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Genotypic Characterization of Methicillin-resistant *Staphylococcus aureus* Strains Recovered from Pneumonia Clinical Trials for Ceftobiprole

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ABSTRACT

Background: This study characterized methicillin-resistant *S. aureus* (MRSA) responsible for hospital (HAP) and community-acquired pneumonia (CAP) collected during Phase 3 trials for ceftobiprole (BPR).

Methods: 56 MRSA were collected from subjects in European countries and Israel, Russia and Ukraine (7 countries, 30 [53.6%] strains), Asia (5 countries, 12 [21.4%] strains), USA (9 [16.1%] strains), Latin America (LA; 2 countries, 3 [5.4%] strains) and South Africa (SAF; 2 [3.6%] strains). One strain per subject collected during the first study visit (baseline) was included, except for 3 patients that had follow up isolates. PVL genes, and SCC*mec* and *agr* types were determined by PCR. Strains were subjected to PFGE and *spa* typing. Selected strains were evaluated by MLST. Clonal complexes (CCs) were assigned based on *spa* and/or MLST.

Results: The vast majority of MRSA (89.3%; 50/56) were CC5-MRSA-I/II/IV (42.9%; 24/56), CC8-MRSA-IV (28.6%; 16/56) or CC239-MRSA-III (17.9%; 10/56). Other clones detected were: CC30 (3.6%; 2/56), CC22 (1.8%; 1/56), CC45 (1.8%; 1/56), CC72 (1.8%; 1/56) and CC1 (1.8%; 1/56). Strains from Asia were CC5-MRSA-II (58.3%; 7/12) or CC239-MRSA-III (33.3%; 4/12), except for 1 strain from Korea (CC72-MRSA-IV). Isolates from EU were CC8-MRSA-IV (50.0%; 15/30), CC5-MRSA-I/II (23.3%; 7/30) or CC239-MRSA-III (20.0%; 6/30), and originated mostly from Russia, Hungary and Romania, respectively. USA strains were CC5-MRSA-II/IV (1 Pediatric clone and 6 USA100 strains; 77.8%; 7/9), and 1 strain each of CC8-MRSA-IV (USA300; 11.1%; 1/9) and CC45-MRSA-II (USA600; 11.1%; 1/9). LA and SAF isolates were CC5-MRSA-I and CC30-MRSA-II (USA200), respectively. PVL-positive strains were not observed, except for 1 USA300 (USA). Major clonal type strains had similar BPR MICs (median 1-2 μg/mL). No significant differences (i.e. $P < 0.05$) were noted in the MRSA lineage distribution between study arms and clonal types did not appear to associate with clinical outcome.

Conclusions: The geographical distribution of MRSA was reflective of the overall distribution of study patients, with >50% being of European origin. Most isolates responsible for HAP and CAP belonged to MRSA lineages responsible for nosocomial infections worldwide.

INTRODUCTION

Staphylococcus aureus remains a leading cause of human bacterial infections worldwide and the incidence of healthcare-associated and community-acquired infections caused by this organism has increased steadily. This species ranks as the main pathogen responsible for nosocomial bloodstream infections, skin and skin-structure infections (SSSI), community- (CAP) and hospital-acquired pneumonia (HAP), including ventilator-acquired pneumonia (VAP). Moreover, one in every five patients with CAP has an infection of a severity that requires hospitalization and antimicrobial treatment, and the mortality rate amongst these patients reaches 8%. In addition, many infections are caused by methicillin-resistant isolates (MRSA) and recent studies have demonstrated an increased MRSA incidence in the hospital and community settings over the last decade.

Ceftobiprole is a novel, broad-spectrum cephalosporin, which has been developed as an intravenous formulation. Ceftobiprole is a potent anti-MRSA compound likely due to its high affinity for the *S. aureus* penicillin-binding protein (PBP) type 2a. This compound has also demonstrated *in vitro* activity against the common bacterial pathogens responsible for pneumonia, including *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae* and extended-spectrum β-lactamase (ESBL)- and carbapenemase-negative *Klebsiella pneumoniae*. The objectives of this study were to characterize the MRSA isolates responsible for HAP and CAP infections collected during Phase 3 trials for ceftobiprole.

MATERIALS AND METHODS

Organisms. A total of 121 *S. aureus* clinical trial isolates collected from 91 subjects were forwarded to JMI Laboratories (North Liberty, Iowa, USA) for further characterization. These isolates originated from double-blind randomized phase 3 studies BAP00248 (36 strains) and BAP00307 (83 strains), together enrolling 781 patients with HAP and VAP, and from CAP-3001 (two strains), enrolling 638 hospitalized patients with CAP requiring i.v. therapy. Among these 91 patients, 20 subjects had multiple isolates. Only one isolate per patient was included in the analysis presented here and these strains were all recovered at the first study visit, except for five strains collected during follow up study visits. Therefore, 57 (42 and 15 from HAP and VAP infections, respectively), 25 (12 and 13 from HAP and VAP infections, respectively) and two (CAP) isolates from studies BAP00307, BAP00248 and CAP-3001, respectively, were part of the analysis.

SCC*mec* typing and detection of PVL genes. SCC*mec* types (I through VI) were characterized using a multiplex PCR strategy. Strains showing inconclusive SCC*mec* typing results were subjected to a secondary strategy proposed by Oliveira et al. (2006). PVL (*lukF-PV* and *lukS-PV*) screening was performed by using a multiplex Real-Time (RT) PCR approach, as previously described.

Epidemiologic typing of MRSA. Chromosomal DNA was subjected to pulsed-field gel electrophoresis (PFGE) after digestion with SmaI. Gel pattern analysis was performed using the GelCompar II software (Applied Math, Kortrijk, Belgium) and the patterns obtained compared to those of the major USA and international clones, which were provided by the Network on Antimicrobial Resistance in *S. aureus* (NARSA, www.narsa.net). All strains were subjected to *agr* and *spa* typing. Clonal complexes (CCs) were assigned based on the *spa* and/or multilocus sequence typing (MLST) results. MRSA strains with *spa* typing results previously associated with specific MLST in the MLST-mapping database (http://spa.ridom.de/mlst) or peer-reviewed publications had the CCs assigned accordingly. Strains with new *spa* typing denominations and unknown MLST associations, but clustering within PFGE types containing strains with known CC results, were assigned the same CCs. MLST was performed in a given strain when showing *spa* type with unknown MLST association and a unique PFGE type.

RESULTS

- MRSA strains will be referred herein according to the CC and *mecA* typing results. Therefore, CC5-MRSA-II indicates that a given strain or group of strains yielded *spa* type(s) associated with MLST results that belong to CC5 and carried SCC*mec* type II.
- **Table 1** describes the overall distribution of MRSA clones detected in this study. The majority of isolates were CC5-MRSA-I/II/IV (44.0%; 37/84), which were followed by CC8-MRSA-IV (22.6%; 19/84) and CC239-MRSA-III (21.4%; 18/84).
- The majority (68.8%; 11/16) of clinical trial strains collected from North America were CC5-MRSA-II/IV, *agr* 2 (**Table 2**). These CC5-MRSA-II/IV isolates grouped within the PFGE NA-D or -G, and the former pattern matched that of USA100 or Canadian MRSA-2. A single CC5-MRSA-IV isolate also clustering within NA-D harbored a SCC*mec* type IV, designated the Pediatric clone.
- Three CC8-MRSA-IV isolates harbored *agr* operon type 1 and were PVL-positive. These strains demonstrated PFGE profiles (NA-C) that matched that of USA300. One strain each of CC45-MRSA-II and CC12-MRSA-IV were detected in subjects from the USA. CC45-MRSA-II displayed a unique PFGE pattern (NA-B), which matched that of USA600 (**Table 2**).
- Isolates from Europe were mostly CC8-MRSA-IV (38.1%; 16/42), CC239-MRSA-III (23.8%; 10/42) or CC5-MRSA-I/II (26.2%; 11/42; **Table 2**). Other MRSA lineages were as follows: CC22-MRSA-IV (4.8%; 2/42), CC1-MRSA-IV (2.4%; 1/42), CC30-MRSA-II (2.4%; 1/42) and CC80-MRSA-IV (2.4%; 1/42; **Table 2**).
- CC8-MRSA-IV (*agr* 1) isolates were mostly from Russia (87.5%; 14/16) and one strain each from Ukraine and Lithuania. These strains clustered within six PFGE types, although a single *spa* type was observed (t008), except for one strain having a t008 variant (t2032, with the same t008 *spa* motif; **Table 2**).
- Isolates clustering within PFGE types EU-D and -E were CC239-MRSA-III, also known as the Brazilian/Hungarian clone, and originated from Romania (80.0%; 8/10) or Serbia and Montenegro (20.0%; 2/10; **Table 2**).
- European CC5-MRSA-II strains (t002/t003; *agr* 2) originated from Germany (two strains), Hungary (four strains) and Israel (one strain), and clustered within EU-G (85.7%; 6/7) or EU-H (14.3%; 1/7). The PFGE profile EU-G matched that of USA100, also known as New York/Japan or UK-eMRSA-3. Three CC5-MRSA-I strains from Hungary and Serbia and Montenegro clustered within *spa* t041, PFGE EU-N, *agr* 2 (South German MRSA; **Table 2**).

Table 1. Overall molecular characteristics of MRSA isolates (unique strains) recovered from patients enrolled in the pneumonia clinical trials.

Clonal complex	*agr* type	SCC*mec* type	PVL	No. (% of total)
CC5	2	I/II/IV	negative	37 (44.0)
CC5-MRSA-II	2	II	negative	26 (31.0)
CC5-MRSA-I	2	I	negative	10 (11.9)
CC5-MRSA-IV	2	IV	negative	1 (1.2)
CC8[a]	1	IV	negative/positive	19 (22.6)
CC239	1	III	negative	18 (21.4)
CC30	3	II	negative	3 (3.6)
CC22	1	IV	negative	2 (2.4)
CC1	3	IV	negative	1 (1.2)
CC12	2	IV	negative	1 (1.2)
CC45	1	II	negative	1 (1.2)
CC72	1	IV	negative	1 (1.2)
CC80	3	IV	positive	1 (1.2)

a. Three PVL-positive strains from the USA and displaying PFGE patterns similar to USA300.

- Two CC22-MRSA-IV isolates (*agr* 1) recovered from Spain and Germany clustered within EU-Q (PFGE profile similar to UK-eMRSA-15; **Table 2**). Other isolates observed were CC1-MRSA-IV from Romania (similar to USA400), CC30-MRSA-II from UK (USA200 or UK-eMRSA-16) and CC80-MRSA-IV from Romania, the epidemic PVL-positive European community-acquired MRSA strain.
- Overall, strains from the APAC region were either CC5-MRSA-II (47.1%; 8/17) or CC239-MRSA-III (47.1%; 8/17), except for a single strain from Korea, which was CC8-MRSA-IV (**Table 2**). CC5 strains originated from Korea, except for one strain from Taiwan, whereas CC239 originated from Australia (one isolate), Taiwan (two isolates), Thailand (two isolates) and China (three isolates).
- CC239-MRSA-III isolates clustered within three PFGE types (AS-A, -B and -D), were t037 (or associated *spa* types) and *agr* 1, features associated with the Hungarian/Brazilian clone. CC5-MRSA-II grouped within PFGE types AS-C and -E, were t002 or related *spa* types and *agr* 2, also known as the New York/Japan clone. The sole strain from South Korea with *spa* type t148, which has been associated with ST72 (CC72), displayed a PFGE profile indistinguishable from that of a representative of USA700 (**Table 2**).

Table 2. Overall epidemiologic data of unique MRSA isolates recovered during the pneumonia clinical trials.

Region/country[a] (no. tested)		No. (%)[b]	SCC*mec*	PVL	*agr*	PFGE	*spa*	CC
NA (16)	USA (14)	1 (6.3)	II	negative	1	NA-B[c]	t004	45
		3 (18.8)	IV	positive	1	NA-C[d]	t008	8
		8 (50.0)	II/IV	negative	2	NA-D[e]	t002/t242	5
		1 (6.3)	IV	negative	2	NA-E	t160	12
		1 (6.3)	II	negative	2	NA-G	t002	5
	Canada (2)	2 (12.5)	II	negative	2	NA-D[e]	t002	5
EU (42)	Germany (3)	2 (4.8)	II	negative	2	EU-G[g]	t003	5
		1 (2.4)	IV	negative	1	EU-Q[f]	t904	22
	Hungary (7)	3 (7.1)	II	negative	2	EU-G[g]	t002	5
		1 (2.4)	II	negative	2	EU-H	t002	5
		3 (7.1)	I	negative	2	EU-N	t041	5
	Israel (1)	1 (2.4)	II	negative	2	EU-G[g]	t002	5
	Lithuania (1)	1 (2.4)	IV	negative	1	EU-F	t008	8
	Romania (10)	1 (2.4)	IV	negative	3	EU-A[g]	t127	1
		4 (9.5)	III	negative	1	EU-D	t030	239
		4 (9.5)	III	negative	1	EU-E	t030	239
		1 (2.4)	IV	positive	3	EU-S	t044	80
	Russia (14)	1 (2.4)	IV	negative	1	EU-I	t008	8
		1 (2.4)	IV	negative	1	EU-K	t008	8
		1 (2.4)	IV	negative	1	EU-L	t008	8
		11 (26.2)	IV	negative	1	EU-M	t008/t2032	8
	Serbia and Montenegro (3)	2 (4.8)	III	negative	1	EU-E	t030, t632	239
		1 (2.4)	I	negative	2	EU-N	t041	5
	Spain (1)	1 (2.4)	IV	negative	1	EU-Q[f]	t717	22
	Ukraine (1)	1 (2.4)	IV	negative	1	EU-J	t008	8
	United Kingdom (1)	1 (2.4)	II	negative	3	EU-P[h]	t012	30
APAC (17)	Australia (1)	1 (5.9)	III	negative	1	AS-A	t037	239
	China (3)	3 (17.6)	III	negative	1	AS-D	t030	239
	Korea (8)	2 (11.8)	II	negative	2	AS-C	t002, t6267	5
		5 (11.8)	II	negative	2	AS-E	t2060	5
		1 (5.9)	IV	negative	1	AS-I[i]	t148	72
	Taiwan (3)	1 (5.9)	III	negative	1	AS-A	t037	239
		1 (5.9)	III	negative	1	AS-B	t037	239
		1 (5.9)	II	negative	2	AS-C	t214	5
	Thailand (2)	2 (11.8)	III	negative	1	AS-B	t037, t654	239
LA (7)	Argentina (6)	4 (57.1)	I	negative	2	LA-B[j]	t149	5
		2 (28.6)	I	negative	2	LA-C	t149	5
	Brazil (1)	1 (14.3)	II	negative	2	LA-A[e]	t002	5
SA (2)	South Africa (2)	2 (100.0)	II	negative	3	AF-A[h]	t012	30

a. APAC, Asia-Pacific region; EU, Europe (including Israel); SA, South Africa; NA, North America; and LA, Latin America.
b. Percentage within each region.
c. PFGE profile similar to USA600.
d. PFGE profile similar to USA300.
e. PFGE profile similar to USA100 or Pediatric clone (NA-D, CC5-MRSA-IV).
f. PFGE profile similar to UK-eMRSA-15.
g. PFGE profile similar to USA400.
h. PFGE profile similar to USA200.
i. PFGE profile similar to USA700.
j. PFGE profile similar to the Cordobes/Chilean clone.

- All isolates recovered from Latin America were CC5-MRSA-I (85.7%; 6/7) or -II (14.3%; 1/7). CC5-MRSA-I strains (Argentina) clustered within PFGE LA-B or -C, and the LA-B profile was associated to the Cordobes/Chilean clone. A single CC5-MRSA-II (Brazil) strain displayed a PFGE pattern similar to that of the USA100 prototype (**Table 2**).
- Two baseline isolates recovered from a subject in South Africa were CC30-MRSA-II (*agr* 3) showed a PFGE profile similar to the isolate originated from the UK (USA200 or UK-eMRSA-16) described above (**Table 2**).
- No major differences were noted between study arms, regardless of patient population analyzed (all pneumonia patients versus HAP and CAP only; **Table 3**). One exception was observed for the CC239-MRSA-III lineage, which was more common among MRSA in the comparator (25.0%) study arm than in the ceftobiprole arm (8.3%) in the HAP and CAP patient populations, although the *P* value (0.107) was not statistically significant (**Table 4**).

Table 3. Distribution of MRSA lineages between pneumonia clinical trial study arms.

	Number of strains (%) by study arm			
Clonal complex	Ceftobiprole	Comparator[b]	No. (% of total)	OR[a]
CC5	17 (45.9)	20 (42.6)	37 (44.0)	1.1 (0.5 - 2.7)
CC8	9 (24.3)	10 (21.3)	19 (22.6)	1.2 (0.4 - 3.3)
CC239	6 (16.2)	12 (25.5)	18 (21.4)	0.56 (0.2 - 1.7)
CC30	0 (0.0)	3 (6.4)	3 (3.6)	NC
CC22	1 (2.7)	1 (2.1)	2 (2.4)	NC
CC1	1 (2.7)	0 (0.0)	1 (1.2)	NC
CC12	0 (0.0)	1 (2.1)	1 (1.2)	NC
CC45	1 (2.7)	0 (0.0)	1 (1.2)	NC
CC72	1 (2.7)	0 (0.0)	1 (1.2)	NC
CC80	1 (2.7)	0 (0.0)	1 (1.2)	NC
Total	37 (44.0)	47 (56.0)	84 (100)	NC

a. Odds ratio and respective 95% CI refer to comparisons of rates for clonal complexes observed between study arms. All *P* values calculated by χ^2 test were >0.05. NC, not calculated.
b. Studies BAP00248 (36 strains) and BAP00307 (83 strains) had linezolid with or without ceftazidime in the comparator arm, while study CAP-3001 (two strains) had ceftriaxone with or without linezolid.

Table 4. Distribution of MRSA lineages between arms in the hospital and community-acquired pneumonia clinical trials (VAP strains were excluded).

	Number of strains (%) by study arm			
Clonal complex	Ceftobiprole	Comparator[b]	No. (% of total)	OR[a]
CC5	11 (45.8)	13 (40.6)	24 (42.9)	1.2 (0.4 - 3.6)
CC8	8 (33.3)	8 (25.0)	16 (28.6)	1.5 (0.5 - 4.8)
CC239[a]	2 (8.3)	8 (25.0)	10 (17.9)	0.3 (0.05 - 1.4)
CC30	0 (0.0)	2 (6.3)	2 (3.6)	NC
CC22	0 (0.0)	1 (3.1)	1 (1.8)	NC
CC1	1 (4.1)	0 (0.0)	1 (1.8)	NC
CC45	1 (4.1)	0 (0.0)	1 (1.8)	NC
CC72	1 (4.1)	0 (0.0)	1 (1.8)	NC
Total	24	32	56 (100)	NC

a. Odds ratio and respective 95% CI refer to comparisons of rates for clonal complexes observed between study arms. All *P* values calculated by χ^2 test were >0.05. NC, not calculated.
b. Studies BAP00248 (36 strains) and BAP00307 (83 strains) had linezolid with or without ceftazidime in the comparator arm, while study CAP-3001 (two strains) had ceftriaxone with or without linezolid.

CONCLUSIONS

- The majority of baseline isolates from the pneumonia trials for ceftobiprole belonged to CC5-MRSA-I/II/IV (44.0%; 37/84), followed by CC8-MRSA-IV (22.6%; 19/84) and CC239-MRSA-III (21.4%; 18/84). These are the most prevalent lineages of MRSA responsible for nosocomial infections worldwide. In addition, CC5 MRSA strains predominated in the Americas (68.8 - 100.0%) and this clone along with CC239-MRSA-III were equally prevalent in the APAC region.
- Overall, the clonal MRSA population within European countries and Israel demonstrated a greater genetic diversity, and isolates belonging to CC8-MRSA-IV (38.1%) prevailed. This lineage seems to be replacing previous commonly detected clones within Europe, such as ST247-MRSA-I (Iberian; CC8), ST228-MRSA-I (South German; CC5), ST239-MRSA-III (CC239; Brazilian/Hungarian), CC22-MRSA-IV (UK-eMRSA-15) and ST45-MRSA-IV (CC45; Berlin).
- Similar clonal MRSA distribution was observed among clinical trial isolates recovered from all pneumonia patients when compared with those from HAP and CAP patients only. In addition, no significant differences (i.e. $P < 0.05$) in the MRSA lineage distribution were observed between study arms (ceftobiprole versus linezolid/ceftazidime), regardless of patient population.

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