm (OD_{600}) of 0.4–0.5. The pellets were resuspended in Tris-EDTA buffer containing 100 mg/L lysostaphin (Sigma-Aldrich) and incubated for 15 min at 37°C. RNA was extracted and purified using an RNeasy Mini Kit (Qiagen) and Turbo DNase I (Ambion Inc., Austin, TX, USA) following the directions provided by the manufacturers. cDNA synthesis was performed using the Verso cDNA Kit (Thermo Fisher Scientific, Rockford, IL, USA) with random hexamers, according to the manufacturer's instructions. qPCR was carried out with SsoFast EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), 1 μL each of 10 μM forward primer and reverse primer (Table 1)[17] and cDNA template in a 20 μL volume using a CFX96 qPCR detection system (Bio-Rad, Hercules, CA, USA). The amplification conditions were as follows: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 5 s at 55°C. Relative expression levels of *norA*, *norB* and *norC* were calculated using the ΔΔCt method, with the expression levels of *gmk* used as internal control. An increase of 4-fold or more compared with that for *S. aureus* SH1000 was considered indicative of overexpression as previously decribed.[18,19]

Effect of efflux pump inhibitor

To investigate the effect of the NorB efflux pump on fluoroquinolone resistance, we tested five control strains: SH1000, RN6390 (with and without vector pSK950), QT5′, a *norB* knockout mutant (RN6390 *norB*::*cat*), and RN6390 pQT8 (pSK950-*norB*), which overexpresses *norB* from plasmid

Table 1. Primers used in this study

Gene	Primer	Primer sequence (5′→3′)	Purpose	Product size (bp)	Comment
gyrA[15]	gyrA-F	AATGAACAAGGTATGACACC	PCR	223	
	gyrA-R	TACGCGCTTCAGTATAACGC	PCR		
parC[15]	parC-F	ACTTGAAGATGTTTTAGGTGAT	PCR	459	
	parC-R	TTAGGAAATCTTGATGGCAA	PCR		
gmk[17]	gmk-F	TCAGGACCATCTGGAGTAGGTAAAG	qRT–PCR	108	internal control
	gmk-R	TTCACGCATTTGACGTGTTG	qRT–PCR		
norA[17]	norA-F	GACATTTCACCAAGCCATCAA	qRT–PCR	102	target gene
	norA-R	TGCCATAAATCCACCAATCC	qRT–PCR		
norB[17]	norB-F	GCTACACCATCAACAGATACAGCAA	qRT–PCR	117	target gene
	norB-R	ACTCAATGCGACGCCAAA	qRT–PCR		
norC	norC-RT-F	TGGGTTGGAGATGGATTTTC	qRT–PCR	130	target gene
	norC-RT-R	ACAATTAGCCCTGCAACGTC	qRT–PCR		
spa[21]	spa-F	TAAAGACGATCCTTCAGTGAGC	PCR		
	spa-R	CAGCAGTAGTGCCGTTTGCTT	PCR		

pQT8.[4] We tested the MICs of moxifloxacin, a more selective substrate for NorB, by the broth microdilution method in the presence and absence of 20 mg/L reserpine (Sigma-Aldrich), a known inhibitor of several efflux pumps. Strains harbouring plasmid pSK950 and pQT8 were grown in the presence of tetracycline (5 mg/L) at 30°C. We also tested moxifloxacin MICs for four clinical strains with the highest level of *norB* transcripts (18- to 22-fold).

spa typing

To exclude the possibility of specific clonal spread, we determined *S. aureus* *spa* types as previously described.[20,21] The polymorphic X region of the protein A gene (*spa*) was amplified using the primers spa-F and spa-R as shown in Table 1. The amplification conditions were as follows: DNA was denatured at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60 s and extension at 72°C for 60 s, followed by a step of final extension at 72°C for 10 min. Purification and DNA sequencing were carried out using the same methods as for the *gyrA* and *parC* genes. The assignment of *spa* type was made employing a web-based application at http://fortinbras.us/cgi-bin/spatyper/spatyper.pl, which utilizes the Ridom numbering scheme as described at http://spa.ridom.de/index.shtml.[18]

Statistical analysis

The relationship between ciprofloxacin resistance and expression of efflux pump genes was assessed by the χ^2 test or Fisher's exact test, and a P value <0.05 (two-sided) was considered as statistically significant. All statistical analyses were done using SAS Enterprise Guide version 4.1 (SAS Institute Inc., Cary, NC, USA).

Results

Susceptibility to ciprofloxacin and moxifloxacin

Among 115 strains, 62 (53.9%) were ciprofloxacin resistant and 53 (46.1%) were ciprofloxacin susceptible. The distribution of strains according to ciprofloxacin or methicillin susceptibility and site of acquisition is detailed in Table 2. All 53 ciprofloxacin-susceptible strains were also susceptible to moxifloxacin, and 2 ciprofloxacin-resistant strains and 1 ciprofloxacin-intermediate strain were susceptible to moxifloxacin, with MICs of 0.25 and 0.125 mg/L, respectively. Among all 115 strains, the MIC_{50} and MIC_{90} (MICs for 50% and 90% of the isolates, respectively) of ciprofloxacin were 8-fold higher (16 and >128 mg/L, respectively) than those of moxifloxacin (2 and 16 mg/L, respectively) (data not shown). In MSSA strains, the MIC_{50} of both ciprofloxacin and moxifloxacin was 32–64 times lower than in MRSA (0.5 and 32 mg/L versus 0.125 and 4 mg/L, respectively) (Table 3). The MIC_{50} values in CA strains showed a similar pattern to those in HA strains (1 and 32 mg/L for ciprofloxacin, 0.125 and 4 mg/L for moxifloxacin, respectively).

Table 2. Distribution of 115 *S. aureus* isolates

	CIP-R ($n=62$)		CIP-S ($n=53$)		
	MRSA	MSSA	MRSA	MSSA	Total
CA	14	2	12	13	41
HA	32	14	13	15	74
Total	46	16	25	28	115

CIP-R, ciprofloxacin resistant; CIP-S, ciprofloxacin susceptible.

Table 3. MICs (mg/L) of ciprofloxacin and moxifloxacin for 115 *S. aureus* strains

	Ciprofloxacin			Moxifloxacin		
	MIC_{50}	MIC_{90}	range	MIC_{50}	MIC_{90}	range
MSSA	0.5	>128	0.25 to >128	0.125	8	≤0.03 to 128
MRSA	32	>128	0.25 to >128	4	32	0.06 to >128
CA	1	>128	0.25 to >128	0.125	8	≤0.03 to >128
HA	32	>128	0.25 to >128	4	16	≤0.03 to 128

Mutations in gyrA and parC in ciprofloxacin-resistant isolates

All 62 ciprofloxacin-resistant strains had either *gyrA* and/or *parC* mutations that caused changes in amino acids. *gyrA* and *parC* mutations were found in 59 (95.2%) and 61 (98.4%) strains, respectively. The S84L mutation of *gyrA* (59/62, 95.2%) and the S80F mutation of *parC* (61/62, 98.4%) were the most common (Table 4). Fifty-eight (93.6%) strains had both the S84L mutation for *gyrA* and the S80F mutation for *parC*. A single mutation in either *gyrA* or *parC* was only found in one strain and three strains, respectively. *gyrA* mutations other than S84L included S85P (9/62, 14.5%), E88K and A126T (1/62, 1.6%, respectively). *parC* mutations other than S80F included E84K (13/62, 21.0%), S81P (2/62, 3.2%), E84G, S108N, P114S and A116Q (1/62, 1.6%). Within *gyrA*, four single or combined alterations were found: S84L single, combination of both S84L and S85P or E88K, and combination of S84L, S85P and A126T. Within *parC*, seven single or combined alterations were found: S80F or A116Q single, and combination of S80F with one of E84K, E84G, S81P, S108N or P144S.

Expression of norA, norB and norC efflux pump genes

The number of strains that overexpressed each evaluated gene compared with *S. aureus* SH1000 is presented in Table 5. Among 115 *S. aureus* strains, the *norB* gene was found to be the most commonly overexpressed, occurring in 49 (42.6%) strains. Only two strains (1.7%) overexpressed *norA* and none overexpressed *norC*. One of the two strains overexpressing *norA* was a ciprofloxacin-resistant HA MRSA, and the other was a ciprofloxacin-resistant HA MSSA. The *norA*-overexpressing MRSA strain also showed increased expression of *norB*. *norB* overexpression was found most often in MRSA (33/49, 67.3%). When *norB*-overexpressing strains were analysed according to methicillin resistance, ciprofloxacin resistance and site of acquisition, *norB* overexpression was significantly more common in ciprofloxacin-resistant *S. aureus* (33/62, 53.2%) than ciprofloxacin-susceptible *S. aureus* (16/53, 30.2%) ($P=0.013$). However, there was no difference in *norB* overexpression between MRSA and MSSA ($P=0.286$) and only a trend in *norB* overexpression in HA versus CA strains

Table 4. QRDR mutations of 62 ciprofloxacin-resistant *S. aureus* strains

Genes and sites of mutation						
gyrA	*parC*	No. of strains, *n*=62	MRSA, *n*=46	MSSA, *n*=16	CA, *n*=16	HA, *n*=46
S84L	S80F	39 (62.9%)	32 (69.6%)	7 (43.8%)	10 (62.5%)	29 (63.0%)
S84L/S85P	S80F/E84K	7 (11.3%)	5 (10.9%)	2 (12.5%)	4 (25.0%)	3 (6.5%)
S84L	S80F/E84K	4 (6.5%)	4 (8.7%)	0	0	4 (8.7%)
S84L	S80F/E84G	2 (3.2%)	1 (2.2%)	1 (6.3%)	1 (6.3%)	1 (2.2%)
S84L	S80F/S81P	1 (1.6%)	1 (2.2%)	0	0	1 (2.2%)
S84L	S80F/S108N	1 (1.6%)	0	1 (6.3%)	0	1 (2.2%)
S84L	A116Q	1 (1.6%)	0	1 (6.3%)	0	1 (2.2%)
S84L/E88K	S80F/E84K	1 (1.6%)	1 (2.2%)	0	1 (6.3%)	0
S84L/S85P	S80F/S81P	1 (1.6%)	1 (2.2%)	0	0	1 (2.2%)
S84L/S85P/A126T	S80F/E84K	1 (1.6%)	1 (2.2%)	0	0	1 (2.2%)
S84L	—	1 (1.6%)	0	1 (6.3%)	0	1 (2.2%)
—	S80F	2 (3.2%)	0	2 (12.5%)	0	2 (4.3%)
—	S80F/P144S	1 (1.6%)	0	1 (6.3%)	0	1 (2.2%)

Table 5. Distribution of 115 clinical isolates of *S. aureus* overexpressing *norA* or *norB* efflux pump genes

	MRSA, *n*=71	MSSA, *n*=44	*P* value	CIP-R, *n*=62	CIP-S, *n*=53	*P* value	CA, *n*=41	HA, *n*=74	*P* value
norA	1 (1.4%)	1 (2.3%)	1.000	2 (3.2%)	0	0.499	0	2 (2.7%)	0.537
norB	33 (46.5%)	16 (36.4%)	0.286	33 (53.2%)	16 (30.2%)	0.013	13 (31.7%)	36 (48.6%)	0.078

CIP-R, ciprofloxacin resistant; CIP-S, ciprofloxacin susceptible.

Table 6. Comparison of strains overexpressing *norB* in subgroups of ciprofloxacin-resistant *S. aureus* and MRSA

	CIP-R *S. aureus* (*n*=62)			MRSA (*n*=71)		
	MRSA	MSSA	*P* value	CIP-R	CIP-S	*P* value
No. of *norB* overexpressers	28/46 (60.9%)	5/16 (31.3%)	0.041	28/46 (60.9%)	5/25 (20.0%)	0.001

CIP, ciprofloxacin; R, resistant; S, susceptible.

(*P*=0.078). When we analysed the 62 ciprofloxacin-resistant *S. aureus* strains, *norB* overexpression was more common among ciprofloxacin-resistant MRSA (28/46, 60.9%) than ciprofloxacin-resistant MSSA (5/16, 31.3%) (*P*=0.041) (Table 6). By contrast, there was no difference in the overexpression of *norB* between ciprofloxacin-susceptible MRSA (11/28, 39.3%) and MSSA (5/25, 20.0%) strains (*P*=0.127). When 71 MRSA strains were analysed, ciprofloxacin-resistant MRSA (28/46, 60.9%) were significantly associated with *norB* overexpression compared with ciprofloxacin-susceptible MRSA (5/25, 20.0%) (*P*=0.001). In 44 MSSA strains, ciprofloxacin-resistant MSSA (5/16, 31.3%) did not differ from ciprofloxacin-susceptible MSSA (11/28, 39.3%) in the overexpression of *norB* (*P*=0.594). Notably for moxifloxacin, which is a more selective substrate for NorB than ciprofloxacin, similar associations were found between moxifloxacin resistance and increased *norB* expression in MRSA strains [28/46 (60.9%) moxifloxacin-resistant versus 5/25 (20.0%) moxifloxacin-susceptible strains, *P*=0.001], but not in MSSA strains [4/13 (30.8%) moxifloxacin-resistant versus 12/31 (38.7%) moxifloxacin-susceptible strains, *P*=0.738].

Distribution of spa types

Among the 115 *S. aureus* strains, 26 *spa* types were found. The distribution of *spa* types is shown in Table 7. Overall, t2460 was the most commonly identified *spa* type (28, 24.3%) followed by t324 (11, 9.6%), t002 (8, 7.0%), t189 and t264 (6, 5.2%). Forty-nine *norB*-overexpressing strains were assigned to 17 *spa* types. The most prevalent *spa* type was t2460 (16/59, 27.1%), which was also the most common among strains not overexpressing *norB* (12/66, 18.2%). The majority (25/28, 89.3%) of all t2460 strains were MRSA. In the *norB*-overexpressing group, t189 and t264 (10.2% each) were the next most common, followed by t324 (15.2%) in the non-overexpressing group. Among the 66 strains

Table 7. *spa* types of 115 *S. aureus* strains

spa type	*norB*-overexpressing strains, *n* (%)	*norB* non-overexpressing strains, *n* (%)	Total, *n* (%)
t2460	16 (32.7)	12 (18.2)	28 (24.3)
t189	5 (10.2)	1 (1.5)	6 (5.2)
t264	5 (10.2)	1 (1.5)	6 (5.2)
t002	3 (6.1)	5 (7.6)	8 (7.0)
t601	2 (4.1)	1 (1.5)	3 (2.6)
t127	1 (2.0)	1 (1.5)	2 (1.7)
t324	1 (2.0)	10 (15.2)	11 (9.6)
t377	1 (2.0)	1 (1.5)	2 (1.7)
t688	1 (2.0)	1 (1.5)	2 (1.7)
t267	2 (4.1)	—	1 (0.9)
t2167	1 (2.0)	—	1 (0.9)
t3280	1 (2.0)	—	1 (0.9)
t1151	1 (2.0)	—	2 (1.7)
t019	1 (2.0)	—	1 (0.9)
t021	1 (2.0)	—	1 (0.9)
t251	1 (2.0)	—	1 (0.9)
t304	1 (2.0)	—	1 (0.9)
t037	—	3 (4.5)	3 (2.6)
t148	—	3 (4.5)	1 (0.9)
t664	—	2 (3.0)	3 (2.6)
t286	—	1 (1.5)	1 (0.9)
t342	—	1 (1.5)	1 (0.9)
t386	—	1 (1.5)	1 (0.9)
t128	—	1 (1.5)	2 (1.7)
t901	—	1 (1.5)	1 (0.9)
t2413	—	1 (1.5)	1 (0.9)
Novel[a]	5 (10.2)	17 (25.8)	22 (19.1)
Untypeable	—	2 (3.0)	2 (1.7)
Total	49 (100)	66 (100)	115 (100)

[a]No matched *spa* type in SpaServer (http://SpaServer.ridom.de); there were five different *spa* repeats for *norB*-overexpressing strains and 15 different *spa* repeats for non-overexpressing strains.

not overexpressing *norB*, 18 different *spa* types were identified. Nine *spa* types were identified in both *norB*-overexpressing and non-overexpressing groups, and the other nine *spa* types were found only in the *norB* non-overexpressing group. For 5 *norB*-overexpressing and 17 non-overexpressing strains, no matched *spa* types were found in SpaServer. These 22 strains had diverse numbers of *spa* repeats, with 5 different *spa* repeats among 5 *norB*-overexpressing strains, and 15 different *spa* repeats among 17 *norB* non-overexpressing strains. A diversity of strain types was thus found in both of the two groups of strains differing in *norB* expression.

Relationship of norB expression and the effect of reserpine on moxifloxacin MICs

Moxifloxacin is a substrate for NorB[4] and NorC,[2] but not NorA. In defined laboratory strains RN6390 (with and without vector plasmid pSK950), QT5′, a *norB* knockout of RN6390, and SH1000, the reference to which pump gene expression was compared, the MIC of moxifloxacin was 0.06 mg/L and unaffected by reserpine, an efflux pump inhibitor. By contrast, the MIC of moxifloxacin for RN6390 containing *norB* cloned in plasmid pSK950 was 0.25 mg/L and was reduced 4-fold in the presence of reserpine. In four *norB*-overexpressing clinical strains (numbers 21, 22, 26 and 31), the moxifloxacin MICs were 8–16 mg/L and were reduced 2- to 4-fold in the presence of reserpine. Thus, increased *norB* expression is associated with a reduction by reserpine of the moxifloxacin MIC and, in the absence of increased *norC* expression noted above, probably contributes to the moxifloxacin resistance phenotype.

Discussion

In *S. aureus*, the chromosomally encoded MDR efflux pumps NorA, NorB and NorC are widely present in different strains and were identified based on their ability to confer resistance to quinolones.[2–4] NorA is the most commonly studied, and its increased expression resulted in low to moderate increases in the MICs of fluoroquinolones to predispose to the appearance of high-level target-based resistance *in vitro*.[22] It has also been shown to play a role even in quinolone-susceptible strains, since *norA* disruption leads to a reduction in the MICs of ciprofloxacin.[23] In this study, *norB* was the most commonly overexpressed MDR pump gene in clinical isolates from Korea. *norA* overexpression was found in only two strains (3.2%) and *norC* overexpression was not seen. Although a previous study described the predominance of *norB* overexpression (25.4%) among a collection of *S. aureus* bloodstream isolates, there were also similar proportions of strains overexpressing *norA* (22.8%) and *norC* (16.7%) when using the same reference strain, SH1000.[19] In that study, when a single efflux pump gene was overexpressed, it was predominantly *norA*, whereas *norB* and *norC* were the most common when two or more efflux pump genes were overexpressed. By contrast, in our study, most clinical isolates that overexpressed efflux pump genes showed overexpression of a single gene, and only one isolate (0.9%) showed overexpression of both *norA* and *norB* genes. *norC* was the only gene for which no overexpression was detected among clinical isolates. Kosmidis *et al*.[18] reported that MDR pump gene overexpression in clinical isolates varied temporally and geographically. Notably in their report, the predominance of *norB*-only overexpression was also seen in strains from San Francisco, USA.

Our collection of isolates represented a diverse set of primary sites of infection. Since *norB* has been shown to contribute to bacterial fitness in murine subcutaneous abscess,[24] it is interesting to note that there was a trend towards increased *norB* expression in the subgroup of patients with primary skin and soft tissue (including surgical site) infections. In this subgroup, 13 of 22 (59.1%) overexpressed *norB* relative to 36 of 93 isolates (38.7%) from other primary sites of infection ($P=0.082$).

Although increased expression of *norB* was not confined to ciprofloxacin-resistant strains, we found that *norB* overexpression was more common in ciprofloxacin-resistant strains (53.2%) than ciprofloxacin-susceptible strains (30.2%) ($P=0.013$). Strains overexpressing *norB* were predominantly MRSA (33/49, 67.3%). A similar but non-significant trend was seen in another geographic area.[18] *norB* overexpression was more common in HA (36/74, 48.6%) than CA strains (13/41, 31.7%); however, the difference was not statistically significant ($P=0.078$). We also found a

higher rate of *norB* overexpression in ciprofloxacin-resistant MRSA (60.9%) than ciprofloxacin-resistant MSSA (31.3%) ($P=0.041$) and ciprofloxacin-resistant MRSA (60.9%) than ciprofloxacin-susceptible MRSA (20.0%) ($P=0.001$). Although the level of ciprofloxacin resistance indicates that multiple mutations contribute to the resistance phenotype, these results implicate *norB* overexpression as a contributing factor to ciprofloxacin resistance in clinical isolates of *S. aureus* and possibly to a higher prevalence of ciprofloxacin resistance in MRSA strains. The role of *norB* expression in the resistance phenotype is further supported by the finding that in defined laboratory strains and clinical strains that overexpressed *norB*, reserpine reduced the level of resistance to moxifloxacin, a quinolone that is a more selective substrate for NorB relative to other pumps.

Among *S. aureus*, the primary target for fluoroquinolones varies depending on the particular drug; first-step mutations selected for resistance to ciprofloxacin are found in *parC*, encoding the ParC subunit of topoisomerase IV, and first-step mutations selected with moxifloxacin occur in *gyrA*, encoding the A subunit of DNA gyrase.[25,26] Most clinical isolates with resistance to fluoroquinolones have had mutations in both *parC* and *gyrA*.[5,15] Notably, the substrate profile for NorB includes both ciprofloxacin and moxifloxacin, whereas that for NorA includes ciprofloxacin but not moxifloxacin.[4,27] In this study, four strains that showed a single mutation in *gyrA* or *parC* were all HA MSSA. Among these strains, three with a single *parC* mutation were ciprofloxacin resistant but moxifloxacin susceptible, and one of the three strains overexpressed *norB*. One strain with a *gyrA* single mutation that did not overexpress *norB* was resistant to both ciprofloxacin and moxifloxacin. In this study, all 62 ciprofloxacin-resistant *S. aureus* had either *gyrA* or *parC* mutations, and 58 (93.6%) strains had both the S84L mutation for *gyrA* and S80F for *parC*, known as common mutations in *gyrA* and *parC*.[28,29] The sites of mutations in *gyrA* and *parC* were similar to previous reports.[5,15,30,31] As all strains had either S84L mutations for *gyrA* and/or S80F for *parC*, there was no difference in QRDR mutations between MRSA and MSSA or CA and HA strains among ciprofloxacin-resistant *S. aureus*. The higher prevalence of fluoroquinolone resistance in MRSA relative to MSSA cannot therefore be attributed to different patterns of QRDR mutations.

To exclude the influence of clonal spread on the results of this study, we tested *spa* types for all 115 strains. The t2460 type was the most common among strains overexpressing *norB*, but it accounted for only 32.7% of the group, and it was also the most common type among the strains not overexpressing *norB* (18.2%). The majority (25/28, 89.3%) of all t2460 strains were MRSA. The two *norA*-overexpressing strains were both t002, and this finding corresponded to a previous report that showed t002 to be the most common *norA*-overexpressing *spa* type, regardless of geographic origin.[18] The low level of *norA* overexpression seen in our study may thus reflect a more diverse set of *spa* types studied. The distributions of major *spa* types are known to differ depending on the study period and geographic origin.[18,21] t2460 is thought to be one of the major *spa* types in Korea on the basis of the results of this and previous studies.[32,33] In this work, however, the *spa* types of clinical isolates were quite diverse, as >46 types (including novel types) and t2460 were found in strains both overexpressing and not overexpressing *norB*. Therefore, clonal spread of a single *norB*-overexpressing strain cannot account for the dominance of *norB* overexpression relative to that of *norA* and *norC*.

Increased expression of *norB* is thus significantly more common in ciprofloxacin-resistant MRSA than ciprofloxacin-resistant MSSA. To our knowledge, this study is the first to investigate the mechanism of higher ciprofloxacin resistance among MRSA than MSSA. This association could not be linked to differences in QRDR mutations or to clonal spread. The ability of NorB to provide a fitness advantage in *S. aureus* abscesses or its ability to confer reduced susceptibility to moxifloxacin and other fluoroquinolones are properties to consider as promoting its dominance among our patient population, but further studies are needed to strengthen these associations.

Acknowledgements

The *norB* knockout mutant QT5′ (RN6390 *norB*::cat) was obtained from Q. C. Truong-Bolduc. We are grateful to Sook-In Jung, Hee-Chang Jang (Chonnam National University Hospital), Kyung Hwa Park (Chunnam National University Hwason Hospital), Na Ra Yun, Dong-Min Kim (Chosun University College of Medicine), Chang-Seop Lee (Chonbuk National University Medical School), Jae Hoon Lee (Wonkwang University College of Medicine), Ae-Jung Huh, Yoon Seon Park (National Health Insurance Corporation Ilsan Hospital), Kkot Sil Lee (Kwandong University College of Medicine) and Seong Yeon Park (Dongguk University Ilsan Hospital) for collecting clinical data and strains.

Funding

This work was supported by the Inje Research and Scholarship Foundation in 2011 (to Y. G. K.) from Inje University College of Medicine, by a grant (to H. B. K. on behalf of KOISA) from the Korea Centers for Disease Control and Prevention, and by a grant R37-AI23988 (to D. C. H.) from the National Institutes of Health, US Public Health Service.

Transparency declarations

None to declare.

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