Slov Vet Res 2015; 52 (3): 155-60
UDC 579.861:577.2:57.083.1

Original Scientific Article

MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT *Staphylococcus aureus*, ST398 (LA-MRSA), FROM HUMAN SAMPLES

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Summary: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major human pathogen and an important cause of hospital-associated (HA-MRSA) infections. MRSA infections significantly increase morbidity and mortality, affect the increased use of antibiotics and the cost of treatment. During the last decade MRSA has emerged as a significant pathogen also in the community (community-associated; CA-MRSA). In recent years, livestock has been proven to be a source of human infections with the MRSA sequence type (ST) 398 (livestock-associated; LA-MRSA). During the year 2010 all the regional microbiological laboratories took part in the task of monitoring CA-MRSA infections in Slovenia. We included all patients harbouring a MRSA strain that was susceptible to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin or gentamicin. Altogether we collected 151 MRSA isolates of which 15 (9.9%) belonged to a spa type known to be associated with the clone ST398 respectively. Among them 12 isolates belonged to *spa* type t011, 2 isolates to t034 and 1 isolate to t108. We found the staphylococcal cassette chromosome - SCC*mec* type IV or V, and regulatory genes - *agr* type I. None of the isolates were positive for Panton - Valentine leukocidin (PVL), the toxic shock syndrome toxin (tst) and leukocidin *Luk*M. All MRSA isolates were resistant to tetracycline and penicillin. Some of them were also resistant to erythromycin and clindamycin. Most of the LA-MRSA ST398 were isolated from screening specimens of patients from Murska Sobota and Maribor, which are the most important agricultural regions with intensive livestock breeding. Evidence of the presence of LA-MRSA in humans requires a close cooperation of human and veterinary microbiologists. Our goal is to find the epidemiological relation between human and animal hosts, to obtain information on the phenotypic and genotypic characteristics and monitor infections caused by LA - MRSA strains.

Key words: LA-MRSA; human samples; ST398; Slovenia

Introduction

Staphylococcus aureus is present in commensally flora of humans and various animal species, where it can also cause a wide variety of infections (1-5). In humans, *S. aureus* is the most important cause of nosocomial infections, from superficial skin and soft tissue infections to life-threatening infections, while in animals, *S. aureus* mostly causes mastitis in cows and infection in chickens (4, 5).

Methicillin-resistant *S. aureus* (MRSA) was first identified in 1961 in the UK (6). The bacteria managed to spread around the world and became the main cause of hospital-associated (HA-MRSA) infections. HA-MRSA can cause different infections in hospitalized patients of all ages with risk factors, namely MRSA infection or colonization, surgery, admission to a nursing home, use of an indwelling catheter or other medical devices. MRSA infections

Received: 12 September.2014
Accepted for publication: 22 April 2015

significantly increase morbidity and mortality, affect the increased use of antibiotics and significantly increase the cost of treatment (6). During the last decade MRSA has emerged as a significant pathogen also in the community (community-associated; CA-MRSA) and has caused infections in young and healthy people lacking contact with healthcare (6, 7). In recent years, livestock has been proven to be a source of another kind of MRSA strains in human infections (livestock-associated; LA-MRSA) (1-5). In Europe, LA-MRSA mainly belongs to the sequence type (ST) ST398, while in Asia ST9 is predominant and in Korea ST72 (1, 3, 4, 5). LA-MRSA, HA-MRSA and CA-MRSA differ phenotypically and genotypically (1-6). LA-MRSA ST398 is non-typeable by PFGE using *Sma*I digestion. It carries a smaller staphylococcal cassette chromosome (SCC*mec*) element IV or V, accessory gene regulator (*agr*) type I and lack the major virulence factor of *S. aureus*, such us Pantone-Valentine Leukocidin (PVL), Leukocidin M (*LukM*), toxic shock syndrome toxin 1 (*tst*) and exfoliative toxins (1). LA-MRSA ST398 is generally resistant to tetracycline and in some cases to other antibiotics, such us macrolides, lincosamides, trimethoprim, fluoroquinolones and aminoglycosides (1, 3, 4). The main reservoirs of LA-MRSA ST398 are pigs, poultry or cattle and other animals (1, 2, 3, 5). Humans in close contact with livestock, such as farmers, veterinarians, are often colonized, so therefore the exposure to animals is a risk factor for LA-MRSA carriage (1, 2, 3). In the last years, human infections caused by MRSA ST398 have increasingly been documented in the Netherland, Belgium, Denmark, Germany and Austria, and MRSA ST398 has also been introduced into the healthcare setting mainly in areas with high density of livestock farming (2, 5).

Little is known about the epidemiology of livestock-associated MRSA in Slovenia. Recently, LA-MRSA was found in pigs, pork and dusts (8), but data from humans remains incomplete. The aim of this study was to investigate the presence of LA-MRSA ST398 in human samples, their antimicrobial resistance pattern, toxin gene profile and molecular characterization. For this purpose, we reviewed presumptive CA-MRSA isolates in the strain collection database of the microbiology department of the National Laboratory for Health, Environment and Food based on phenotypic characteristics retrospectively during a 12-month period in the year 2010. Only tetracycline resistant MRSA were included in further analyses.

Material and methods

National collection of presumptive CA-MRSA

Inclusion criteria for presumptive CA-MRSA were based on the antibiotic resistance profile (screening phenotypic pattern). In our national collection, we included only *S. aureus* isolates resistant to cefoxitin and oxacillin, and susceptible to at least two of the following four antibiotics: ciprofloxacin, erythromycin, clindamycin or gentamicin (9).

MRSA isolates

In the year 2010, we investigated 151 MRSA isolates with a positive screening phenotypic pattern isolated from asymptomatic carriers or clinical specimens in Slovenian microbiological laboratories. Thirty-one (20.5%) MRSA isolates were tetracycline resistant and were isolated from unrelated patients. All isolates were identified by mass spectrometry (MALDI-TOF MS, Biotyper, Bruker Daltonic GmBH, Bremen, Germany).

Susceptibility testing

The susceptibility testing was performed on Mueller–Hinton agar with the disk diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) (10). The antibiotics tested were penicillin, cefoxitin, vancomycin, gentamicin, tobramycin, kanamycin, erythromycin, clindamycin, tetracycline, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, linezolid, mupirocin and fusidic acid (BD, Maryland). The minimal inhibitory concentration (MIC) for oxacillin and vancomycin was performed using the E-test (bioMerieux, France).

Molecular analysis

All MRSA isolates were molecularly characterized. The SCC*mec* typing was determined by multiplex PCR described previously (11). Methicillin resistance was confirmed by *mec*A and *mec*C PCR (12). Genes encoding accessory gene regulator (*agr*), staphylococcal enterotoxin (*sea* to *see, seg, seh, sei, sek, sel, sen, seo, seu, seq*), toxic shock syndrome toxin-1 (*tst*), staphylococcal exfoliative toxins (*eta, etb, etd*), leukocidin (*luk*M) and Panton-Valentine

leukocidin (*luk*S-*luk*F) were detected by multiplex PCR (13, 14). Amplification, sequencing and analyses of the polymorphic region of the protein A (*spa* typing) and MLST were performed according to a method described previously (15, 16).

Results

Among 31 tetracycline resistant MRSA isolated in the year 2010 from single patients, 15 (48%) isolates belonged to ST398 and presented three different *spa* types: t011 (12 isolates), t034 (2 isolates) and t108 (1 isolate). The SCC*mec* type V was predominant in 14 isolates and the SCC*mec* type IV was found only in one isolate. The *agr* type I and the *mec*A gene were determined in all 15 isolates. 10 isolates with the *spa* type t011 were resistant to penicillin, cefoxitin and tetracycline, 1 isolate with the *spa* type t108 was resistant to penicillin, cefoxitin, tetracycline and chloramphenicol, 2 isolates with the *spa* type's t011 and t034 were resistant to penicillin, cefoxitin, erythromycin, clindamycin and tetracycline. None of the isolates were positive for PVL leukocidin, the toxic shock syndrome toxin and leukocidin *Luk*M. The gene for enterotoxin type O was detected in 6 isolates, the enterotoxin type U was detected in 4 isolates and the enterotoxin type K or I in 1 isolate (table 1). 14 isolates ST398 were isolated from asymptomatic carriers and 1 isolate from a clinical specimen, wound swab. Most isolates were from patients from Murska Sobota and Maribor.

The remaining 16 tetracycline resistant MRSA isolates belonged to 11 different *spa* types: t127 (4 isolates), t015 (3 isolates), t002, t091, t174, t595, t701, t791, t1094, t1218, t11983 (1 isolate of each). The SCC*mec* type IV was predominant in 10 isolates, the SCC*mec* type V was found in 5 isolates and 1 isolate was non-typeable. Five isolates with the *spa* type t015 (3 isolates), t701 and t791 were resistant to penicillin, cefoxitin and tetracycline. One isolate with the *spa* type t791 was PVL positive and belonged to ST72. Isolates with the *spa* type t127 (4 isolates) and t174 (1 isolate) belonged to ST1. These isolates were resistant to penicillin, cefoxitin, erythromycin, kanamycin and tetracycline, while one isolate was also resistant to clindamycin.

Table 1: Characterization of 15 tetracycline resistant MRSA isolated from humans in Slovenia, in the year 2010

Isolate number	Isolate area in Slovenia	Patient gender	Origin	Resistance pattern to antibiotics	PCR		*Spa* type
					SCC*mec* type	Toxin profile	
10-20	MS	F	Screening swab	P	V	-	t011
10-29	MS	F	Screening swab	P, C	V	-	t108
10-39	MS	F	Wound swab	P	V	*selo, selu*	t011
10-40	MS	M	Screening swab	P, E, CC	V	*selo, selu*	t011
10-41	MS	M	Screening swab	P	IV	*selo, selu*	t011
10-46	KP	F	Screening swab	P, E, CC	V	*selo, selu*	t034
10-58	MS	M	Screening swab	P	V	-	t011
10-59	MS	F	Screening swab	P	V	-	t011
10-60	MS	F	Screening swab	P	V	-	t011
10-61	MS	M	Screening swab	P	V	-	t011
10-62	MS	M	Screening swab	P	V	sek	t011
10-67	LJ	F	Screening swab	P, E, CC	V	-	t034
10-115	MB	M	Screening swab	P, E, CC	V	*selo*	t011
10-121	MB	M	Screening swab	P	V	*sei, selo*	t011
10-140	MB	F	Screening swab	P	V	-	t011

Legend:
MS Murska Sobota, KP Koper, LJ Ljubljana, MB Maribor, F female, M male, P penicillin, E erythromycin, C chloramphenicol, CC clindamycin, PCR polymerase chain reaction, SCC*mec* Staphylococcal cassette chromosome *mec*, selo / selu staphylococcal enterotoxin like type O / U, sek / sei staphylococcal enterotoxin type K / I

Discussion

The data about the epidemiology of MRSA among humans in Slovenia are limited. Only few data are available about *spa* types for individual hospitals within a fixed period (9, 17). During the last years, CA-MRSA was found in a low, but increasing number in Slovenian patients. We documented outbreaks of four cases of skin and soft tissue infections due to a CA-MRSA strain obtained from one hospital in 2003 and 2004 (*spa* type t044, ST80) (18). In the year 2005, Mueller-Premru *et al*. reported of PVL-positive CA-MRSA strains among football players (*spa* type t002, ST5 and *spa* type t454, ST152) (19). We confirmed *mec*C-positive MRSA in humans, but no epidemiological connection between humans and animals were found (20). To date, LA-MRSA has not been documented among human isolates. Antimicrobial susceptibility patterns of LA-MRSA are similar to CA-MRSA, and ST398 is one of the predominant clones of CA-MRSA in Europe (21). Therefore, we checked a national collection of presumptive CA-MRSA isolates collected in the year 2010. As we suspected, we found MRSA belonging to a pig-associated clone ST398. MRSA ST398 was mainly detected in colonized patients from rural areas, Murska Sobota and Maribor, where livestock breeding is an important agricultural activity in our country. Human carriage of LA-MRSA is strongly related to direct contact to livestock, but recent studies confirmed the presence of LA-MRSA also in people without risk factors (1, 2, 5). Epidemiological information about animal contact in the patients included in our study was lacking, so the connection between humans and animals could not be confirmed. Despite of that, human colonization upon admission to healthcare indicates strong connection between high pig-farming density and their probably close contact with livestock. Carriage of MRSA ST398 could also be a risk factor in the development of possible infection (1, 2, 4). MRSA ST398 is mainly associated with skin and soft-tissue infections (4, 5), while in our study the MRSA ST398 was detected from wound swab only in one isolate.

The majority of LA-MRSA ST398 lacks many virulence factors that are found in HA-MRSA or CA-MRSA (1, 2, 3, 5). One exception was PVL positive ST398, found in several Chinese isolates (4, 21). In our study, none of the isolates in our study belonging to ST398 carried the leukocidine PVL and *Luk*M or *tst*. The data on the presence of staphylococcal enterotoxin genes in isolates of ST398 are limited. Small number of porcine ST398 isolates carried the enterotoxin genes *seb* or *sek* and *seq* (22). We confirmed different enterotoxins genes (*sek, sei, selo, selu*) in 7 (46.7%) isolates.

In addition to tetracycline resistance, 27% of the MRSA ST398 also presented resistance to macrolides and lincosamides. The resistance to chloramphenicol was detected in one MRSA ST398. Tetracycline resistance could be a good phenotypic marker for the detection of potential LA-MRSA, but we should be aware that different HA-MRSA and CA-MRSA clones are circulating in Slovenia and some of them are also resistant to tetracycline (17-22). Other STs (*spa* type - number of isolate) detected in this study, which genetically are not associated to LA-MRSA ST398, were ST1 (t127-4, t174-1), ST5 (t002, t1094, t11983-1), ST6 (t701-1), ST7 (t091-1), ST22 (t1218-1), ST45 (t015-3), ST72 (t791-1) and ST152/377 (t585-1). Most of these isolates were also resistant to kanamycin and other antimicrobial agents.

Evidence of the presence of LA-MRSA in humans requires a close cooperation of human and veterinary microbiologists. In the future, we all want to find the epidemiological relation between both hosts, to acquire information on the phenotypic and genotypic characteristics and to monitor infections caused by LA - MRSA strains.

Acknowledgements

The authors are gratefull to Iztok Štrumbelj, Martina Kavčič, Tatjana Harlander, Tjaša Žohar-Čretnik, Slavica Lorenčič Robnik, Živa Petrovič, Ljudmila Sarjanović, Ingrid Berce (National Laboratory for Health, Environment and Food) and Jerneja Fišer, General Hospital dr. Franc Derganc, for providing the presumptive CA-MRSA isolates.

None conflict of interest statement and no funding sources.

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MOLEKULARNA OPREDELITEV PROTI METICILINU ODPORNE BAKTERIJE *Staphylococcus aureus*, KI PRIPADA KLONU ST398 (LA-MRSA) IZ HUMANIH VZORCEV

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Povzetek: Proti meticilinu odporna bakterija *Staphylococcus aureus* (MRSA) ima v zdravstvu zelo velik pomen. Okužbe s to bakterijo pomembno povečajo obolevnost, umrljivost, vplivajo na povečano rabo antibiotikov in pomembno povečujejo stroške zdravljenja. MRSA povzroča okužbe v bolnišničnem (angl. *hospital-acquired*; HA-MRSA) in v domačem okolju (angl. *community-acquired*; CA-MRSA), v zadnjih letih pa novo grožnjo za okužbe pri ljudeh predstavljajo rejne živali. Te so z LA-MRSA (angl. livestock-associated) večinoma kolonizirane, obravnavane bakterije pa pri njih le redko povzročajo okužbe. LA-MRSA najpogosteje povezujejo s sekvenčnim tipom (ST) ST398 oz. klonskim kompleksom (CC) CC398.
Od 1.1.2010 do 31.12.2010 smo v mikrobioloških laboratorijih po Sloveniji, ki opravljajo mikrobiološko diagnostiko, zbirali seve CA-MRSA. V raziskavo smo vključili le izolate, ki so bili odporni proti oksacilinu oz. cefoksitinu in občutljivi vsaj za dva antibiotika, in sicer za ciprofloksacin, eritromicin, klindamicin ali gentamicin. Po tem kriteriju smo zbrali 151 izolatov MRSA, od katerih jih je 15 (9,9 %) pripadalo tipom *spa*, ki jih povezujejo s klonom ST398 oz. MRSA rejnih živali. V sekvenčni tip ST398 smo uvrstili 12 izolatov s tipom *spa* t011, 2 izolata s tipom *spa* t034 in 1 izolat s tipom *spa* t108. Dokazali smo stafilokokni kromosom kasete - SCC*mec* tip IV ali V in regulatorni tip genov - *agr* I. Noben izolat ni imel zapisov za levkocidin Panton-Valentine (PVL), toksin toksičnega šok sindroma (tst) in levkocidin *Luk*M. Vsi izolati MRSA so bili odporni proti tetraciklinu in penicilinu, nekateri tudi proti eritromicinu in klindamicinu. Večino LA-MRSA s sekvenčnim tipom ST398 smo dokazali iz nadzornih kužnin pri ljudeh s področja Murske Sobote in Maribora, kar nakazuje kolonizacijo oz. nosilstvo predvsem pri prebivalcih, ki živijo na območju, kjer sta razvita poljedelstvo in živinoreja.
Dokaz prisotnosti LA-MRSA tudi pri ljudeh predstavlja medicinskim in veterinarskim mikrobiologom velik izziv za medsebojno sodelovanje, katerega cilj je ugotoviti epidemiološko povezavo med obema gostiteljema, pridobiti informacije o fenotipskih in genotipskih lastnostih teh sevov LA-MRSA ter slediti pogostosti okužb, povzročenih s sevi LA-MRSA.

Ključne besede: LA-MRSA; humani vzorci; ST398; Slovenija