ORIGINAL ARTICLE BACTERIOLOGY

A variant of the Southern German clone of methicillin-resistant *Staphylococcus aureus* is predominant in Croatia

A. Budimir[1], R. H. Deurenberg[2], Z. Bošnjak[1], E. E. Stobberingh[2], H. Cetkovic[3] and S. Kalenic[1]

1) *Department of Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb, Croatia,* 2) *Department of Medical Microbiology, Maastricht University Medical Centre, Maastricht, The Netherlands and* 3) *Division of Molecular Biology, Rudjer Boskovic Institute, Zagreb, Croatia*

Abstract

The aim of the present study was to investigate the antibiotic susceptibility patterns and molecular epidemiology of clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolates recovered in 24 hospitals in 20 cities in Croatia from October to December 2004. A total of 1815 consecutive *S. aureus* isolates were recovered, 248 of which were MRSA. The MRSA isolates were analysed using *spa* typing, multilocus sequence typing and SCC*mec* typing. Furthermore, the presence of Panton–Valentine leukocidin (PVL) genes was determined as a genetic marker for community-associated MRSA. The MRSA prevalence was 14%. Ninety-six per cent of the MRSA isolates were resistant to ciprofloxacin, 95% to clindamycin and azithromycin, 94% to gentamicin, and 93% to erythromycin. The majority of the MRSA isolates (78%) was associated with the ST111-MRSA-I clone. In addition, various other endemic MRSA clones were observed, such as the ST247-MRSA-I (4%), the ST45-MRSA-IV (2%), the ST5-MRSA-I (2%), the ST239-MRSA-III (2%), the ST5-MRSA-II (1%), the ST8-MRSA-IV (1%) and the ST5-MRSA-IV (<1%) clones. Furthermore, we observed one PVL-negative ST80-MRSA-IV isolate. Four PVL-positive MRSA isolates were found, associated with ST8-MRSA-IV, ST80-MRSA-IV and ST80-MRSA-I. The ST111-MRSA-I clone was predominant in Croatia. Future surveillance studies of MRSA are important to elucidate whether changes in the clonal distribution of MRSA will occur, and if the minor endemic MRSA clones observed in the present study will replace the ST111-MRSA-I clone on a large scale.

Keywords: Croatia, MRSA, PVL, SCC*mec*, *spa* typing
Original Submission: 8 April 2009; **Revised Submission:** 10 August 2009; **Accepted:** 24 August 2009
Editor: G. Lina
Article published online: 2 September 2009
Clin Microbiol Infect 2010; **16**: 1077–1083
10.1111/j.1469-0691.2009.03042.x

Corresponding author and reprint requests: R. H. Deurenberg, Department of Medical Microbiology, Maastricht Infection Centre (MINC) University Hospital Maastricht, PO Box 5800, 6202 AZ Maastricht, The Netherlands
E-mail: ruud.deurenberg@mumc.nl

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most significant hospital-associated (HA) pathogens, causing a wide range of infections. The first report on MRSA appeared in 1960, and, since then, various HA-MRSA clones have disseminated worldwide [1]. The prevalence of MRSA bacteraemia varies from 1% in Norway to 67% in Japan [1,2].

In addition to HA infections, MRSA causes community-associated (CA) infections, especially skin and soft tissue infections and necrotizing pneumonia. The increased rate of CA-MRSA infections is a public health concern, and a challenge for infection control, as MRSA is observed in nursing homes, kindergartens and schools. However, studies on CA-MRSA prevalence are rare. HA-MRSA and CA-MRSA can be distinguished on the basis of a combination of the genetic background, the staphylococcal cassette chromosome *mec* (SCC*mec*) and the presence of Panton-Valentine leukocidin (PVL) [1].

Strategies to control the transmission of MRSA require knowledge about the prevalence, nature and number of MRSA clones [3]. In Croatia, antibiotic resistance surveillance studies for relevant clinical bacteria are performed and the results are published yearly (http://www.amzh.hr/eng/index-eng.htm). However, only limited information on the nationwide spread of MRSA clones in Croatian hospitals is available [4].

The aim of the present study was to investigate the prevalence and the molecular epidemiology of MRSA isolated from ambulatory and hospitalized patients in Croatia during a 3-month period in 2004, using SCC*mec* typing, multilocus

sequence typing (MLST) and *spa* typing. PVL was detected as a marker for CA-MRSA [5].

Materials and Methods

Clinical isolates

From October to December 2004, 24 hospitals, with 104–1724 beds (mean 490 beds), in 20 Croatian cities collected 1815 consecutive clinical *S. aureus* isolates, ranging from six to 224 (mean 56) isolates per hospital. One isolate per patient was included in the study. The isolates were sent to the Clinical Hospital Centre Zagreb, Croatia for molecular analyses. All MRSA isolates were sent to the Maastricht University Medical Centre, The Netherlands, for *spa* typing.

Antimicrobial susceptibility testing

Susceptibility testing was performed according to the guidelines of the CLSI [6], using the microbroth dilution method for the following antibiotics: penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, azithromycin, gentamicin, amikacin, tetracycline, rifampicin, trimethoprim–sulphamethoxazole, linezolid, ciprofloxacin and vancomycin. Susceptibility testing for fusidic acid and mupirocin was performed according to the guidelines of the French Society of Microbiology, using the disk diffusion method [7]. The oxacillin-resistant isolates were tested for the presence of the *mecA* gene [8].

SCC*mec* typing

SCC*mec* typing was performed using the multiplex PCR described by Oliveira *et al.* [9]. In addition, *ccr* typing was performed according to the method of Ito *et al.* [10,11], using modified primer β2 [12].

Typing of the *spa* locus

Real-time amplification of the *spa* locus, followed by sequencing of the short sequence repeat region, was performed as described previously [13]. The *spa* types were clustered into *spa* clonal complexes (CCs), using the algorithm based upon repeat pattern (BURP) with Ridom StaphType 1.4 software (http://www.ridom.de). As it has been shown that *spa* typing/BURP data are in accordance with results obtained using MLST and pulsed-field gel electrophoresis, the putative MLST CC was allocated through the Ridom SpaServer (http://spaserver.ridom.de) [14,15].

MLST analyses

To confirm the association between MLST and *spa* typing/BURP results, MLST was performed for representative isolates of each major *spa* type [12,16].

Detection of PVL

The detection of the genes coding for PVL was performed as described previously by Lina *et al.* [17].

Results

MRSA prevalence

During the 3-month study period, 248 MRSA isolates were recovered, giving a prevalence of 14%. The MRSA isolates were from various body sites, i.e. 109 (44%) from wound infections, 39 (16%) from tracheal aspirates, 14 (6%) from nasal swabs, 13 (5%) from bloodstream infections, 12 (5%) from urinary tract infections, ten (4%) from central venous catheters and 51 (21%) from various other body sites. The MRSA prevalence varied among hospitals, from 2% in Split to 95% in Zagreb. In the three small county hospitals of Čakovec, Nasice and Ogulin (117, 150 and 351 beds, respectively) and the Clinical Hospital Centre Rijeka (a 1191-bed tertiary-care hospital), no MRSA isolate was detected during the study. The MRSA prevalence was related neither to the number of beds per hospital, nor to a specific geographical area, nor to hospital infection control policy, nor to the patient population. The absence of wards for orthopaedic, plastic, cardiac and vascular surgery could contribute to the low MRSA prevalence in the latter four hospitals.

Antimicrobial susceptibility patterns

All MRSA isolates were resistant to the β-lactam antibiotics tested, i.e. penicillin, oxacillin and cefoxitin, and all isolates were susceptible to linezolid and vancomycin. Ninety-six per cent of the MRSA isolates were resistant to ciprofloxacin, 95% to clindamycin and azithromycin, 94% to gentamicin, 93% to erythromycin, 10% to rifampicin, 9% to mupirocin and to tetracycline, 8% to amikacin, 3% to trimethoprim–sulphamethoxazole and 0.4% to fusidic acid.

Distribution of SCC*mec* elements

The most prevalent SCC*mec* element was type I (n = 224; 90%), followed by SCC*mec* type IV (n = 15; 6%), SCC*mec* type II (n = 5; 2%) and SCC*mec* type III (n = 4; 2%). Six of the SCC*mec* elements, four SCC*mec* type IV and two SCC*mec* type III, could not be typed with the method of Oliveira *et al.* [9] but could be typed with the method of Ito *et al.* [10].

The four non-typeable SCC*mec* type IV elements all harboured *ccrAB2*, and lacked *mecI* and the *dcs* region. However, one element harboured the *pls* region, which is characteristic for SCC*mec* type I, and another harboured locus F, which is characteristic for SCC*mec* type III. The two non-typeable SCC*mec* type III elements harboured *mecA* and loci C, D, E

and F. Therefore, they differ from SCC*mec* type III with regard to the presence of locus D, the *dcs* region.

Distribution of *spa* CCs and MLST analyses

Thirty-one *spa* types were found among the 248 MRSA isolates, and were grouped into four *spa* CCs and eight singletons. Two isolates could not be *spa* typed (Table 1). One main *spa* CC, *spa* CC 041, comprising 82% of the isolates, was associated with MLST CC5. Within MLST CC5, 13 new *spa* types were found (Table 1). The distribution of the *spa* types among *spa* CC 041 is presented in Table 2. In addition, three *spa* CCs with no founder, associated with MLST CC45, CC239 and CC8, were observed, accounting for 4%, 2% and 9% of the isolates, respectively (Table 1). Of the eight isolates that were classified as singletons, two were associated with MLST CC1 and four with MLST CC80. MLST analyses confirmed the association between *spa* CC and MLST CC (Table 1).

Distribution of MRSA clones

The combination of the genetic background and SCC*mec* was used to define the MRSA clones in Croatia [16]. The majority of the MRSA isolates (n = 193; 78%) were associated with a single-locus variant (ST111-MRSA-I) of the Southern German (ST228-MRSA-I) clone. Other MRSA clones observed were the Iberian (ST247-MRSA-I) clone (n = 11; 4%), the Berlin (ST45-MRSA-IV) clone (n = 5; 2%), the UK EMRSA-3 (ST5-MRSA-I) clone (n = 5; 2%), the Brazilian/Hungarian (ST239-MRSA-III) clone (n = 4; 2%), the New York/Japan (ST5-MRSA-Ii) clone (n = 3; 1%), the UK EMRSA-2/-6 (ST8-MRSA-IV) clone (n = 3; 1%) and the Paediatric (ST5-MRSA-IV) clone (n = 1; <1%). Figure 1 shows the distribution of the MRSA clones in Croatia.

Nine isolates could not be associated with endemic MRSA clones: four isolates (2%), observed in Pula, Split and Vukovar, were associated with ST45-MRSA-I, and single isolates were associated with ST1-MRSA-I, ST1-MRSA-IV, ST228-MRSA-II, ST239-MRSA-I and ST80-MRSA-IV. The PVL-negative ST80-MRSA-IV isolate did not harbour collagen adhesion or toxic shock syndrome toxin-1 [4]. Six isolates harbouring SCC*mec* type I and associated with MLST CC8 (*spa* type t008) could not be associated with a particular MRSA clone, but they were most likely associated with the Archaic or the Iberian clone. Four of the 248 MRSA isolates could not be related to an MRSA clone, either because the *spa* type was not related to an MRSA clone, or because they could not be *spa* typed.

Prevalence of PVL and distribution of CA-MRSA clones

Four of the 248 MRSA isolates (1.6%) harboured PVL; three of these harboured SCC*mec* type IV, and the other harboured SCC*mec* type I. These isolates had a genetic background associated with CA-MRSA. One isolate had *spa* type t008, associated with MLST CC8, and three isolates had *spa* type t044, associated with MLST CC80. Of the four PVL-positive MRSA isolates, one was from the Dubrovnik area and associated with ST8-MRSA-IV, two were from the Split and Zadar area and associated with the ST80-MRSA-IV (European CA-MRSA) clone, and one was from Bjelovar and associated with ST80-MRSA-I.

Discussion

To control the MRSA transmission in and among hospitals, it is important to study the prevalence, nature and number of MRSA clones present. This study was performed to

TABLE 1. Composition of the *spa* clonal complexes (CC)s

spa CC	No. (%) of isolates	No. (%) of *spa* types	*spa* types[a]	ST[b]	Putative CC
spa CC[c] 041	203 (82)	21 (68)	**t001**, t003, **t041**, t811, t892, t1003, t1185, t2521, t2625, t2687, t2688, t2692, t2746, t2766, t2822, t2823, t2824, t2825, t2827, t2836, t2837	111, 1410[d]	5
No founder 2	9 (4)	2 (6)	**t015**, t2682	45	45
No founder 3	5 (2)	2 (6)	t030, t037		239
No founder 4	21 (8)	2 (6)	**t008**, **t051**	8, 247[e]	8
Singletons	8 (3)	4 (13)	**t044**, t127, t1605, t2608	80	1, 80[f]
NT	2 (1)				
Total	248 (100)		31 (100)		

MLST, multilocus sequence typing; NT, not typeable; ST, sequence type.
[a]Bold type indicates *spa* types for which MLST analysis was performed. The new *spa* types are underlined.
[b]As determined with MLST.
[c]As determined with MLST and eBURST.
[d]Two isolates *spa* typed as t003 and t041 had ST111, a single-locus variant of ST228, at the *pta* locus. A second isolate *spa* typed as t041 had ST1410 (MLST profile 1-4-1-8-46-24-29), a double-locus variant of ST228, at the *gmk* locus and *pta* locus.
[e]One isolate *spa* typed as t008 had ST8, and one isolate *spa* typed as t051 had ST247.
[f]*spa* type t044 is associated with CC80, and *spa* type t127 is associated with CC1.

TABLE 2. Composition of *spa* clonal complex (CC) 041

spa type	No. (%) of isolates
t001	34 (17)
t003	7 (3)
t041	117 (58)
t811	1 (0.5)
t892	9 (4)
t1003	3 (1)
t1185	1 (0.5)
t2521	1 (0.5)
t2625	1 (0.5)
t2687	1 (0.5)
t2688	13 (6)
t2692	4 (2)
t2746	2 (1)
t2766	1 (0.5)
t2822	2 (1)
t2823	1 (0.5)
t2824	1 (0.5)
t2825	1 (0.5)
t2827	1 (0.5)
t2836	1 (0.5)
t2837	1 (0.5)
Total	203 (100)

investigate the antibiotic susceptibility patterns and the population structure of MRSA in Croatia. The majority of the MRSA isolates were associated with a variant of the Southern German clone. Furthermore, MRSA isolates associated with the Berlin, UK EMRSA-3, Brazilian/Hungarian, New York/Japan, UK EMRSA-2/-6 and Paediatric clones were observed. Four PVL-positive MRSA isolates were likewise identified.

Enright *et al.* [16] proposed that the combination of the genetic background and SCC*mec* type should be used to describe MRSA clones. Typing of the *spa* locus has been shown to be a reliable method for long-term epidemiological studies, and *spa* typing/BURP data are in accordance with results obtained by MLST. Nevertheless, *spa* typing should preferably be used in combination with additional genetic markers, e.g. SCC*mec* [1].

The difference between the MRSA prevalence observed in this study (14%) and that of 37% previously observed [2] might be due to differences in the origin of the isolates, i.e. clinical isolates from various sources rather than bloodstream isolates. When the antibiotic susceptibility pattern of the MRSA isolates of this study (October to December 2004) was compared with that of isolates from between 1999 and 2003, there were increases in the levels of resistance to most antibiotics, i.e. azithromycin (64–95%), ciprofloxacin (57–96%), clindamycin (49–95%), gentamicin (65–94%) and muprirocin (7–12%), whereas the rifampicin resistance level decreased (from 50% in 1999 to 11% in 2003). The fusidic acid resistance level decreased from 1999 to 2001 (from 5.3% to 2%), increased between 2002 and 2003 (from 2% to 6%) and decreased to 0.4% in 2004 (http://www.amzh.hr/eng/index-eng.htm). The data on antibiotic susceptibility were consistent with data from a previous study on MRSA bloodstream isolates, showing that the vast majority of MRSA isolates in Croatia were multiresistant and belonged to ST111-MRSA-I, a single-locus variant of ST228-MRSA-I (Southern German clone) [4]. Until 1995, multiresistance of MRSA strains associated with ST228-

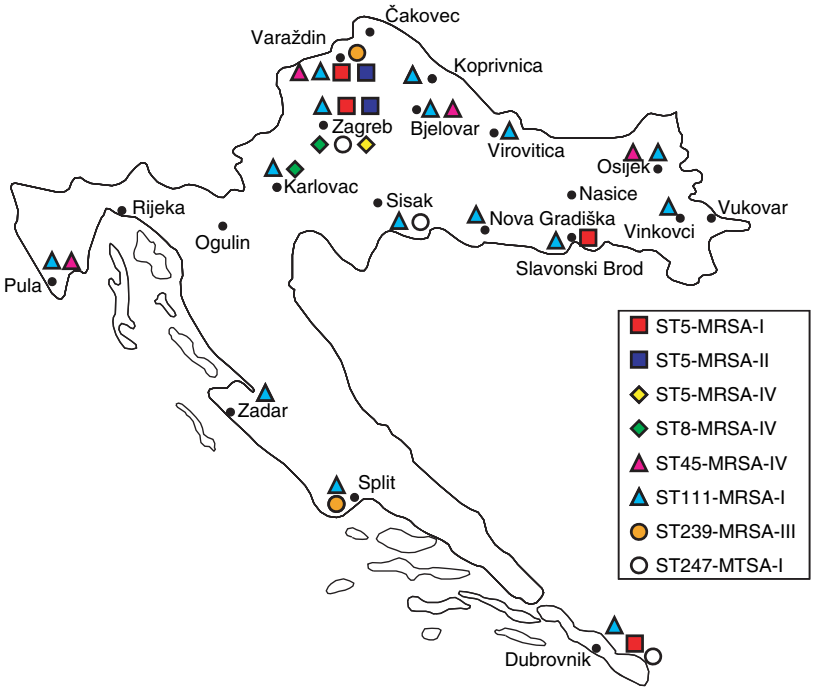

FIG. 1. Map of Croatia showing the distribution of the endemic methicillin-resistant *Staphylococcus aureus* (MRSA) clones.

MRSA-I had been observed in various countries. Nonetheless, recent ST228-MRSA-I isolates in Germany were resistant to both β-lactam antibiotics and one or two antibiotics [18].

MRSA strains related to the Southern German clone have been previously observed in Croatia [4], and in the neighbouring countries of Austria, Hungary and Slovenia [1]. In Hungary, the Southern German clone, together with the New York/Japan clone, represented 85% of the MRSA isolates between 2001 and 2004 [19]. ST111-MRSA-I was found in 21 of the 24 Croatian hospitals. The high prevalence of ST111-MRSA-I is particular to Croatia, as, to the best of our knowledge, no other study has found such a high prevalence of ST111-MRSA-I. However, a recent study of MRSA isolates in France reported the predominance of the Lyon (ST8-MRSA-IV) clone, which was observed in nearly 70% of the MRSA isolates [20]. This suggests that ST111-MRSA-I has as yet undiscovered features that promote its fitness and interaction with hosts in Croatia, which may include searching for an entry site, targeting a place for multiplication in the host, becoming persistent in the host, and reaching the next host. It has been observed that individuals with nasal *S. aureus* colonization are at increased risk of developing an *S. aureus* infection. Furthermore, *S. aureus* of any genotype can become a life-threatening pathogen under favourable circumstances, and some clones are more virulent than others [21]. In the past decade, it has been shown that several *S. aureus* lineages are highly epidemic [1]. However, whether certain *S. aureus* lineages are more able to colonize or to infect humans is unknown [21].

The Iberian (ST247-MRSA-I) clone, which was the second most frequent clone in Croatia, has been observed previously in Croatia as well as in the neighbouring countries of Austria, the Czech Republic, Hungary and Slovenia [1,4]. Although the Berlin (ST45-MRSA-IV) clone, the UK EMRSA-3 (ST5-MRSA-I) clone, the Brazilian/Hungarian (ST239-MRSA-II) clone, the New York/Japan (ST5-MRSA-Ii) clone, the UK EMRSA-2/-6 (ST8-MRSA-IV) clone, the Paediatric (ST5-MRSA-IV) clone, the PVL-negative ST1-MRSA-IV clone and the ST80-MRSA-IV clone have not been observed before in Croatia [1], these MRSA clones have been reported previously in the countries neighbouring Croatia. ST45-MRSA-IV has been observed in Austria and Hungary, ST5-MRSA-I in Slovenia, ST239-MRSA-III in Austria, the Czech Republic, Hungary and Slovenia, ST5-MRSA-II in Hungary, ST8-MRSA-IV in Austria and Hungary, and ST5-MRSA-IV in Austria [1]. PVL-negative ST1-MRSA-IV has been described in Australia and Portugal [22,23], whereas PVL-negative ST80-MRSA-IV has not been described previously. The observation that these minor MRSA clones were observed in the large cities, such as the capital Zagreb, and those on the coast, such as Dubrovnik, might suggest that these MRSA clones were imported from abroad (Fig. 1).

ST45-MRSA-I, ST1-MRSA-I, ST228-MRSA-II and ST239-MRSA-I were not associated with the major MRSA clones [1]. The observation of an uncommon SCC*mec* element in an uncommon *S. aureus* lineage can be explained by the exchange of the SCC*mec* element through homologous recombination among MRSA strains [24]. For example, studies have described ST45-MRSA-I in The Netherlands, ST45-MRSA-II in Poland, ST45-MRSA-III in Belgium, and ST45-MRSA-V in Portugal [23,25–27].

CA-MRSA isolates usually harbour SCC*mec* type IV or type V, and often PVL genes, but may differ in their genetic background (CC1, CC8, CC30, CC59 or CC80) [5]. PVL-positive MRSA strains with a heterogeneous genetic background were observed in Croatia. PVL-positive ST8-MRSA-IV has been observed previously in countries neighbouring Croatia, such as Austria and Bulgaria [1], whereas ST80-MRSA-IV (European CA-MRSA) has been found before in both Croatia and the neighbouring countries of Austria, Bulgaria, Romania and Slovenia, as well as in former Yugoslavia [1]. PVL-positive ST80-MRSA-I has not been observed previously.

Two non-typeable SCC*mec* type IV elements lacked locus D (*dcs* region) and these could be SCC*mec* type IVE/F, previously observed in Ireland [28]. The other two non-typeable SCC*mec* type IV elements, one harbouring the *pls* region and the other locus F, have, to our knowledge, not been reported earlier. Two MRSA isolates contained a non-typeable SCC*mec* type III element that harboured locus D (*dcs* region), which is normally observed in SCC*mec* types I, II and IV. Similar SCC*mec* elements have been observed previously in Croatia and Portugal [4,29]. These novel SCC*mec* elements may have originated through recombination among existing SCC*mec* elements [30].

In summary, ST111-MRSA-I is widely disseminated in Croatia. Furthermore, other MRSA clones, such as ST5-MRSA-I, ST239-MRSA-III, ST5-MRSA-II, ST8-MRSA-IV and ST5-MRSA-IV, were observed. Future surveillance studies of MRSA isolates are important to elucidate whether changes in the clonal distribution of MRSA will occur in the future and whether the minor MRSA clones observed in the present study will replace ST111-MRSA-I.

Acknowledgements

A. Budimir and R. H. Deurenberg contributed equally to this study.

We would like to thank M. Rijnders for excellent technical assistance with the MLST analyses, and the participants of the study and the members of the Croatian Academy of Medical Sciences: T. Tambić (Croatian Academy of Medical Sciences, Zagreb), A. Tambić Andrašević (Infectious Diseases Clinic, Zagreb), N. Kučišec-Tepeš (GH Sveti Duh, Zagreb), M. Payerl-Pal (IPH, Čakovec), S. Krešić (IPH, Bjelovar), M. Vodnica-Martucci (IPH, Dubrovnik), J. Magdić and D. Milanović-Martinović (IPH, Karlovac), V. Janeš-Poje (IPH, Koprivnica), L. Hodak (CH Našice and GH Nova Gradiška), N. Juričić (GH Ogulin), D. Vuković (IPH Osijek), M. Vranić-Ladavac (IPH Pula), D. Ambrožić (CHC Rijeka), S. Heitmanek (IPH Sisak), M. Petanović and M. Tomić Paradžik (IPH Slavonski Brod), V. Zoranić (IPH, Split), S. Sardelić (CHC Split), E. Sušić (IPH Šibenik), N. Tkalec Makovec and I. Koščak (PHI Varaždin), L. J. Potočić and S. Nađ (PHI Vukovar, Vinkovci), Emira Buljan and S. Baranjec (HIP 'Sveti rok' Virovitica), V. Gilić (HIP Zadar), J. Vraneša Tunuković (CPHI Zagreb), N. Jarža Davila (IPH, Zagreb), A. Lukić Grlić (Children's Diseases Clinic Klaićeva, Zagreb) and S. Marinković (Pulmonary Diseases Clinic Jordanovac, Zagreb). Furthermore, we would like to thank T. Ito (Department of Bacteriology, Juntendo University; Tokyo, Japan) and D. Oliveira (Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal) for providing the reference strains for SCC*mec* typing.

Transparency Declarations

All authors have no conflict of interest to declare.

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