

CHANGING EPIDEMIOLOGY OF PRESUMPTIVE COMMUNITY-ASSOCIATED-METHICILLIN-RESISTANT *Staphylococcus aureus* IN SLOVENIA IN 2014-2015 COMPARED TO 2010

SPREMENBA EPIDEMIOLOGIJE PRI PROTI METICILINU ODPORNEM *Staphylococcus aureus* DOMAČEGA OKOLJA V SLOVENIJI V LETIH 2014-2015 V PRIMERJAVI Z LETOM 2010

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ABSTRACT

Keywords:

Slovenia, CA-MRSA, LA-MRSA, *mecC*, *spa* types, clones

Introduction: Although the distinction between the Community-Associated-Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) and Hospital-Associated-Methicillin-Resistant *S. aureus* (HA-MRSA) has blurred in recent years, the CA-MRSA is an important group because of its potential to cause fulminant and severe infections. Its importance has further increased with the emergence of Livestock-Associated-Methicillin-Resistant *S. aureus* (LA-MRSA).

Methods: In the present study we analysed clonal distributions and virulence factors in presumptive CA-MRSA isolated from January 2014 to December 2015 and compared the results with our previous study from 2010. Phenotypic definition for presumptive CA-MRSA was based on resistance to ceftiofuran and oxacillin and susceptibility to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin and gentamicin.

Results: In 2014 and 2015 altogether 304 MRSA isolates fulfilled our screening phenotypic definition, 45 isolates were cultivated from clinical specimens and 259 from screening specimens. Sequence types ST398, LA-MRSA and *mecC* MRSA increased significantly in 2015 compared to 2010 (*p*-value <0.05) and were spread over Slovenia.

Conclusion: The clonal distribution of presumptive CA-MRSA has changed within the study period in Slovenia. In 2015 the most frequent clone among clinical and screening specimens was a pig-associated clone, ST398, but the number of confirmed ST398 infections remains low. While previously ST398 and *mecC* positive MRSA strains were geographically limited, they have spread throughout the country since 2010.

IZVLEČEK

Ključne besede:

Slovenija, CA-MRSA, LA-MRSA, *mecC*, tipi *spa*, kloni

Uvod: Čeprav je ločevanje med proti meticilinu odporno *Staphylococcus aureus* domačega okolja (CA-MRSA) in proti meticilinu odporno *Staphylococcus aureus* bolnišničnega okolja (HA-MRSA) oteženo, je v zadnjih letih CA-MRSA pomembna skupina, ki povzroča resne in ogrožajoče okužbe. Njen pomen se je povečal še s pojavom proti meticilinu odpornim *Staphylococcus aureus* rejnih živali (LA-MRSA).

Metode: V tej raziskavi smo analizirali zastopanost klonov in prisotnost virulenčnih dejavnikov pri sevih, sumljivih za CA-MRSA, ki so bili osamljeni od januarja 2014 do decembra 2015, in rezultate primerjali s študijo iz leta 2010. Fenotipska definicija za sumljive CA-MRSA je temeljila na odpornosti proti cefoksitinu in oksacilinu ter občutljivosti za vsaj dva od naslednjih štirih antibiotikov: ciprofloksacin, eritromicin, klindamicin in gentamicin.

Rezultati: V letih 2014 in 2015 je skupno 304 izolatov MRSA ustrezalo naši fenotipski definiciji. 45 izolatov je bilo osamljenih iz kliničnih kužnin, 259 iz nadzornih kužnin. Sekvenčni tip ST398, LA-MRSA in *mecC* MRSA, sta se v letu 2015 v primerjavi z letom 2010 signifikantno povečala (*p*-vrednost <0,05) in se razširila po Sloveniji.

Zaključki: Zastopanost klonov pri sevih, sumljivih za CA-MRSA, se je v obdobju raziskave v Sloveniji spremenila. V letu 2015 je bil najpogostejši klon med kliničnimi in nadzornimi kužninami klon ST398, MRSA rejnih živali, vendar je število potrjenih okužb s ST398 še vedno nizko. V letu 2010 so bili ST398 in *mecC* pozitivni sevi MRSA geografsko omejeni, v kasnejših letih pa so se razširili po vsej državi.

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1 INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major pathogens responsible for hospital-associated (HA-MRSA) and community-associated (CA-MRSA) infections. The epidemiological distinction between HA-MRSA and CA-MRSA is blurred, because CA-MRSA is increasingly invading into the health care setting and consequently the epidemiology of MRSA has changed (1-3). MRSA infections in the community can also be caused by livestock-associated MRSA (LA-MRSA) (1, 4-7). In recent years, LA-MRSA, specifically sequence type (ST) ST398 has emerged in farm animals, and human infections caused by MRSA ST398 have been increasingly documented in Europe (1, 4-9). In 2011 a novel divergent *mecA* gene homologue (*mecA*_{LG251}), designated *mecC*, was discovered. *mecC* MRSA was reported in humans, in animals (farm, companion and wildlife animals), and also from non-animal sources (e.g. water and urban wastewater), indicating the existence of *mecC* MRSA in several different reservoirs (1, 10, 11).

No systematic surveillance of MRSA isolates and epidemiology of MRSA clone distribution in humans has been performed in Slovenia, and only the surveillance of presumptive CA-MRSA has been established. Antibiotic susceptibility patterns with partially available epidemiological data (colonization or infection with MRSA and hospitalization history in the previous year) was used as a screening tool to select the presumptive CA-MRSA in an earlier study (12), and isolates were analysed on a national level for the first time in 2010 (13). To be consistent with inclusion criteria and for comparative purposes we have retained the previous antibiotic susceptibility based criteria for strain selection and are referring to the strains as presumptive CA-MRSA.

Among 92 presumptive CA-MRSA in 2010 the most prevalent sequence types (STs) were ST45 (n=35, 38%), ST398 (n=14, 15.2%), ST5 (n=9, 9.8%) and ST22 (n=8, 8.7%) (13). *mecC* MRSA was confirmed previously in Slovenia and seven of 359 isolates (1.7%) were positive for *mecC* in a retrospective study covering the years 2006 to 2013 (14).

The aim of the present study was to determine possible changes in the clonal distribution of presumptive CA-MRSA in Slovenia. We performed a prospective study and compared the phenotypic and genotypic characteristics of presumptive CA-MRSA isolates collected from seven laboratories throughout Slovenia collected in 2014 and 2015 with isolates obtained from the same laboratories in 2010.

2 MATERIALS AND METHODS

2.1 Study Setting

The Centre of Medical Microbiology at the National Laboratory for Health, Environment and Food (NLZOH) covers seven laboratories throughout Slovenia. The same NLZOH laboratories that participated in the study in 2010 were invited to collect prospective MRSA isolates from routine diagnostics that fulfilled the screening phenotypic pattern for presumptive CA-MRSA during a two-year sampling period (1 January 2014 to 31 December 2014 and 1 January 2015 to 31 December 2015). Isolates were classified as presumptive CA-MRSA if they were resistant to oxacillin and ceftiofloxacin and susceptible to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin and gentamicin. Each MRSA isolate, with additional information (gender, sample material and date of sampling), was sent to the NLZOH Kranj for molecular analyses. Information was extracted from the laboratory information system (MBL, Src Infonet, Kranj). All isolates were identified by MALDI-TOF mass spectrometry (Bruker Daltonic GmbH, Bremen, Germany).

2.2 Antimicrobial Susceptibility Testing

The susceptibility patterns of the MRSA isolates were performed by the agar disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (15, 16). The antibiotics tested were penicillin, ceftiofloxacin, gentamicin, tobramycin, kanamycin, erythromycin, clindamycin, tetracycline, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, linezolid, mupirocin and fusidic acid (BD, Sparks, USA). Minimal inhibitory concentration (MIC) determination of oxacillin, ceftiofloxacin and vancomycin was performed using the E-test (bioMérieux, Marcy-l'Etoile, France).

2.3 Molecular Characterisation

The presence of *mecA* or *mecC* and Panton-Valentine leukocidin (PVL) genes was tested by PCR using Genotype MRSA (Hain Lifescience GmbH, Nehren, Germany). Genes encoding for staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed* and *see*), toxic shock syndrome toxin (*tst*), locus enterotoxin gene cluster (*egc*) and staphylococcal exfoliative toxins (*eta*, *etb*, *etd*) were detected by multiplex PCR (17-19). SCC*mec* typing was performed by a multiplex PCR strategy previously described by Chen et al. and Petersdorf et al. (20, 21).

The DNA sequence-based methods, *spa* typing, is based on the number of tandem repeats and sequence variation in region X of the *S. aureus*-specific staphylococcal protein A gene. Amplification, sequencing and analysis of *spa* typing were performed according to a method described previously and analysed with Ridom SpaServer

(22). *Spa* clustering was performed using the *spa*-plugin in BioNumerics, and the results displayed with a minimum spanning tree. Multilocus sequence typing (MLST) is a highly discriminatory method of DNA sequence-based typing that relies on analysis of relatively conserved genes that encode essential proteins (seven housekeeping genes). MLST was performed for 19 MRSA isolates according to a method described by Enright et al. (23) and sequence types (ST) were determined with BioNumerics MLST-plugin (version 7.6; Applied Maths). All other STs were assigned based on *spa* types as published on <http://spaserver.ridom.de> and Kinross et al. (6).

2.4 Statistical Analyses

Data were analysed with the free online software MedCalc (www.medcalc.org). Categorical variables were compared using the Chi-square test. A p-value ≤ 0.05 was considered as statistically significant.

3 RESULTS

3.1 Bacterial Isolates - Distribution Between Clinical and Screening Specimens

In 2010, 2014 and 2015, 92, 122 and 182 MRSA isolates had a positive susceptibility pattern of presumptive CA-MRSA, respectively. Of those, 335 MRSA isolates were cultivated from screening specimens and 61 from clinical specimens (year 2010, n=16, 17.4%; year 2014, n=20, 16.4%; year 2015, n=25, 13.7%) (Table 1).

3.2 Distribution of *Spa* Types among Presumptive CA-MRSA

Presumptive CA-MRSA strains isolated during 2014 were associated with 46 different *spa* types and two (1.6%) strains were non-typable. Strains from 2015 were associated with 71 different *spa* types and one (0.5%) strain was non-typable. Based on *spa* types were strains in 2014 and 2015 were assigned to 18 MLST STs (Table 2, Figure 1).

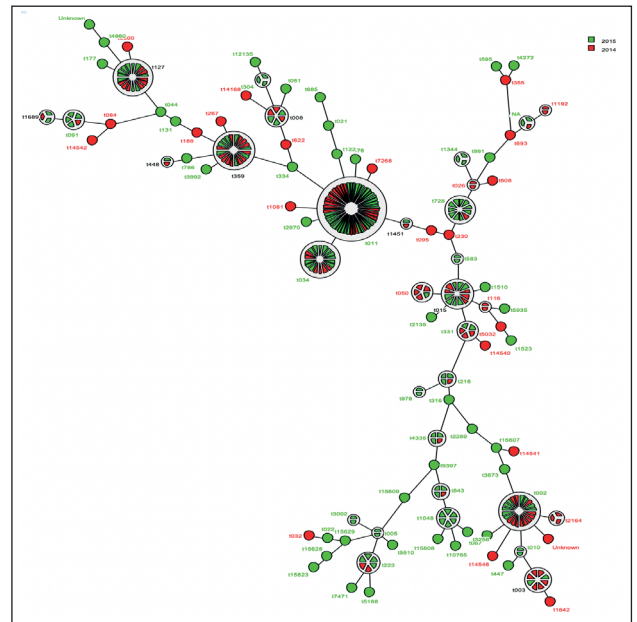


Figure 1. Distribution of CA-MRSA *spa* types circulating in Slovenia in 2014 and 2015.

Cluster analysis was performed using the *spa* typing plug-in tool of the BioNumerics program. Nodes indicate *spa* types and the number of sections within the node indicate number of strains for each *spa* type. Tree is color-coded according the year of isolation.

Table 1. Distribution of presumptive CA-MRSA isolates among clinical specimens.

Specimen	Results ^a for MRSA patients from		
	2010 (n=16)	2014 (n=20)	2015 (n=25)
Skin and soft tissue	12 (75)	13 (65)	13 (52)
Aspirate tracheae	1 (6.3)	4 (20)	4 (16)
Knee joint puncture	-	1 (5)	1 (4)
Urine	1 (6.3)	1 (5)	2 (8)
Nose swab	-	1 (5)	1 (4)
Blood culture	-	-	1 (4)
Bone	-	-	1 (4)
Faeces	-	-	1 (4)
Nasopharynx	-	-	1 (4)
Ear swab	2 (12.4)	-	-

^a value is number (%)

Table 2. Most frequent *spa* types and sequence types of presumptive CA-MRSA isolates from year 2010, 2014 and 2015.

Rank	2010*			2014			2017		
	<i>spa</i> type	Frequency % (number)	MLST ^a	<i>spa</i> type	Frequency % (number)	MLST ^a	<i>spa</i> type	Frequency % (number)	MLST ^a
1	t015	21.8 (20)	ST45	t011	17.1 (21)	ST398 ^b	t011	17.0 (31)	ST398 ^b
2	t011	13.0 (12)	ST398 ^b	<u>t359</u>	9.8 (12)	ST97	t034	6.6 (12)	ST398 ^b
3	<u>t728</u>	6.5 (6)	ST45	t002	8.1 (10)	ST5	t127	5.5 (10)	ST1
4	t091	6.5 (6)	ST7	t127	6.5 (8)	ST1	<u>t728</u>	5.5 (10)	ST45
5	t008	5.4 (5)	ST8	t034	4.9 (6)	ST398 ^b	t002	4.9 (9)	ST5
6	t002	4.3 (4)	ST5	<u>t003</u>	4.9 (6)	ST5	t015	4.4 (8)	ST45
7	t005	4.3 (4)	ST22	t015	4.1 (5)	ST45	<u>t359</u>	3.8 (7)	ST97
8	t026	4.3 (4)	ST45	t050	3.3 (4)	ST45	<u>t1048</u>	3.3 (6)	ST130
9	t127	3.3 (3)	ST1	t008	2.4 (3)	ST8	t091	2.2 (4)	ST7
10	t020	2.2 (2)	ST22	t2164	2.4 (3)	ST5	t223	2.2 (4)	ST22
>10	<u>t003</u> , t006, t034, t041, t105, t108, t116, t174, t355, t595, t737, t791, <u>t843</u> , t846, <u>t1079</u> , t1094, <u>t1111</u> , <u>t1179</u> , <u>t1218</u> , t1231, t1509, t1911, <u>t2032</u> , <u>t3824</u> , <u>t4365</u> , <u>t13070</u>	28.4 (26)	ST1, ST5, ST7, ST8, ST22, ST30, ST45, ST72, ST88, ST130, ST152/377, ST225, ST228, ST398	t026, t032, t091, t095, t116, t189, t216, t223, t230, t267, t331, t355, t448, t622, t693, <u>t728</u> , t808, <u>t843</u> , t1081, t1094, t1192, t1340, t1451, t1689, t1842, t1943, <u>t4335</u> , t5032, t5500, t7268, t14168, t14540, t14541, t14542, t14546**	36.5 (45)	ST5, ST7, ST8, ST15, ST22, ST30, ST45, ST59, ST88, ST97, ST152/377, ST130, ST188, ST398, ST627, ST1774, ST3138	<u>t003</u> , t005, t008, t010, t021, t022, t044, t050, <u>t051</u> , t067, t122, t131, t177, t216, t304, t316, t331, t334, t447, t448, t583, t589, t595, t685, t786, <u>t843</u> , t976, t991, t1344, t1451, t1510, t1523, t1689, t1943, t2032, t2135, t2289, t2576, t2970, t3002, t3213, <u>t3256</u> , t3380, t3673, t3992, t4272, t4335, t4960, t5168, t5510, <u>t9397</u> , <u>t10765</u> , t12135, t15607**, <u>t15608</u> , t15609**, t15610**, t15628**, t15629**, t15773**, t15823**	44.6 (81)	ST1, ST5, ST8, ST22, ST30, ST45, ST59, ST80, ST88, ST97, ST152/377, ST130, ST188, ST247, ST398, ST913, ST3138

Legend: *Previously published (9); **New *spa* type; ^aAssociated multilocus sequence types published on <http://spaserver.ridom.de>;

^bPreviously published (15). Underlined *spa* type multilocus sequence types are those determined in this study.

The distribution of the most prevalent STs varied in different geographic regions (Figure 2). In 2014 12 *spa* types were determined among clinical specimens (t002, t008, t011, t015, t034, t050, t091, t223, t267, t359, t1842, t2164) while in 2015 20 *spa* types were determined (t002, t005, t008, t010, t011, t015, t034, t051, t122, t127, t131, t359, t1048, t1344, t2576, t3213, t4335, t15608, t15610, t15773).

The first major clone, ST45, detected in 2010 (n=35, 38%) decreased significantly overall to 15.5% (n=19) in 2014 (p-value=0.0003) and to 15.9% (n=29) in 2015 (p-value=0.0001).

The second major clone confirmed in 2010 and associated with LA-MRSA, ST398 (*spa* types t011, t034, t1344, t1451,

t2576, t2970), increased significantly (p-value <0.05) from 15.2% (n=14) in 2010 to 27.5% (n=50) in 2015 (Figure 3). The association of these LA-MRSA with infections increased, as in 2010 it was reported for only one isolate from skin and soft tissue infection, while in 2015 LA-MRSA were associated with skin and soft tissue infection (n=3) and lower respiratory tract infection (n=1).

mecC positive MRSA, ST130 and ST3138 increased significantly (p-value <0.05) from 1.1% (n=1) in 2010 to 8.2% (n=15) in 2015 (Figure 3). In 2010 *mecC* was isolated from skin/mucosal (screening) swabs for MRSA colonization, in 2015 *mecC* MRSA were recovered from sputum (n=1) and blood culture (n=1), and all the remaining strains were recovered from skin/mucosal (screening) swabs for MRSA colonization.

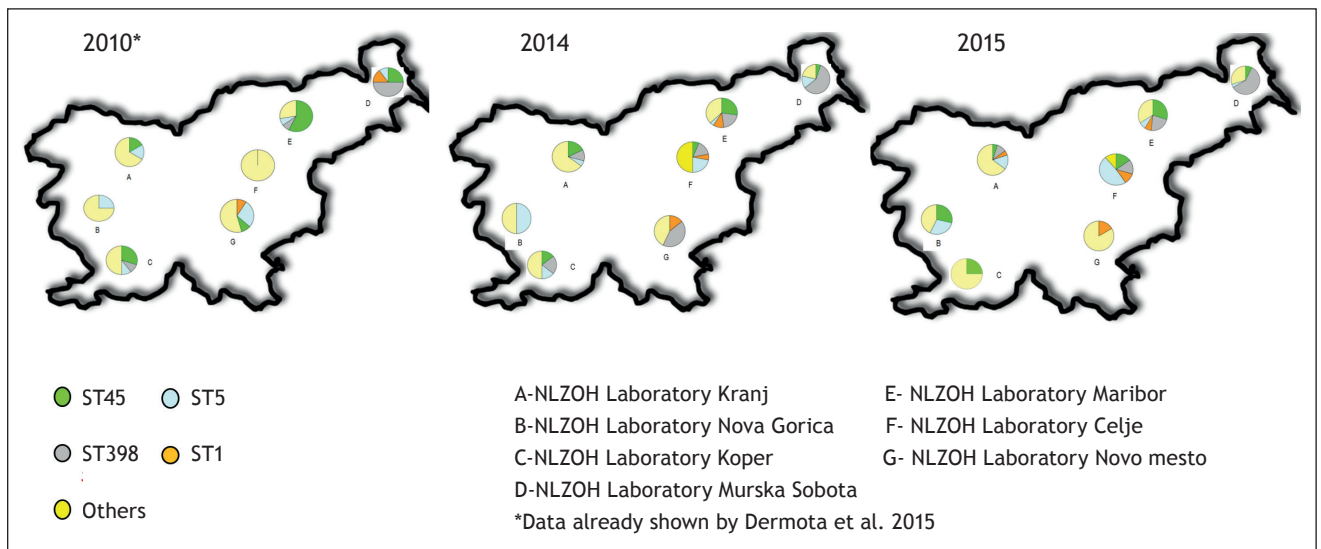


Figure 2. The distribution of presumptive CA-MRSA clones circulating in Slovenia for 2010, 2014 and 2015.

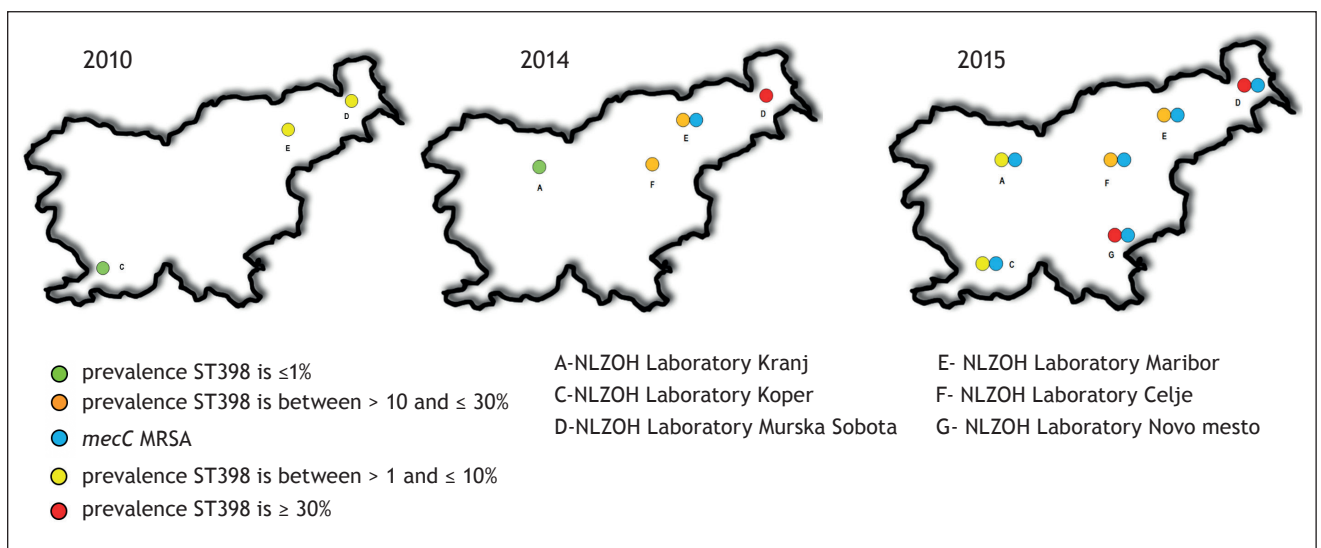


Figure 3. The distribution and prevalence of ST398 clone and *mecC* MRSA in Slovenia for 2010, 2014 and 2015.

Table 3. Frequencies of virulence associated genes and *spa* types in presumptive CA-MRSA isolates from 2010, 2014 and 2015.

Rank	2010*			2014			2017		
	Number of isolates	Frequency %	<i>spa</i> type (number)	Number of isolates	Frequency %	<i>spa</i> type (number)	Number of isolates	Frequency %	<i>spa</i> type (number)
<i>sea</i>	6	6.5	t008 (4), t041 (1), t2032 (1)	3	2.5	t008 (2), t003 (1)	15	8.2	t002 (5), t021 (1), t122 (1), t127 (1), t304 (3), t976 (2), t4335 (1), t12135 (1)
<i>seb</i>	2	2.2	t002 (1), t026 (1)	2	1.6	t216 (1), t1340 (1)	6	3.3	t216 (3), t976 (2), t2289 (1)
<i>sec</i>	33	35.8	t002 (1), t015 (18), t026 (3), t116 (1), t728 (5), t737 (1), t791 (1), t1079 (1), t1231 (1), t13070 (1)	13	10.7	t015 (2), t026 (1), t050 (1), t095 (1), t116 (2), t230 (1), t331 (3), t622 (1), t14540 (1)	41	22.5	t002 (4), t011 (1), t015 (6), t050 (1), t223 (2), t331 (2), t359 (1), t448 (1), t583 (2), t589 (1), t728 (10), t786 (1), t1048 (2), t1510 (1), t1523 (1), t3213 (1), t4335 (1), t5168 (1), t15628 (1), t15773 (1)
<i>sed</i>	14	15.2	t002 (2), t003 (1), t008 (4), t015 (2), t020 (1), t846 (1), t1094 (1), t1179 (1), t2032 (1)	18	14.7	t002 (9), t003 (3), t015 (1), t026 (1), t1192 (1), t2164 (2), t14541 (1)	17	9.3	t002 (4), t003 (2), t008 (2), t010 (2), t011 (1), t050 (1), t067 (1), t359 (1), t447 (1), t2032 (1), t15607 (1)
<i>see</i>	none	0	-	none	0	-	none	0	-
Locus <i>egc</i>	50	54.3	t002 (4), t003 (1), t005 (3), t006 (1), t015 (20), t020 (1), t026 (4), t041 (1), t105 (1), t116 (1), t728 (5), t737 (1), t791 (1), t1079 (1), t1094 (1), t1111 (1), t1231 (1), t3824 (1), t13070 (1)	53	43.4	t002 (10), t003 (6), t015 (5), t026 (2), t032 (1), t050 (4), t095 (1), t116 (2), t223 (2), t230 (1), t331 (3), t359 (2), t448 (1), t622 (1), t728 (1), t808 (1), t1081 (1), t1192 (2), t1340 (1), t1842 (1), t2164 (2), t5032 (1), t14540 (1), t14541 (1)	74	40.7	t002 (9), t003 (2), t005 (2), t010 (1), t011 (3), t015 (7), t021 (1), t022 (1), t050 (1), t067 (1), t091 (1), t122 (1), t127 (1), t216 (1), t223 (4), t331 (2), t359 (1), t447 (1), t583 (2), t589 (1), t685 (1), t728 (10), t1048 (1), t1510 (1), t1523 (1), t2135 (1), t2289 (1), t3002 (2), t3213 (1), t3673 (1), t4335 (1), t5168 (1), t5510 (1), t15607 (1), t15608 (1), t15609 (1), t15610 (1), t15628 (1), t15629 (1), t15773 (1), t15823 (1)
PVL	8	8.7	t002 (2), t005 (1), t008 (1), t091 (1), t355 (1), t791 (1), t4335 (1)	13	10.7	t002 (9), t050 (1), t127 (1), t2164 (1), t355 (1)	10	5.5	t002 (4), t010 (1), t044 (1), t067 (1), t127 (1), t131 (1), t595 (1)
<i>tst</i>	8	8.7	t026 (1), t105 (1), t728 (5), t1111 (1)	2	1.6	t015 (1), t728 (1)	23	12.6	t002 (4), t015 (1), t122 (1), t223 (3), t359 (1), t685 (1), t728 (10), t4335 (1), t15610 (1)
<i>eta</i>	none	-	-	1	0.8	t015 (1)	1	0.5	t991 (1)
<i>etb</i>	none	-	-	none	-	-	none	-	-
<i>etd</i>	none	-	-	none	-	-	3	1.6	t044 (1), t131 (1), t991 (1)

Legend: *Data already shown by Dermota et al. 2015; *sea* staphylococcal enterotoxin gene type A, *seb* staphylococcal enterotoxin gene type B, *sec* staphylococcal enterotoxin gene type C, *sed* staphylococcal enterotoxin gene type D, *see* staphylococcal enterotoxin gene type E, locus *egc* enterotoxin gene cluster, PVL Pantone-Valentine leukocidin, *tst* toxic shock syndrome toxin gene, *eta* exfoliative toxin type A, *etb* exfoliative toxin type B, *etd* exfoliative toxin type D

3.3 Virulence Factors Among All Presumptive CA-MRSA

Two out of 11 tested virulence genes were not detected in the studied strain collection (*see*, *etb*). Enterotoxin genes (*sec*, *sed* and *egc*) were commonly present, while exfoliative toxins were rarely detected (Table 3). PVL was more common in 2014 (n=13, 10.7%) than in 2015 (n=10, 5.5%) and 2010 (n=8, 8.7%), but this result was not statistically significant (Table 4).

Associations of PVL positive MRSA with infections were reported in all study years. In 2010 PVL were detected in five presumptive CA-MRSA strains among 16 clinical specimens. All PVL positive strains were isolated from skin and soft tissue infections (n=5, 31.2%) and belonged to *spa* types t002 (n=1), t008 (n=1), t355 (n=1), t791 (n=1) and t4365 (n=1). Among 20 clinical specimens, six PVL positive presumptive CA-MRSA strains were isolated in 2014 from skin and soft tissue infections, belonging to *spa* types t002 (n=4), t050 (n=1) and t2164 (n=1). In 2015 four PVL positive out of 25 presumptive CA-MRSA strains were isolated from skin and soft tissue infections (n=3, 12%) and faeces (n=1, 4%). These PVL positive strains belonged to *spa* types t002 (n=1), t010 (n=1), t127 (n=1) and t131 (n=1).

3.4 SCCmec Types Among All Presumptive CA-MRSA

The most frequently confirmed type was SCCmec IV (2014, n=62, 50.1%; 2015, n=88, 48.4%) and SCCmec V (2014, n=42, 34.4%; 2015, n=66, 36.3%). SCCmec I was detected in 1.6% (n=2) isolates in 2014, and in 1.6% (n=3) in 2015, while SCCmec II was detected in 5.7% (n=7) isolates in

2014, and in 1.1% (n=2) in 2015. 5.7% (n=7) isolates in 2014 and 3.2% (n=6) isolates in 2015 were non-typable. SCCmec XI was detected in 1.6% (n=2) isolates in 2014, and in 8.2% (n=15) in 2015.

4 DISCUSSION

Several changes were observed in the clonal distribution and virulence properties of presumptive CA-MRSA strains during the years 2014 and 2015 in comparison to 2010 (13). Similar to as in 2010, clones ST5, ST45 and ST398 were most common during 2014 and 2015 study, but with different rankings.

In 2014 and 2015, the first major clone (2014, 28 strains, 22.9%; 2015, 50 strains, 27.5%) was related to a pig-associated clone, ST398, while in 2010 ST398 was found in 15.2% (n=14) and was the second most common clone. In 2010, most strains belonging to ST398 were predominantly found in rural areas of north eastern and southern Slovenia (regions D and E), where livestock breeding is an important agricultural activity. In 2014 and 2015, the density of ST398 was also confirmed in north eastern and southern Slovenia (regions D and E), but ST398 was also detected in non-agricultural regions, namely A, C, F and G (Figure 3). Since LA-MRSA has spread throughout Europe, the number of colonized people and infections is increasing (4, 7). The main reservoirs of LA-MRSA ST398 are pigs, poultry, cattle, and companion animals, and close contact with animals is a risk factor for LA-MRSA carriage (4, 7).

Table 4. Virulence factors of Slovenian presumptive CA-MRSA isolates from 2010, 2014 and 2015.

Virulence factor	2010*	2014	2015	p-value (Chi-square test)	
	(n=92)	(n=122)	(n=182)	Year 2014 vs. 2010	Year 2015 vs. 2010
	% (number) of presumptive CA-MRSA isolates				
<i>sea</i>	6.5 (6)	2.5 (3)	8.2 (15)	0.15	0.61
<i>seb</i>	2.2 (2)	1.6 (2)	3.3 (6)	0.77	0.60
<i>sec</i>	35.8 (33)	10.7 (13)	22.5 (41)	< 0.0001	0.01
<i>sed</i>	15.2 (14)	14.7 (18)	9.3 (17)	0.92	0.15
<i>see</i>	0	0	0	-	-
Locus <i>egc</i>	54.3 (50)	43.4 (53)	40.7 (74)	0.11	0.03
PVL	8.7 (8)	10.7 (13)	5.5 (10)	0.63	0.31
<i>tst</i>	8.7 (8)	1.6 (2)	12.6 (23)	0.02	0.33
<i>eta</i>	0	0.8 (1)	0.5 (1)	0.61	0.79
<i>etb</i>	0	0	0	-	-
<i>etd</i>	0	0	1.6 (3)	-	0.39

Legend: *Data already shown by Dermota et al. 2015; *sea* staphylococcal enterotoxin gene type A, *seb* staphylococcal enterotoxin gene type B, *sec* staphylococcal enterotoxin gene type C, *sed* staphylococcal enterotoxin gene type D, *see* staphylococcal enterotoxin gene type E, locus *egc* locus enterotoxin gene cluster, PVL Panton-Valentine leukocidin, *tst* toxic shock syndrome toxin gene, *eta* exfoliative toxin type A, *etb* exfoliative toxin type B, *etd* exfoliative toxin type D

In recent years, LA-MRSA carriage was also reported in humans without any animal contact (7-9). MRSA ST398 has also been introduced into the health care setting, mainly in areas with a high density of livestock farming (5).

The second major clone (2010, 9 strains, 9.8%; 2014, 21 strains, 17.2%; 2015, 17 strains, 9.3%) was related to the ST5. Despite the difference in percentages, no significance in ST5 distribution was observed.

The third major clone (2010, 35 strains, 38.0%; 2014, 19 strains, 15.5%; 2015, 29 isolates, 15.9%) was related to the ST45. *Spa* type t015, *SCCmec* type IV decreased from 17.4% (n=16) in 2010 to 3.3% (n=4) in 2014 and 2.7% (n=5) in 2015. *Spa* type t728, *SCCmec* type IV also decreased from 6.5% (n=6) in 2010 to 0.8% (n=1) in 2014 and 5.5% (n=10) in 2015. Isolates with *spa* type t728 were found in 2010 in only one region in Slovenia, E, but in 2015 they were also found in two other regions, A and D.

European clone ST80 and Balkan clone ST152/377, which are circulating in Europe and our neighbouring countries (Italy, Austria, Croatia), are rare in Slovenia (24, 25). In 2010, PVL gene was detected in eight (8.7%) strains that belonged to ST5, ST7, ST8, ST22, ST72, ST88, ST152/377 and ST772. In 2014 and 2015, 23 (7.6%) PVL positive strains were associated with ST1, ST5, ST22, ST80 and ST152/377. PVL positive ST5 (t002) significantly increased from 1.3% (n=2) in 2010 to 7.4% (n=9) in 2014 (p-value=0.0466). PVL positive MRSA strains were also found in 2014 and 2015 in 10 clinical samples (nine samples from wounds, one from faeces), but in Slovenia PVL positive clones are surprisingly rarely distributed (25).

mecC positive MRSA strains were found in 1.1% in 2010 (14), while in 2014 and 2015 we observed an increase to 1.6% and 8.2%, respectively. *mecC* positive MRSA, associated with ST130 and ST3138, were observed in 2014 in the rural area of Southern Slovenia (region E), but in 2015 *mecC* MRSA were distributed throughout Slovenia, also in non-rural regions A, C, G and F (Figure 3). Most *mecC* positive MRSA were detected in asymptomatic carriers; in two patients, *mecC* were detected from clinical specimens (sputum, blood culture) (*spa* type t1048, t15608). In the literature, *mecC* MRSA have mainly been reported in humans from screening specimens, as well as from clinical specimens, and the range of infections caused by *mecC*-carrying MRSA is the same as seen in other *S. aureus*, including life-threatening diseases such as bacteraemia (7, 10, 26).

Our study has the following limitations. The first is that the definition of presumptive CA-MRSA is based on the susceptibility pattern. As we have written in the manuscript, in Slovenia no systematic surveillance of MRSA isolates is performed. To the best of our knowledge the HA-MRSA strains in our country are resistant to beta-lactam antibiotics, fluoroquinolones, macrolides and

lincosamides (27). Because of the emerging CA-MRSA in Europe, in 2006 a definition for presumptive CA-MRSA as a screening tool, the same as in other countries, was set based on the susceptibility pattern. To compare the data from 2014 and 2015 with that from 2010, the same definition for presumptive CA-MRSA was used. The authors are aware, however, that our definition is not reliable without epidemiological data, as in 2010, due to incomplete epidemiological data of health care risk factors. The authors are also aware that our definition based on susceptibility pattern does not cover all CA-MRSA strains (e.g. more resistant CA-MRSA) and also includes more sensitive HA-MRSA strains. Another limitation of our study is that most isolates were recovered from screening specimens at mucocutaneous site.

In this work we confirmed the changes in clonal distribution of presumptive CA-MRSA in Slovenia. The most frequent sequence type during the 2014/2015 study period was ST398, a pig-associated clone, which is mostly distributed among asymptomatic carriers. While previously ST398 and *mecC* positive strains were geographically limited, they have spread throughout the country since 2010.

Future surveillance studies of molecular epidemiology with improved molecular methods and whole genome sequencing (WGS) of systematically collected MRSA are very important in Slovenia in order to monitor changes in clonal distribution.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist in relation to this research.

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ETHICAL APPROVAL

Not required.

REFERENCES

- Bal AM, Coombs GW, Holden MTG, Lindsay JA, Nimmo GR, Tattevin P, et al. Genomic insights into the emergence and spread of international clones of healthcare-, community- and livestock-associated methicillin-resistant *Staphylococcus aureus*: blurring of the traditional definitions. *J Glob Antimicrob Resist*. 2016;6:95-101. doi: 10.1016/j.jgar.2016.04.004.
- Otto M. Community-associated MRSA: what makes them special? *IJMM*. 2013;303:324-30. doi: 10.1016/j.ijmm.2013.02.007.
- Uhlemann AC, Otto M, Lowy FD, DeLeo FR. Evolution of community- and healthcare-associated methicillin-resistant *Staphylococcus aureus*. *Infect Genet Evol*. 2014;21:563-74. doi: 10.1016/j.meegid.2013.04.030.
- Cuny C, Wieler LH, Witte W. Livestock-associated MRSA: the impact on humans. *Antibiotics*. 2015;4:521-43. doi: 10.3390/antibiotics4040521.
- Larsen J, Petersen A, Larsen AR, Sieber RN, Stegger M, Koch A, et al. Emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* bloodstream infections in Denmark. *CID*. 2017;65:1062-75. doi: 10.1093/cid/cix504.
- Kinross P, Petersen A, Skov R, Van Hauwermeiren E, Pantosti A, Laurent F, et al. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) among human MRSA isolates, European Union/European Economic Area countries, 2013. *Euro Surveill*. 2017;22. doi: 10.2807/1560-7917.ES.2017.22.44.16-00696.
- Aires-de-Sousa M. MRSA among animals: current overview. *Clin Microbiol Infect*. 2017;23:373-80. doi: 10.1016/j.cmi.2016.11.002.
- Zomer TP, Wielders CC, Veenman C, Hengeveld P, van der Hoek W, de Greeff SC, et al. MRSA in persons not living or working on a farm in a livestock-dense area: prevalence and risk factors. *J Antimicrob Chemother*. 2017;72:893-9. doi: 10.1093/jac/dkw483.
- van Rijen MM, Bosch T, Verkade EJ, Schouls L, Kluytmans JA, CAM Study Group. Livestock-associated MRSA carriage in patients without direct contact with livestock. *PlosOne*. 2014;9:e100294. doi: 10.1371/journal.pone.0100294.
- Becker K, Ballhausen B, Köck R, Kriegeskorte A. Methicillin resistance in *Staphylococcus* isolates: the "mec alphabet" with specific consideration of mecC, a mec homolog associated with zoonotic *S. aureus* lineages. *Int J Med Microbiol*. 2014;304:794-804. doi: 10.1016/j.ijmm.2014.06.007.
- Concepcion P, Harrison EM, Fernández-Garayzábal JF, Paterson GK, Díez-Guerrier A, Holmes MA, et al. Detection of mecC-Methicillin-resistant *Staphylococcus aureus* isolates in river water: a potential role for water in the environmental dissemination. *Environ Microbiol Rep*. 2014;6:705-8. doi:10.1111/1758-2229.12191.
- Grmek-Košnik I, Dermota U, Ribic H, Ravnik M, Kavcic M, Harlander T, et al. Analysis of Slovenian MRSA strains with susceptibility patterns suggestive of CA-MRSA. *Wien Klin Wochenschr*. 2009;121:552-7. doi: 10.1007/s00508-009-1178-7.
- Dermota U, Mueller-Premru M, Švent-Kučina N, Petrovič Ž, Ribič H, Rupnik M, et al. Survey of community-associated-methicillin-resistant *Staphylococcus aureus* in Slovenia: Identification of community-associated and livestock-associated clones. *Int J Med Microbiol*. 2015;305:505-10. doi: 10.1016/j.ijmm.2015.05.002.
- Dermota U, Zdovc I, Štrumbelj J, Grmek-Košnik I, Ribič H, Rupnik M, et al. Detection of methicillin-resistant *Staphylococcus aureus* carrying the mecC gene in human samples in Slovenia. *Epidemiol Infect*. 2014;18:1-4. doi: 10.1017/S0950268814001861.
- The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Accessed January 28th, 2020 at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf
- The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Accessed January 28th, 2020 at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf
- Chiefari AK, Perry MJ, Kelly-Cirino C, Egan CT. Detection of *Staphylococcus aureus* enterotoxin production genes from patient samples using an automated extraction platform and multiplex real-time PCR. *Mol Cell Probes*. 2015;29:461-7. doi: 10.1016/j.mcp.2015.06.004.
- Fusco V, Quero GM, Morea M, Blaiotta G, Visconti A. Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (egc) and quantitative detection in raw milk by real time PCR. *Int J Food Microbiol*. 2011;144:528-37. doi: 10.1016/j.ijfoodmicro.2010.11.016.
- Shi D, Ishii S, Sato T, Yamazaki H, Matsunaga M, Higuchi W, et al. *Staphylococcal* scalded skin syndrome in an extremely low-birth-weight neonate: molecular characterization and rapid detection by multiplex and real-time PCR of methicillin-resistant *Staphylococcus aureus*. *Pediatr Int*. 2011;53:211-7. doi: 10.1111/j.1442-200X.2010.03246.x.
- Chen L, Mediavilla JR, Oliveira DC, Willey BM, de Lencastre H, Kreiswirth BN. Multiplex Real-Time PCR for Rapid *Staphylococcal* Cassette Chromosome mec Typing. *J Clin Microbiol*. 2009;47:3692-706. doi: 10.1128/JCM.00766-09.
- Petersdorf S, Herma M, Rosenblatt M, Layer F, Henrich B. A Novel *Staphylococcal* cassette chromosome mec Type XI Primer for detection of mecC-harboring methicillin-resistant *Staphylococcus aureus* directly from screening specimens. *J Clin Microbiol*. 2015;53:3938-941. doi: 10.1128/JCM.02328-15.
- Harmsen D, Claus H, Witte W, Rothgänger J, Claus H, Turnwald D, et al. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J Clin Microbiol*. 2003;41:5442-8. doi:10.1128/jcm.41.12.5442-5448.2003.
- Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol*. 2000;38:1008-15.
- Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, et al. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. *PLoS One*. 2010;6:e17936. doi: 10.1371/journal.pone.0017936.
- Dermota U, Jurca T, Harlander T, Košir M, Zajc U, Golob M, et al. Infections caused by community-associated methicillin-resistant *Staphylococcus aureus* European clone (ST80) in Slovenia between 2006 and 2013. *Zdr Varst*. 2016; 55:121-5. doi: 10.1515/sjph-2016-0018.
- Paterson GK, Harrison EM, Holmes MA. The emergence of mecC methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol*. 2014;22:42-7. doi: 10.1016/j.tim.2013.11.003.
- Štrumbelj I, Pirš M, Berce I, Fišer J, Golle A, Harlander T, Jeverica S, et al. Pregled občutljivosti bakterij za antibiotike - Slovenija 2015. Ljubljana: Slovenska komisija za ugotavljanje občutljivosti za protimikrobna zdravila (SKUOPZ), 2016. Accessed September 13th, 2020 at: <http://www.imi.si/strokovna-zdruzenja/skuopz>.