ORIGINAL ARTICLE

Methicillin-resistant S. aureus (MRSA), extended-spectrum (ESBL)- and plasmid-mediated AmpC ß-lactamase -producing Gram-negative bacteria associated with skin and soft tissue infections in hospital and community settings

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ABSTRACT

Aim To investigate the characteristics of meticillin-resistant *S. aureus* (MRSA), extended-spectrum (ESBL), and plasmid-mediated AmpC beta-lactamase producing Gram-negative bacteria causing skin and soft tissue infections (SSTIs) in hospital and outpatient settings of Zenica-Doboj Canton, Bosnia and Herzegovina.

Methods Antibiotic susceptibility was determined by disc-diffusion and broth microdillution methods according to CLSI guidelines. *MecA* gene was detected by PCR, and genetic characterization of MRSA was performed using *spa*-typing and the algorithm based upon repeat patterns (BURP). Double-disk-synergy test was used to screen for ESBLs. PCR was used to detect bla_{ESBL} alleles. Genetic relatedness of the strains was tested by PFGE.

Results Seventeen in-patients with MRSA, 13 with ESBL-producing Gram-negative bacteria and three patients co-infected with both, were detected. Five MRSA and 16 ESBL-producing Gram-negative bacteria were found in outpatient samples. *Klebsiella* spp. was isolated in 11 in- and seven outpatients. MLST CC152 was the most prevalent MRSA. Seven (38.9%) *Klebsiella* spp. yielded amplicons with primers specific for SHV, TEM-1 and CTX-M group 1 β-lactamases. Eight *K. pneumonia* (44.4%) and 16 (64%) MRSA (including the in- and outpatient) strains were clonally related.

Conclusion The presence of MRSA and ESBL-producing organisms causing SSTIs in the community poses a substantial concern, due to the high morbidity and mortality associated with possible consequent hospital infections.

Key words: surgical wound infections, CTX-M beta-lactamase, MLST CC152, antibiotic resistance

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INTRODUCTION

According to Edelsberg's classification skin and soft tissue infections (SSTIs) include eighteen types of infections (1). With regard to predominanted (microbial etiology) pathogens and risk of mortality (severity of local and systemic signs) there are superficial SSTIs caused by *Staphylococcus aureus* or *Streptococcus pyogenes,* deeper or healthcare-associated infections caused by anaerobic or gram-negative organisms, and gangrenous or necrotizing infections (or "often fatal infections") (1,2). The practice guidelines of the Infectious Diseases Society of America (IDSA) for the diagnosis and management of skin and soft tissue infections (3) classifies SSTIs into five categories, including superficial, uncomplicated infection (impetigo, erysipelas and cellulitis), necrotizing infection, infections associated with bites and animal contact, surgical site infections and infections in the immunocompromised host. The purpose of all SSTI definitions is to develop the useful guidelines for the clinical management and treatment options for patients with SSTIs (1,3).

The annual frequency of visits to physicians' offices for SSTIs have an increasing trend (4). The predominant pathogens associated with SSTIs in hospitalized patients include *S. aureus* (ranked first in all geographical regions), *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus* spp. (5).

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes many infections, but most frequently SSTIs, such as cutaneous abscesses, furuncles and cellulitis. Thus, the prevalence of these infections has increased dramatically (6). Risk factors for MRSA SSTIs include the presence of an abscess, previous MRSA colonization/infection, antibiotic prescriptions within 8 weeks, diabetes mellitus and hospital admission within the preceding year (6).

Extended-spectrum beta-lactamase (ESBL) production is one of the main mechanisms of resistance to beta-lactam antibiotics in Enterobacteriaceae, so the therapeutic choices in infections caused by such strains are limited (7,8). Most ESBLs belong to SHV and TEM family, but recently a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamase) has been reported (8). In contrast to TEM or SHV-ESBLs, CTX-M β-lactamases are native ESBLs and are derived from the chromosomal β-lactamases of the genus *Kluyvera* (9). In many countries CTX-M β-lactamases are the most prevalent type of ESBLs (9,10). Plasmid-mediated AmpC β-lactamases are derived from chromosomal *ampC* genes of the family *Enterobacteriaceae*. AmpC enzymes encoded by both chromosomal and plasmid genes are also evolving to hydrolyze broad-spectrum cephalosporins more efficiently (11).

Since most skin and soft tissue infections in outpatient settings are treated with empiric antimicrobial therapy, it is very important to estimate the prevalence of causative agents associated with skin and soft tissue infections, as well as their antimicrobial resistance patterns and mechanisms (3-5) .

The aim of this study was to determine prevalence and molecular epidemiology of SSTIs caused by MRSA, ESBL- and plasmid-mediated AmpC-producing β-lactamase Gram-negative bacteria in the in- and outpatient settings in Zenica-Doboj Canton, Bosnia and Herzegovina (B&H).

MATERIALS AND METHODS

Setting, bacterial isolates and study design

The Cantonal Hospital Zenica, B&H, is a 849-bed tertiary level hospital admitting about 25.000 patients/year, with 240.000 patient days, and covers a population of 331.229 in Zenica-Doboj Canton, B&H.

All consecutive, non-duplicate strains identified as MRSA and/or ESBL- or plasmid-mediated AmpC β-lactamase-producing Gram-negative bacteria obtained from SSTIs of the in- and outpatients during the period December 2009–May 2010 were analyzed. The SSTIs comprise surgical wound infections (SWIs) (postoperation and postraumatic wound infection) and ''other SSTIs'' (oSSTIs) (including furuncles/abscesses, cellulitis, folliculitis) documented by the clinical provider/physician).

Clinical and epidemiological data recorded for the patients involved in the study included: age, gender, occupation, place of residence at admission to the hospital (e.g. at home, other hospital, nursing home), contact with person(s) with history of hospitalization in the past 12 months, hospital department, antibiotic usage in the past

four months, isolated causative agent (MRSA and/or ESBL or plasmid-mediated AmpC β-lactamase-producing Gram–negative bacteria).

An institutional review board approval had been obtained from the Ethics Committee in the Cantonal Hospital of Zenica prior to the initiation of the study, and all the participants read and signed informed consents about the purpose of the study (participation was voluntary and anonymous) as well.

Identification of MRSA and susceptibility testing

Staphylococcus aureus isolates were identified according to standard microbiological methods. The strains were tested for oxacillin and cefoxitin sensitivity/resistance by disk-diffusion method at Mueller-Hinton (MH) agar (Oxoid, Basingstoke, UK) (growth zone inhibition around 1 μg and 30 μg oxacillin and cefoxitin disk, respectively) in accordance with CLSI (Clinical Laboratory Standards) guidelines (12).

All *S. aureus* isolates were analyzed for the presence of the *S. aureus*-specific *femA* gene as well as the MRSA-specific *mecA* gene using a multiplex real-time PCR assay (13).

The disc diffusion method using Mueller-Hinton agar (Oxoid, Besingstoke, UK) was used to test susceptibility to 11 antimicrobials (Oxoid, Basingstoke, UK): mupirocin, MUP (200 μg), imipenem, IPM (10 μg), erythromycin, ERY (15 μg), vancomycin, VAN (30 μg), gentamicin, GEN (10 μg), amikacin, AMK (30 μg), ciprofloxacin, CIP (5 μg), clindamycin, CLI (2 μg), trimethoprim/sulfamethoxazole, SXT (25 μg), chloramphenicol, CHL (30 μg), and rifampicin, RIF (5 μg). The susceptibility testing results were interpreted according to CLSI (12). Staphylococcus aureus ATCC 25923 control strain was used for quality control. Multidrug resistance (MDR) was defined as resistance to three or more groups of antibiotics.

Susceptibility testing of ESBL and AmpC producing bacteria

The susceptibility testing to cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), cefotaxime (CTX), cefoxitin (FOX), tazobactam (TZP), cefepime (FEP), gentamicin (FEP), ciprofloxacin (CIP), and piperacillin (PIP) was performed by a twofold microdilution technique according to CLSI standard procedures (12). Susceptibility to imipenem (10 μg), meropenem (10 μg), tetracycline (30 μg), chloramphenicol (30 μg) and sulphametoxazole /trimethoprim (23.75 /1.25 μg) was performed by disk diffusion test (12).

Phenotypic detection of ESBLs and plasmid-mediated AmpC ß-lactamases

A double-disk-synergy test (DDST) using the combination of amoxycillin/clavulanate with cefotaxime, ceftriaxone, ceftazidime, and cefepime was performed to detect the production of ESBLs. Distortion of the inhibition zones around cephalosporin and aztreonam disks towards central disk was considered as a positive result (14). Production of ESBLs was confirmed by CLSI combined disk test.

Production of plasmid-mediated AmpC β-lactamase was tested in *E. coli*, *Klebsiella* spp. and *P. mirabilis* by combined disk test using 3-amino phenyl boronic acid. An overnight Mueller-Hinton (MH) broth culture of the strains was adjusted and swabbed on MH agar and disks of cefotaxime, ceftriaxone, ceftazidime and cefepime were placed on the surface of the agar plate. 10 μl of 3-amino phenyl boronic acid (20 mg/mL) was dropped on the disks containing cefotaxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg) and cefepime (30 μg). Control plate contained disks of the same cephalosporins without phenyl boronic acid. Augmentation of the inhibition zones around cephalosporin disks for ≥ 5 mm in the presence of boronic acid was indicative for production of AmpC β-lactamases (15).

Transfer of resistance determinants

The transferability of cefotaxime resistance was tested by conjugation (broth mating method). Enterobacteriaceae were investigated for the transferability of their resistance determinants. Conjugation experiments were set up employing plasmid-free and rifampin-resistant *E. coli* A15 R^- recipient strain (15). Transconjugants were selected on the combined plates containing cefotaxime (1 mg/L) and rifampicin (256 mg/L). The frequency of conjugation was expressed relatively to the number of donor cells.

Typing of the *spa* locus of MRSA isolates

Real-time amplification of the *spa* locus followed by sequencing was performed as des-

cribed above (16). The *spa* types were clustered into *spa* CCs (clonal complexes) using the algorithm based upon repeat pattern (BURP) with the Ridom Staph Type, version 1.5, software package (http://www.ridom.de) (17). The default settings recommended by the manufacturer were used. Since it has been shown that *spa* typing, together with the algorithm BURP, yields results consistent with typing results obtained by MLST (17-19), the associated CCs, as determined with MLST, were allocated through the Ridom SpaServer (http://spaserver.ridom.de).

Molecular characterization of ESBL and plasmid-mediated AmpC ß-lactamases

PCR was used to detect alleles encoding ESBL enzymes. Extended-spectrum β-lactamases were characterized at the molecular genetic level. The presence of bla_{TEM}, $bla_{SHV,}$ $bla_{CTX-M,}$ ESBL genes was investigated by polymerase chain reaction (PCR) using primers and conditions as described previously (9,20,21). Template DNA was extracted by boiling method. PCR mix (50 µl) contained 25 µl of master mix (Roche), 20 µl of ultrapure water, 1 µl of each primer (10 pmol) and 3 µl of template DNA. Strains positive for CTX-M beta-lactamases were further tested by multiplex PCR with primers specific for CTX-M groups 1, 2, 8, 9 and 25 (22). Amplicons were column-purified (Quiagen DNA purification kit) and sequenced directly using ABI PRISM 377 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using BioEdit v.7.0.9. (Ibis Biosciences) program. Designation of bla genes based on identified mutations was done according to Bush and Jacoby (23).

Primers IS26F (5'-GCG-GTA-AAT-CGT-GGA-GTG-AT-3) and IS26R (5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3') were used to amplify 400 bp fragment spanning the link between IS26 insertion sequence and bla_{CTX-M} gene in CTX-M producing isolates (22). Primers ISEcp1L1 (CAGCTTTTATGACTCG) and ALA-5 (CCTAAATTCCACGTGTGT) were applied to amplify the IS*Ecp1* insertion sequence (10).

Multiplex PCR with primers specific for MOX, CMY, DHA, and FOX β-lactamases was used to detect plasmid-mediated Amp β-lactamases in *E. coli*, *Klebsiella* spp. and *P. mirabilis* strains resistant to cefoxitin and β-lactam/inhibitor combinations (11).

Typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA

Isolation of genomic DNA, its digestion with the *Xba*I restriction enzyme (Invitrogen) and PFGE of the resulting fragments was performed as described by Kaufman et al. (24,25). The electrophoresis was carried out with a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA). The PFGE patterns were compared following the criteria of Tenover et al. (26) and analyzed by the GelComparII software (Applied Maths, St Martens, Belgium). The patterns obtained were compared by clustering methods (unweighted pair group methods with arithmetic averages) using the Dice coefficient. The optimization of 0.5% and position tolerance of 3% were applied.

RESULTS

During the period December 2009 – May 2010, 33 hospitalized patients with SSTIs caused by MRSA or/and ESBL-producing Gram-negative bacteria were identified: 17 patients had infection caused by MRSA, 13 patients had infection caused by ESBL-producing Gram-negative bacteria, and three patients had co-infection with MRSA and ESBL-producers.

MRSA infections

Among 20 in-patients infected with MRSA, six (30%) had surgical wound infections (SWI) and 14 (60%) had oSSTIs. Three patients with MRSA (two were *spa*-type t355) infection had co-infection with MSSA (one patient with oSST at Dermatology, and two patients with SWI at surgery and orthopaedic department, respectively). Five (25%) in-patient and two (out of five) outpatient isolates were susceptible to all antibiotic tested, respectively. None of the isolates were multidrug-resistant. Most in-patient isolates have shown gentamicin resistance phenotype, 13 (65.0%).

In outpatient settings five SSTIs were noted, all were oSSTIs; one was MRSA MLST CC152 (newborn).

Among 14 oSSTIs, all but one MRSA belonged to *spa*-clonal-complex (CC) 355/595 associated with MLST CC152. Among six MRSA isolated

from SWIs, MLST CC152 was found in three cases (Table 1).

Most in-patients with MRSA SSTIs were admitted to the hospital from home, with an exception of five patients who were transferred from other hospital or from nursing home (four MRSA belonged to MLST CC152) (Table 1). All hospitalized patients with SSTIs had contact with persons with positive history of hospitalization. A history of β-lactam antibiotics usage in combination with glycosides was positive in 16 patients (data not shown).

Table 1. Characteristics of patients with MRSA infections and susceptibility /resistance to antibiotics

Protocol No	Gender	Isolate origin	Age (years)	HospItal department	Spa-type (Spa CC) (MLST CC)	Residance before hospitalization	Susceptibility/resistance to antimicrobial agents*										
							IMP	ERY	VAN	GEN	AMK	CIP	CLI	SXT	CHL	RA	MUP
3196	F	oSSTI	42	Dermatology	t355 (355/595) (152)	Home	S	S	S	R	S	S	S	NT	NT	S	S
21441†	M	oSSTI	40	Dermatology	t355 (355/595) (152)	Home	NT	S	S	R	NT	S	S	S	S	S	S
245	F	SWI	54	ICU	t355 (355/595) (152)	Other hospital	S	S	S	R	S	S	S	NT	NT	S	S
13549	M	SWI	60	Internal	t1855 (singleton)	Home	NT	S	S	S	NT	S	S	S	NT	S	S
13476 †	F	SWI	45	Orthopedics	t041 (002) (005)	Other hospital	NT	S	S	S	NT	S	S	S	NT	S	S
4357	F	oSSTI (umbilicus)	<01	Pediatrics	t355 (355/595) (152)	Health care center	S	S	S	S	S	S	S	NT	NT	S	S
2236	M	oSSTI	01	Pediatrics	t355 (355/595) (152)	Health care center	S	S	S	R	S	S	S	NT	NT	S	S
17304	F	oSSTI	01	Pediatrics	t355 (355/595) (152)	Home	NT	S	S	S	NT	S	S	R	S	S	S
2822	F	oSSTI	01	Pediatrics	t355 (355/595) (152)	Home	S	S	S	R	S	R	S	NT	NT	S	S
33733	M	oSSTI	01	Pediatrics	t355 (355/595) (152)	Home	NT	S	S	R		S	S	S	S	S	S
4189	M	oSSTI	<01	Pediatrics	t355 (355/595) (152)	Home	S	S	S	R	S	S	S	NT	NT	S	S
39027	M	oSSTI	<01	Pediatrics	t355 (355/595) (152)	Home	NT	S	S	R	NT	S	S	S	S	S	S
8723	M	oSSTI	<01	Pediatrics	t355 (355/595) (152)	Home	S	S	S	R	S	S	S	NT	NT	S	S
5928	M	oSSTI (umbilicus)	<01	Pediatrics	t355 (355/595) (152)	Home	S	S	S	R	S	S	S	NT	NT	S	S
9522	F	oSSTI	<01	Pediatrics	t355 (355/595) (152)	Home	S	S	S	R	S	S	S	NT	NT	S	S
25621	F	oSSTI	02	Pediatrics	t595 (355/595) (152)	Home	NT	S	S	R	NT	S	S	S	S	S	S
16578	M	oSSTI	<01	Pediatrics	t919 (008/024) (008)	Home	NT	S	S	S	NT	S	S	S	S	S	S
26267 †	F	SWI	01	Surgery	t355 (355/595) (152)	Home	NT	S	S	R	NT	S	S	S	S	S	S
32913	M	SWI	<01	Surgery	t355 (355/595) (152)	Health care center	NT	S	S	R	NT	S	S	S	S	S	S
129/U	M	SWI		Urology	t7250 (singleton)	Home	S	S	S	S	S	S	S	S	S		S
7559	M	oSSTI	01	Outpatient	t728 (015) (045)	DM	S	S	S	S	S	S	S	S	S	S	S
13802	F	oSSTI (umbilicus)	01	Outpatient	t355 (355/595) (152)	DM	NT	S	S	S	NT	S	S	S	NT	S	S
20733	F	oSSTI	<01	Outpatient	Not typeable	DM	NT	R	S	R	NT	R	S	R	S	S	S
33005	M	oSSTI	29	Outpatient	t451 (008/024) (008)	DM	NT	R	S	R	NT	S	S	R	S	S	S
16548	M	oSSTI	01	Outpatient	Not typeable		NT	S	S	R	NT	S	S	S	S	S	S

*IMP, imipenem (10 μg); ERY, erythromycin (15 μg); VAN, vancomycin (30 μg); GEN, gentamicin (10 μg), AMK, amikacin (30 μg); CIP, ciprofloxacin (5 μg); CLI, clindamycin (2 μg); SXT, trimethoprim/sulfamethoxazole (25 μg); CHL, chloramphenicol (30 μg); RIF,. rifampicin (5 μg); MUP, mupirocin (100 μg); †Patients with MRSA and ESBL-producing Gram-negative bacteria coinfection Spa- CC, spa clonal complex; MLST CC, MLST clonal complex; SWI, surgical wound infection; oSSTI, other skin and soft tissue infection (other than SWI); NT, not tested; DM, data missing;

Among 23 MRSA strains analyzed by PFGE, 15 (65.2%) belonged to the same clonal complex (Figure 1).

ESBL infections

Among 16 in-patients with infection caused by ESBL-producing Gram-negative bacteria two (12.5%) had SWI and 14 (87.5%) had oSSTI. Most oSSTI hospital infections were registered at pediatric department, nine (56.3%) (all were newborns). ESBL-producing *Klebsiella* spp. was the most frequently isolated Gram-negative bacteria, in 12 (75%) in-patients of which *K. pneumonia* was isolated in 11 cases.

The total number of 18 outpatients were infected with ESBL-producing Gram-negative bacteria, of which 17 (94.4%) had SWI and one had oSSTI. Two outpatients had co-infection with two Gram-negative bacteria (*K. pneumoniae* in both cases with *K. pneumoniae* and *Pseudomonas aeruginosa*, respectively). *Klebsiella* spp. was the most isolated, in seven (38.9%) cases. The four in-patients were transferred from another hospital. Eleven (61.1%) outpatients were ≥60 years of age (Table 2).

Almost all in-patient ESBL-producing *Klebsiella* spp. isolates were resistant to gentamicin. Resistance to ciprofloxacin was noted in six *Klebsiella* spp. from in-patients, and in four outpatients. Two *E. coli* isolates have shown high-level resistance to almost all tested antibiotics. From one of these, MRSA was isolated too. All but one (*Pseudomonas*) isolates remained susceptible to carbapenems (Table 2).

Eight strains transferred cefotaxime resistance to *E. coli* recipient strains with frequency ranging from 10^{-7} to 10^{-4}. Resistance to gentamicin, tetracycline, chloramphenicol and cotrimoxazole was cotransferred alongside with cefotaxime resistance in four strains (data not shown).

Seven (out of 18, 38.9%) *K. pneumoniae* isolates (all were from the in-patients) yielded amplicons with primers specific for all three SHV, TEM-1 and CTX-M group 1 β-lactamases, one of which

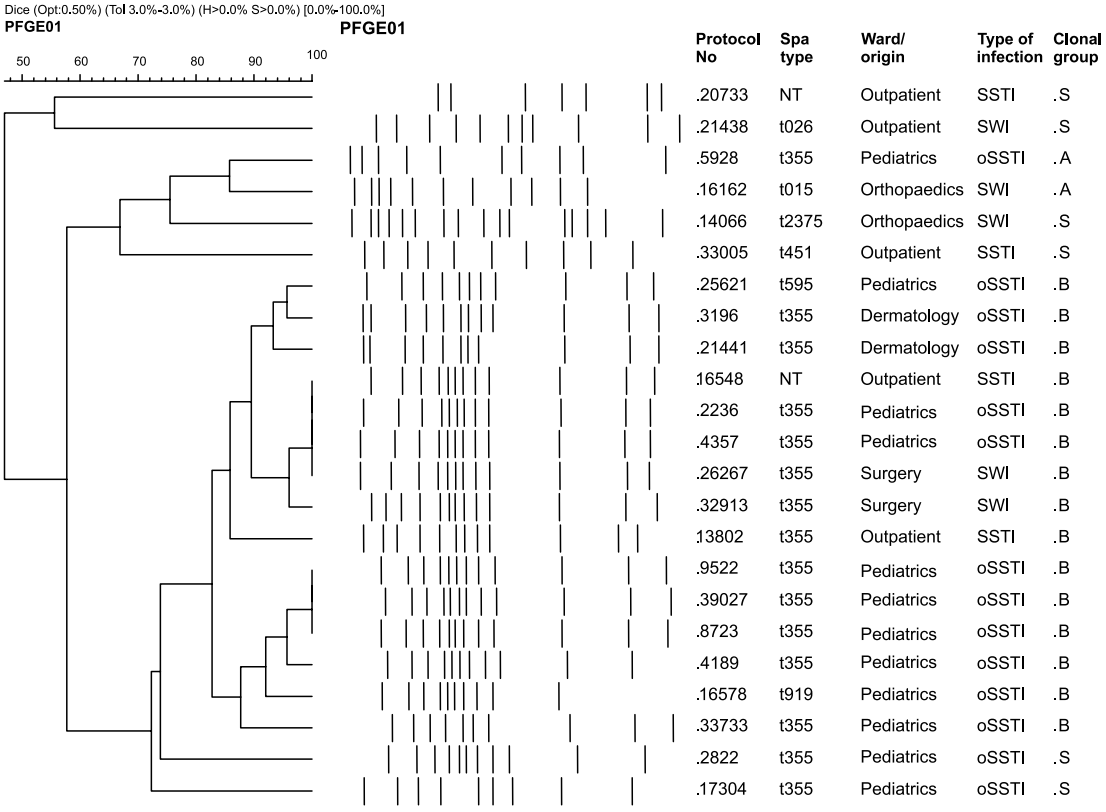

Figure 1. Dendogram showing the genetic relatedness of the 23 MRSA isolates. Two groups were identified by PFGE typing (A and B) by using 80% similarity. Group A consisted of two, group B of 15 isolates and they appeared to be clonally related S, singleton; oSSTI, other skin and soft tissue infections; SWI, surgical wound infections; NT, non-typeable;

Table 2. Characteristics of patients with ß-lactamase producing Gram-negative bacteria causing skin and soft tissue infections and antibiotic susceptibility

Protocol No	gender	Age (years)	Isolate origin/ diagnosis	Hospital department	Causative agent isolated	Residance before hospitalization	Antibiotic (MIC in µg/mL)*												Type of ß-lactamase†
							AMX	PIP	TZP	AMC	CZ	CXM	CAZ	CTX	CRO	FOX	FEP	GM	CIP	
11284	M	60	SWI	outpatient	E. coli	Home	≥256	32	16	NT	NT	>256	32	128	32	>256	16	64	1	TEM
30047	M	53	SWI	outpatient	E. coli	Home	≥256	>256	16	16	>256	>256	>256	64	256	256	64	256	8	+
11511	M	72	SSTI	outpatient	E.coli	Home	≥256	16	8	4	>256	>256	1	1	<0.12	256	0,25	64	16	TEM, CTX-M
22853	F	71	SWI	outpatient	E.coli	Home	≥256	64	64	4	4	>256	16	32	128	256	128	32	2	TEM
8851	F	73	SWI	ICU	Enterobacter cloacae	Other hospital	≥256	>256	8	>128	>128	>256	8	32	32	>128	8	>256	2	+
13819	F	85	SWI	outpatient	Enterobacter cloacae	Home	≥256	8	2	>128	>128	32	128	32	4	128	16	16	1	AmpC
22040‡	F	<01	oSSTI (umbilicus)	Pediatrics	Enterobacter cloacae	Home	≥256	>256	64	>128	>128	>256	>256	>256	>256	>128	64	>256	256	SHV-1, TEM-1, CTX-M 15, OXA1
28268	M	46	SWI	outpatient	Enterobacter cloacae	Home	≥256	4	2	>128	>128	16	128	32	8	128	16	16	1	TEM, CTX-M, OXA1
14754	M	39	oSSTI	Internal	K. oxytoca	Home	≥256	128	32	16	>256	>256	>256	16	256	>128	16	16	16	SHV-1, TEM, CMY-2
32049	F	63	oSSTI (combustio, gangrena)	Surgery	K. pneumoniae	Home	≥256	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	SHV-1, TEM, CTX-M 1
1360	M	01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	>256	16	16	>256	>256	128	128	128	>128	8	128	4	SHV-1, TEM-1, CTX-M 15, OXA1
2671	F	01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	>256	64	16	>256	64	8	128	128	>128	32	>256	32	SHV-1
4357	F	<01	Other SSTI (umbilicus)	Pediatrics	K. pneumoniae	Other hospital	≥256	128	1	16	>256	>256	>256	16	32	>128	2	4	≤0.12	SHV-1, TEM
5139	F	<01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	>256	64	16	>256	>256	>256	>256	>256	128	32	0,5	64	SHV-1, TEM-1, CTX-M 15
9474	M	28	SWI	outpatient	K. pneumoniae	Home	≥256	16	16	16	>256	64	64	128	16	128	64	>256	128	SHV-1
21438§	M	47	SWI	outpatient	K. pneumoniae	Home	≥256	64	64	16	>256	>256	16	64	128	256	128	128	2	SHV-1
21534	F	<01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	128	16	16	>256	>256	>256	128	>256	>128	8	64	1	SHV-1, TEM, CTX-M 1
22050	F	01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	128	128	4	<0.12	>256	>256	32	64	>128	32	32	4	SHV-1
22063	F	74	oSSTI	Internal	K. pneumoniae	Other hospital	≥256	NT	NT	8	>256	NT	NT	NT	NT	NT	NT	NT	NT	SHV-1, TEM-1, CTX-M 15
24805§	M	82	SWI	outpatient	K. pneumoniae	Home	≥256	32	32	16	>256	>256	<0,12	<0,12	4	4	<0,12	16	16	SHV-1
24848	F	76	SWI	outpatient	K. pneumoniae	Home	>256	>256	16	16	>256	>256	>256	64	32	128	8	>256	>128	SHV-1
30396	F	<01	oSSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	>256	16	8	>256	>256	>256	256	>256	64	8	32	1	SHV-1, TEM-1, CTX-M 15
30398	M	<01	Other SSTI (umbilicus)	Pediatrics	K. pneumoniae	Home	≥256	>256	16	2	>256	>256	>256	128	>256	>128	16	16	2	SHV-1, CTX-M 1, OXA1
33014§	M	82	SWI	outpatient	K. pneumoniae	Home	≥256	4	4	16	>256	>256	>256	128	128	>256	16	32	2	SHV-1
33369	M	64	SWI (Coxarthrosis)	Ortopedics/ traumatology	K. pneumoniae	Home	≥256	>256	64	16	>256	128	<0.12	64	<0,12	>256	16	32	2	SHV-1, TEM-1, CTX-M 15, OXA1
51978	M	60	SWI	outpatient	K. pneumoniae	Home	≥256	>256	8	8	>256	>256	16	<0,12	<0.12	4	<0,12	32	128	SHV-1, TEM-1
52055	M	<01	oSSTI (umbilicus)	outpatient	K. pneumoniae	Home	≥256	>256	64	4	256	32	32	32	16	1	32	8	≤0.12	SHV-1, CTX-M
21441‡	M	40	oSSTI	Dermatology	P. aeruginosa	Home	≥256	128	16	16	>256	>256	>256	64	64	>128	16	>256	256	VIM
22222§	M	47	SWI	outpatient	P. aeruginosa	Home	≥256	4	4	NT	NT	>256	8	16	4	>256	8	64	2	+
2721‡	F	45	oSSTI	Orthopedics/ traumatology	P. mirabilis	Other hospital	≥256	>256	8	16	>256	>256	>256	256	256	>128	32	>256	256	+
22367	M	28	SWI	outpatient	P. mirabilis	Home	≥256	128	16	16	>256	>256	32	>256	>256	128	128	>256	>128	+
6627	M	68	SWI	outpatient	P. mirabilis	Home	≥256	>256	1	1	8	>256	<0.12	<0,12	<0,12	1	<0.12	8	0.25	TEM
84874	F	65	SWI	outpatient	P. vulgaris	Home	≥256	>256	16	16	>256	>256	256	128	256	>256	16	32	16	+
52158	M	67	SWI	outpatient	Proteus vulgaris	Home	≥256	64	16	16	>256	>256	4	32	64	4	16	32	0.5	CTX-M, OXA1

*AMX, amoxycillin; PIP, piperacilin; TZB tazobactam; AMC, amoxycillin+clavulanic acid; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; FOX, cefoxitin; FEP, cefepime; GEN, gentamicin; CIP, ciprofloxacin; NT, non-tested; †Type of ß-lactamase or AmpC, or positive phenotypic test for ESBL; ‡Patients with MRSA-ESBL coinfection; §Patient with two ESBL-producing strains coinfection;

additionally produced plasmid-mediated AmpC β-lactamase. One of four *E. coli* (all from outpatients) coproduced both TEM and CTX-M β-lactamase. In one *Pseudomonas aeruginosa* isolate VIM β-lactamase was found. CTX-M beta-lactamases were most prevalent with 13 positive isolates (*K. pneumoniae*, *E. coli*, *E. cloacae*, *Proteus vulgaris*), and in five cases they were accompanied by both TEM-1 and OXA-1 beta-lactamase (Table 2).

Insertion sequence IS26 was located upstream of bla_{CTX-M} gene in two *Enterobacter cloacae* strains (data not shown).

PFGE typing of *K. pneumoniae* using the 80% breakpoint for clonal relatedness revealed dominant cluster A which contained two subclusters: the clone A comprised 4 outpatient and the clone B two outpatient *K. pneumoniae* strains; three strains from pediatric and one from surgery hospital units were allocated in the dominant cluster A (Figure 2 A).

Two *E. coli* strains were clonally related with 90% similarity of their banding patterns and assigned to cluster A, and one strain was singleton (Figure 2 B).

MRSA/ESBL coinfection

Among 17 and 16 in-patients with MRSA and ESBL infections, respectively, three patients were coinfected with both (Table 3).

DISCUSSION

S. aureus is a causative agent of large number of ambulatory healthcare visits for skin and soft tissue infections each year (6). The prevalence of MRSA-positive SSTIs has increasing trend, and up to 46-72% prevalence was noted (4,6,27,28). Of the SSTI cultures negative for MRSA, almost half are usually caused by methicillin-sensitive *S. aureus* (MSSA), 6% by gram-negative organisms, and 3% infections are polymicrobial (6). The Gram-negative ESBL producing bacteria were identified more commonly as a causative agent of post-surgical than other skin and soft tissue infections (7), and both *Escherichia coli* and *Klebsiella* spp. are among the most frequent

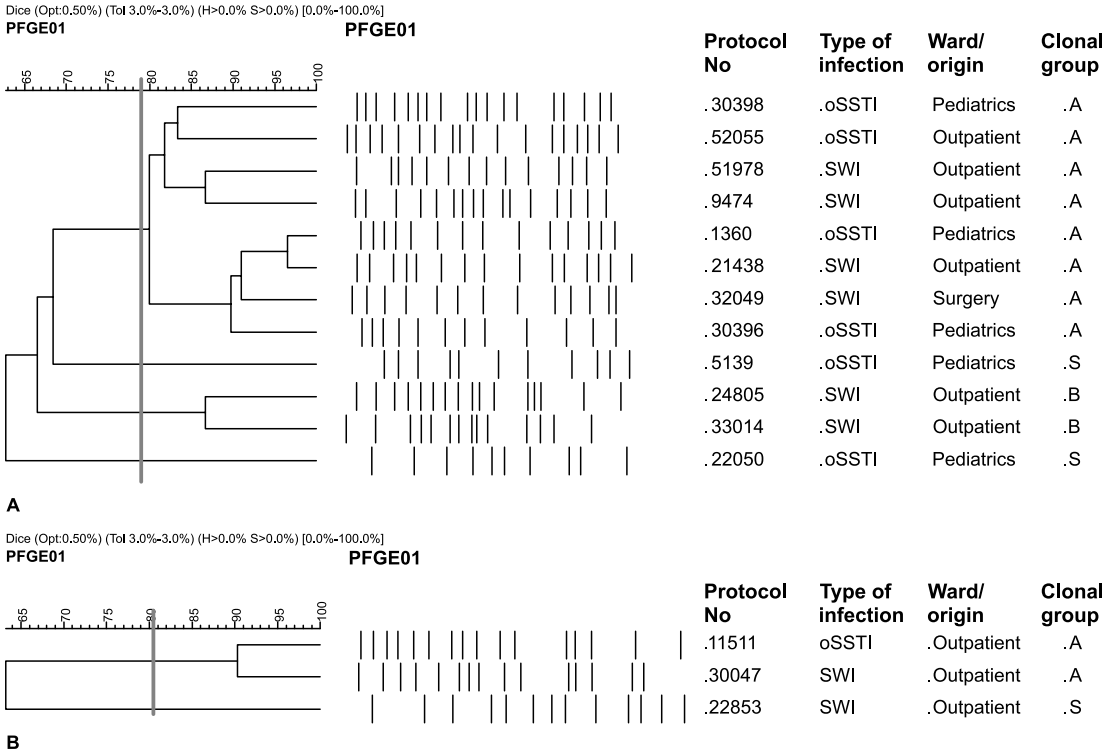

Figure 2. Dendogram showing the genetic relatedness of the *K. pneumoniae* and *Escherichia coli* strains by PFGE typing. A) The clone A comprised four *K. pneumoniae* outpatient strains and the clone B comprised two outpatient strains using the 80% similarity; three strains from pediatric and one from surgery hospital units were allocated in dominant cluster A, which contained two subclusters; B) Two *E. coli* strains were clonally related with 90% similarity of their banding patterns and assigned to cluster A; one strain was singleton. SSTI, skin and soft tissue infection; SWI, surgical wound infection;

Table 3. Patients with MRSA and ESBL-producing Gram-negative bacteria coinfection

Patient	Protocol No	Age (years)	Isolate origin	Hospital department	Causative agent	Residance before hospitalization	ATB used
Patient 1	13476	45	SWI	Orthopedics	MRSA (*spa*-type t041; *spa*-CC 002; MLST CC5)	Other hospital	Fluoroquinolones Glycosides
	2721		Other SSTI	Orthopedics	*Proteus mirabilis* (ESBL+)		
Patient 2	26267	<01	SWI	Surgery	MRSA (*spa*-type t355; *spa*-CC 355/595; MLST CC152)	Home	Beta lactam+beta lactamase inhibitors
	22040		Other SSTI (umbilicus)	Pediatrics	*Enterobacter cloacae* (TEM-1, CTX-M-15, SHV-1)		Glycosides, penicillins
Patient 3	21441	40	Other SSTI	Dermatology	MRSA (*spa*-type t355; *spa*-CC 355/595; MLST CC152)	Home	Fluoroquinolones
	21441				*P. aeruginosa* (VIM)		

* Patient with MRSA-ESBL coinfection; SWI, surgical wound infection; Other SSTI, skin and soft tissue infection other than SWI;

enterobacteria producing ESBLs in these infections (7). Almost equal proportion of both SSTI caused by MRSA and MSSA was noted in this study (among 43 SSTIs identified during the study period caused by *Staphylococcus* spp., 39.5% were infected with MRSA, 41.9% with MSSA, 9.3% with *S. epidermidis*, and 9.3% of patients had co-infection with MRSA and MSSA) (Uzunović S, unpublished data).

According to the results of this study ESBL-producing Gram-negative bacteria in outpatients were more frequently causative agents of SWIs, in contrast to the in-patients in which they more frequently caused other SSTIs. *K. pneumoniae* was dominant ESBL-producing pathogen of the in-patient SSTIs in this study, while the causative agents obtained from outpatient SSTIs were much more heterogeneous. A co-infection in SSTIs occurred frequently, mainly with *Pseudomonas aeruginosa* and MRSA (7), as demonstrated in this study. Moreover, two patients had co-infection with two ESBL-producing Gram-negative bacteria. Chronic infections, especially in patients previously treated with antibiotics, tend to be polymicrobial. Such mixed infections additionally complicate the antibiotic treatment and the outcome (29).

Community-associated methicillin-resistant *S. aureus* (CA-MRSA) is the most common cause of SSTIs, especially in closed populations with frequent skin-to-skin contact (4,21). It is well known that skin infections occurred predominantly in children and young adults without risk factors, where family members can serve as a reservoir of CA-MRSA, so, the epidemic MRSA clone might be propagated in the community (30). As reported previously, an outbreak of CA-MRSA infections in a neonatal intensive care unit was initiated by a mother with CA-MRSA wound infection and mastitis (31). In the present study, it was not possible to differentiate between hospital-associated MRSA (HA-MRSA) and CA-MRSA. However, we found that all patients with MRSA infection in this study had contacts with persons recently hospitalized and who used antibiotics in the previous 4 months, which were identified as risk factors for HA-MRSA (6). Based on antimicrobial susceptibility testing, MRSA in this study was susceptible to a wide range of antibiotics, which is typical for CA-MRSA (27).

From the genotyping results in this study, a MLST CC152 MRSA strains (Balkan clone) found in the in-patients (at multiple hospital departments), as well as in the outpatients, suggesting clonal spread by cross-transmission following introduction in the hospital (32,33), which was previously described in other reports (31,34). Indeed, PFGE results have shown that 80% of the MRSA strains belonged to one clone. Similar to MRSA, ESBL-producing *K. pneumoniae* were also clonally related indicating a common source. TEM- and SHV-type β-lactamases, mainly produced by *K. pneumoniae*, have spread throughout hospital settings, while CTX-M enzymes, mainly produced by *E. coli*, have become predominant in the community (35,36). ESBL-producing organisms are increasingly prevalent worldwide, and represent an emerging infectious threat**,** thus indicating that ESBL-producing organisms may be an emerging problem not only in hospital, but also in outpatient settings (35-37). The gut colonization of in-patients was identified as a risk factor for developing self and cross infections due to ESBL-producers, and further dissemination of ESBL-producing clones was a consequence of the transfer of patients between various units of the same hospital, but also between hospitals in the same countries, as well as across the borders (38).

MRSA colonization of patients or their household members represents an important risk for subsequent MRSA infection (38). Nares and umbilicus were the two most common sites of MRSA colonization, and sampling of these two sites is necessary and might be adequate for surveillance cultures (24). Some authors suggest MRSA screening of family members, but others recommend the screening only for some categories of patients (40). Our study confirmed the importance of umbilicus as a possible site of the future infection and screening of mothers as possible sources of the future infection.

The three most commonly described groups of SSTIs are "other cellulitis or abscess", "decubitus ulcer" and "post-operation wound infection" (superficial infections), and they accounted for 76.5% of all hospitalized cases (41), which is confirmed in this study.

The presence of MRSA and ESBL-producing organisms in outpatients is a substantial concern, due to the high morbidity and mortality associated with possible consequent hospital infections and their emergence poses a significant threat (37).

There are some limitations of this study. Firstly, it could not be ascertained whether these SSTIs were community-acquired or healthcare-associated. Secondly, this retrospective report has been based on the results obtained in the 5-month period resulting in a small number of MRSA or ESBL-producing bacteria causing SSTIs. Despite these shortcomings, this study underlines the importance of surveillance and improving identification of MRSA and ESBL-producing bacteria in hospitals, as well as in community settings, not only in hospitalized patients but in healthy people too.

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TRANSPARENCY DECLARATIONS

Conflict of interest: none to declare.

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Meticilin-rezistentni *S. aureus* (MRSA) i gram-negativne bakterije koje proizvode ß-laktamaze proširenog spektra (ESBL) i plazmidom-posredovane AmpC ß-laktamaze kao uzročnici bolničkih i vanbolničkih infekcija kože i mekih tkiva

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ABSTRACT

Cilj Istražiti meticilin-rezistentni *S. aureus* (MRSA) i gram-negativne bakterije koje proizvode ß-laktamaze proširenog spektra (ESBL) i plazmidom-posredovane AmpC ß-laktamaze kao uzročnike bolničkih i vanbolničkih infekcija kože i mekih tkiva (SSTI).

Metode Osjetljivost na antibiotike određivana je disk-difuzijskom i mikrodilucijskom metodom u skladu s CLSI. *MecA* gen je određivan pomoću PCR-a, a genetička karakterizacija MRSA-e pomoću *spa*-tipizacije i BURP-a (*algorithm based upon repeat patterns*). Dvostruki sinergistički disk-test korišten je za probir ESBLs. bla_{ESBL} aleli su detektirani pomoću PCR-a. Genetska srodnost između sojeva testirana je pomoću PFGE-a.

Rezultati Kod bolničkih pacijenata izolirano je 17 MRSA, 13 ESBL-producirajućih gram-negativnih bakterija, kod tri pacijenta zabilježena je koinfekcija obje bakterije, a kod vanbolničkih pacijenata pet MRSA i 16 ESBL-producirajućih gram-negativnih bakterija. *Klebsiella* spp. izolirana je u 11 bolničkih i sedam vanbolničkih pacijenata. MLST CC152 bio je najprevalentniji MRSA. Kod sedam (38,9%) *Klebsiella* spp. detektirani su amplikoni s početnicama specifičnim za SHV, TEM-1 i CTX-M grupu 1 β-laktamaza. Osam (44,4%) sojeva *K. pneumonia* i 16 (64%) MRSA (bolničkih i vanbolničkih) pripadali su klonovima.

Zaključak MRSA i ESBL-producirajuće gram-negativne bakterije koje uzrokuju infekcije kože i mekih tkiva vrijedne su pažnje zbog toga što usljed visokog morbiditeta i mortaliteta predstavljaju rizik za nastanak bolničkih infekcija.

Ključne riječi: infekcije kirurških rada, CTX-M beta-laktamaze, MLST CC152, otpornost na antibiotike