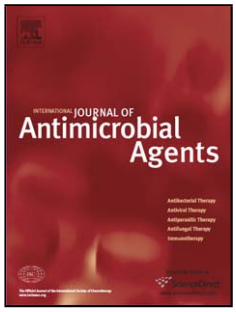
Molecular analysis and susceptibility patterns of meticillin-resistant (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones

Anna Marchese, Laura Gualco, Elisabetta Maioli, Eugenio Debbia



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Authors: Anna Marchese, Laura Gualco, Elisabetta Maioli, Eugenio Debbia

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Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones

Anna Marchese *, Laura Gualco, Elisabetta Maioli, Eugenio Debbia

Sezione di Microbiologia del DISCMIT, University of Genoa, Largo R. Benzi 10, 16132 Genoa, Italy

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* Corresponding author. Tel.: +39 010 353 7502; fax: +39 010 353 7651.

E-mail address: anna.marchese@unige.it (A. Marchese).

ABSTRACT

For many years meticillin-resistant *Staphylococcus aureus* (MRSA) has been considered a typical nosocomial pathogen. Recently, MRSA has emerged as a frequent cause of infections in the community. A multicentre surveillance study was carried out in the Ligurian area of Italy to evaluate the incidence, molecular nature and susceptibility patterns of MRSA strains circulating among outpatients. The genetic background of MRSA strains was analysed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Determination of antimicrobial susceptibility patterns, staphylococcal cassette chromosome *mec* (SCC*mec*) type, accessory gene regulator (*agr*) group and Panton–Valentine leukocidin (PVL) production was also performed. In total, 12 (6.4%) of 188 *S. aureus* isolates collected during 2006–2007 were found to be MRSA by phenotypic and genotypic methods. Analysis of isolates by PFGE showed that the majority of strains (11/12) belonged to two well known international clones (EMRSA-15 and USA300) and their variations. High variability regarding SCC*mec* IV subtypes, susceptibility patterns and PVL toxin production was found among members of the USA300 clonal group, even when displaying the same PFGE profiles. The remaining MRSA strain belonged to sequence type (ST) 8, *agr* group I and carried SCC*mec* type I. Both community-associated MRSA and healthcare-associated MRSA epidemic international clones circulate among outpatients in our region. It is alarming that members of the most represented clonal group in our collection (USA300) can acquire multiresistance as well as PVL genes. Infection control measures in our area should be improved to avoid the selection of microorganisms displaying both traits simultaneously as well as the spread of these epidemic international clones.

1. Introduction

Meticillin-resistant *Staphylococcus aureus* (MRSA) is a key nosocomial pathogen. However, in recent years MRSA has been seen with increasing frequency in the community worldwide [1]. It was assumed that community-associated (CA)-MRSA might have disseminated from the hospital to the community [2], although evidence has accumulated from molecular studies indicating that CA-MRSA and healthcare-associated (HA)-MRSA isolates have a number of distinct phenotypic and genotypic features [3–5]. CA-MRSA isolates have historically had the following traits: they carry smaller staphylococcal cassette chromosome *mec* (SCC*mec*) (i.e. types IV and V); they harbour genes encoding Panton–Valentine leukocidin (PVL) toxin; and they are susceptible to clindamycin and other non-β-lactam antibiotics [6]. Recently, CA-MRSA clones have been shown to cause infections in hospitalised patients, and HA-MRSA clones have caused infection in the general community [7–10]. As reported by other investigators, this suggests that clinical epidemiology is increasingly less useful in stratifying patients with community-acquired and hospital-acquired MRSA infection than molecular strain typing [11–13].

To our knowledge, no epidemiological surveillance studies in Italy have investigated the molecular nature of MRSA strains circulating in the community.

Given the high incidence of meticillin-resistant staphylococci circulating in Italian hospitals (>50%) [14], the aim of this study was to define the prevalence of MRSA among outpatients in the Ligurian area of Italy and to characterise the molecular profile of the strains isolated from these subjects.

2. Materials and methods

2.1. Bacterial isolates

This study was conducted during 2006–2007 with the collaboration of 11 clinical microbiology laboratories evenly distributed across the Ligurian area (see Acknowledgments).

Overall, 188 consecutive isolates of *S. aureus* from ambulatory outpatients were collected and sent to the co-ordinating laboratory (Sezione di Microbiologia del DISCMIT, University of Genoa, Italy). Strains isolated from hospitalised patients and residents in community long-term care facilities were excluded. Only one isolate per patient was collected and shipped to the co-ordinating laboratory. Participating laboratories also provided susceptibility data obtained by their routine method.

Clinical information regarding patient's risk factors for healthcare acquisition, such as recent hospitalisation and antimicrobial therapy, was not available.

At the co-ordinating laboratory, all isolates were subcultured onto trypticase soy agar, re-identified by biochemical tests (API Staph identification strip; bioMérieux, Marcy-l'Etoile, France) and coagulase production, and then stored at –70 °C.

2.2. Susceptibility tests

Minimum inhibitory concentrations of ciprofloxacin, erythromycin, clindamycin, trimethoprim/sulfamethoxazole (SXT), tetracycline, vancomycin, teicoplanin, daptomycin, quinupristin/dalfopristin (Q/D) and linezolid were determined by the broth microdilution method, whilst oxacillin, cefoxitin, chloramphenicol and gentamicin susceptibility was

determined using the disk diffusion test. For isolates identified as resistant to erythromycin but susceptible to clindamycin, a D-test was performed to detect inducible clindamycin resistance. All assays were performed in accordance with Clinical and Laboratory Standards Institute guidelines [15–17]. *Staphylococcus aureus* ATCC 29213 and 25923 were included as control strains. Antimicrobial multiresistance was defined as resistance to three or more different classes of non-β-lactam antimicrobials.

2.3. Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA was prepared and digested with *Sma*I endonuclease (New England Biolabs, Italian distributor Celbio S.r.l., Milan, Italy) and the DNA fragments were separated by PFGE with a CHEF-DRII apparatus (Bio-Rad Laboratories S.r.l., Milan, Italy) as previously described [18]. International reference strains (USA100, USA200, USA300-0114, USA-400, WA-1, WB49, WB81 and EMRSA-15 NCTC 13142) were included in the analysis [19–22]. The banding patterns were interpreted visually following published guidelines [23].

2.4. Sequence type (ST) determination

Multilocus sequence typing (MLST) was performed on all MRSA strains following the recommended procedure at the *S. aureus* MLST website (http://saureus.mlst.net/misc/info.asp).

2.5. Polymerase chain reaction (PCR)

Presence of the *mecA* gene was investigated by PCR using primers and conditions described by Oliveira and de Lencastre [24].

Multiplex PCR was performed to determine SCC*mec* types I to V [25]. If an SCC*mec* subtype could not be identified using this method, an additional PCR assay to distinguish SCC*mec* subtypes IVa–h was carried out using primers and PCR conditions described by Milheiriço et al. [26].

Detection of the PVL toxin genes *lukS-PV* and *lukF-PV* as well as accessory gene regulator (*agr*) typing were performed for all MRSA isolates [27,28].

3. Results

3.1. Identification of MRSA isolates

Agreement of species identification between participating laboratories and the co-ordinating laboratory was 100%.

Twelve (6.4%) of 188 *S. aureus* isolates were confirmed to be meticillin-resistant by the oxacillin and cefoxitin disk diffusion test and by *mecA* PCR at the co-ordinating laboratory.

Three strains categorised as meticillin-resistant by the local laboratories using the oxacillin disk diffusion test were not confirmed by the co-ordinating laboratory by both phenotypic (oxacillin and cefoxitin disk diffusion tests) and genotypic (*mecA* PCR) methods. On this basis, these three isolates were excluded from further characterisation.

Overall, confirmed MRSA strains were isolated from six of eleven participating laboratories.

3.2. Pulsed-field gel electrophoresis

Three different main profiles could be distinguished by PFGE (Table 1). Profile A was the same as that displayed by the MRSA USA300 reference isolate. Strains belonging to the profile designated A and its variations (8/12) came from all laboratories providing MRSA isolates. Strains belonging to PFGE profile B (the same profile as that displayed by EMRSA-15 reference isolate) and its variations (3/12) were isolated in two laboratories. Only one strain belonged to profile C.

3.3. Sequence typing

To assess more rigorously the phylogenetic relationships within this group of isolates, all strains underwent MLST analysis. The majority of strains (9/12), belonging to PFGE type C, A and A-related profiles, were found to belong to ST8. The remaining three strains (PFGE type B and B-related profiles) belonged to ST22.

3.4. PCR

SCC*mec* type IV was found in 11/12 isolates. The most common SCC*mec* subtype IV was SCC*mec* IVc (five strains), followed by SCC*mec* IVh (three strains), SCC*mec* IVb (two strains) and SCC*mec* IVa (one strain). SCC*mec* IVa, IVb and IVc were carried by strains belonging to clonal group A (USA300). Subtype SCC*mec* IVh was related to strains belonging to clonal group B (EMRSA-15). Only one strain carried SCC*mec* type I (PFGE profile C, ST8).

PVL was detected in only three isolates belonging to clonal group A. All strains belonged to *agr* group I.

3.5. Antimicrobial susceptibility

All isolates were susceptible to SXT, vancomycin, teicoplanin, linezolid, daptomycin and Q/D (Table 2). Two isolates (PFGE profile A, ST8, PVL+, SCC*mec* IVb) were susceptible to all antimicrobials tested. Erythromycin resistance was detected in the majority of isolates (9/12) as well as resistance to ciprofloxacin (8/12).

A D-test was performed on five isolates classified as clindamycin-susceptible and erythromycin-resistant by the microdilution method. Two of these five strains were positive for inducible clindamycin resistance.

Antimicrobial multiresistance was detected in 41.7% of the studied strains.

4. Discussion

Staphylococcus aureus, the most virulent *Staphylococcus* species, is also the most prevalent pathogen isolated from hospitalised patients and the second most common from patients in outpatient settings [29]. The main resistance problem related to this pathogen is resistance to meticillin and other β-lactams.

MRSA strains are typically associated with nosocomial infections, however emergence of MRSA in the community has become a major threat [30].

It is clear that the epidemiology of MRSA is continuously evolving, with CA-MRSA increasingly reported as causing hospital-associated infections and with the dissemination into the community of HA-MRSA strains, typically associated with nosocomial acquisition [12,31].

MRSA strains are globally spread but their prevalence and distribution in the hospital and in the community vary from country to country.

This study represents the first epidemiological survey in the Ligurian area to evaluate the incidence of MRSA strains circulating in the community and to characterise the genetic background of such strains.

Our data show that in our region, MRSA isolated from outpatients do not represent an uncommon finding (6.4%). In addition, the majority of these strains were also resistant to erythromycin and ciprofloxacin, posing a further threat.

The emergence of MRSA in our geographic area has not resulted from the dissemination of a single clone, but rather from the simultaneous circulation of two international clones (EMRSA-15 and USA300) and their variations. Only one strain, displaying typical nosocomial characteristics, SCC*mec* type I and multiresistance, had a profile not related to these two clones.

EMRSA-15 and USA300 are two well known international clones associated with hospital-acquired and community-acquired infections, respectively.

Staphylococcus aureus USA300 is the predominant CA-MRSA strain in the USA. This clone has recently spread to European countries and Japan [32,33].

At present, in Italy three cases of severe pneumonia, one case of skin and soft-tissue infection and one case of severe sepsis sustained by different CA-MRSA strains have

been documented [34–38]. In particular, a variant of the SWP clone, one strain belonging to ST88 (SCC*mec* V) and three strains belonging to ST8 (SCC*mec* IV) have been isolated as causative agents of the abovementioned infections.

To our knowledge, this is the first detailed description of the USA300 clone in Italy. Valentini et al. [38] classified one CA-MRSA causing severe pneumonia as USA300 on the basis of some characteristics (ST8, *spa* type 008, SCC*mec* IV and *agr* group I), but further tests to assess more rigorously the clonal origin of the strain (PFGE) and SCC*mec* subtyping were not carried out by these authors.

The fact that the great majority of our strains belong to the USA300 clonal group and that they can differ in virulence potential and antimicrobial susceptibility, even when indistinguishable by PFGE, is further confirmation of the great ability of this clone to spread and diversify [19].

EMRSA-15 emerged in the UK in 1991 and is still one of the most dominant epidemic clones in that country [39,40]. In Europe it was found in a number of hospitals throughout Germany and Portugal [41–43] and it is currently established as the major clone in the Czech Republic and in the Majorcan islands [44,45]. In Asia, EMRSA-15 represented 66.1% of all MRSA isolated in local hospitals in Singapore in the first half of 2006, replacing the ST239 clone island-wide [46]. The fact that in Italy MRSA belonging to ST22 have been detected (http://saureus.mlst.net), coupled with the evidence provided here that EMRSA-15 and its variants circulate in our community, suggest a possible more wide distribution of this clone in our country. Further local studies are needed to evaluate the incidence and distribution of this epidemic MRSA, both in the hospital and in the community setting.

It is alarming that irrespective of their origin (nosocomial or community-acquired) at present, different epidemic MRSA clones circulate among outpatients in our area. The spread of an epidemic clone able to acquire multiple antibiotic resistance as well as important virulence factors such as the PVL toxin is a further threat because the selection of strains combining both resistance and virulence treats simultaneously could occur in our area.

With the exception of SXT, the only antimicrobial agents active against all our strains were the so-called last resort antibiotics. However, these drugs are almost exclusively recommended for the treatment of hospital-acquired MRSA infections.

Better control of MRSA within the community setting is necessary to prevent dissemination of epidemic and/or multiresistant MRSA clones among outpatients in Italy.

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MRSA NCTC 10441 (SCC*mec* type I), MRSA N315 (SCC*mec* type II), MRSA 85/2082 (SCC*mec* type III), MRSA CA05 (SCC*mec* type IVa), MRSA 8/6-3P (SCC*mec* type IVb), MRSA MR 108 (SCC*mec* type IVc), MRSA JCSC 4469 (SCC*mec* type IVd) and MRSA WIS (SCC*mec* type V), and to Prof. Agnes Marie Sà Figueiredo, Instituto de Microbiologia, Centro de Ciências de Saúde (CCS), Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, for providing MRSAUSA100–400, MRSA WA-1, MRSA WB49 and MRSA WB81.

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Table 1

Clinical origin, antimicrobial resistance and molecular characteristics of meticillin-resistant *Staphylococcus aureus* (MRSA) isolated from outpatients in Liguria, Italy

Isolate no.	Laboratory no. (location)	Date of isolation	Sex/age (years) of patient	Clinical origin	Antimicrobial resistance markers [a]	SCC*mec* type	PVL toxin gene	*agr* group	PFGE type [b]	MLST type
35	2 (Savona)	07/02/2006	M/7	Pharynx swab	ERY-TET	IVa	+	I	A	ST8
99	3 (Genoa)	28/04/2006	n.a.	Urine	ERY-CIP-CLI	IVc	–	I	A5	ST8
24	5 (Genoa)	08/03/2006	F/87	Urine	ERY-CIP	IVh	–	I	B5	ST22
28	5 (Genoa)	17/03/2006	M/80	Urine	CIP	IVc	–	I	A3	ST8
29	5 (Genoa)	20/03/2006	M/71	Urine	ERY-CIP-CLI-GEN	I	–	I	C	ST8
160	7 (Genoa)	10/05/2006	F/31	Skin swab		IVb	+	I	A3	ST8
211	7 (Genoa)	21/10/2006	F/29	Skin swab		IVb	+	I	A3	ST8
216	7 (Genoa)	15/02/2007	F/32	Skin swab	ERY-CIP-CLI-CHL-GEN	IVc	–	I	A4	ST8
87	9 (Chiavari)	02/03/2006	F/72	Ear swab	ERY-CLI	IVh	–	I	B	ST22
151	9 (Chiavari)	13/04/2006	M/82	Urine	ERY-CIP-(CLI)	IVh	–	I	B2	ST22

85	9 (Chiavari)	01/03/2006	M/84	Skin swab	ERY-CIP-(CLI)-CHL-GEN	IVc	–	I	A5 [c]	ST8
101	10 (La Spezia)	25/03/2006	M/69	Skin swab	ERY-CIP	IVc	–	I	A4 [c]	ST8

SCC*mec*, staphylococcal cassette chromosome *mec*; PVL, Panton–Valentine leukocidin; *agr*, accessory gene regulator; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; n.a., not available; ERY, erythromycin; TET, tetracycline; CIP, ciprofloxacin; CLI, clindamycin; GEN, gentamicin; CHL, chloramphenicol.

[a] Inducible clindamycin resistance is reported in parenthesis.

[b] PFGE profiles A and B were identical to the reference strains MRSA USA300 and EMRSA-15, respectively [19–21].

[c] PFGE profiles of strains 101 and 85 differ by four and five bands from PFGE profile A, respectively, but they are not equal to profiles A4 (strain 216) and A5 (strain 99).

Table 2

Susceptibility profiles of the 12 meticillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from outpatients in Liguria

Isolate no.	MIC (μg/mL)										Other resistance markers
	ERY	CLI	CIP	SXT	TET	VAN	TEIC	LZD	DAP	Q/D	
35	>64	0.12	0.5	≤0.06	16	1	0.25	2	1	0.5	
99	>64	2	16	≤0.06	≤0.06	2	0.25	2	0.5	0.25	
24	16	0.12	64	≤0.06	≤0.06	2	0.5	2	1	0.5	
28	0.12	≤0.06	16	0.12	≤0.06	2	2	1	1	0.5	
29	>64	>64	64	0.12	≤0.06	1	4	2	1	1	GEN
160	0.5	0.12	0.25	≤0.06	≤0.06	2	0.25	2	0.5	0.25	
211	0.5	0.12	0.25	≤0.06	≤0.06	2	0.25	2	0.5	0.25	
216	>64	1	64	≤0.06	≤0.06	1	0.25	2	0.5	0.5	CHL-GEN
87	>64	2	0.25	≤0.06	≤0.06	1	0.12	1	0.5	≤0.06	
151	>64	0.25	64	≤0.06	≤0.06	2	0.12	2	0.5	0.5	CHL-GEN
85	>64	0.25	64	0.25	≤0.06	2	0.25	2	1	0.5	
101	16	0.12	16	≤0.06	≤0.06	2	0.25	2	0.25	0.5	

MIC, minimum inhibitory concentration; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; VAN, vancomycin; TEIC, teicoplanin; LZD, linezolid; DAP, daptomycin; Q/D, quinupristin/dalfopristin; GEN, gentamicin; CHL, chloramphenicol.