Antibiotic resistance, virulence and clonal diversity of methicillin resistant and sensitive *Staphylococcus aureus* isolates

PhD theses

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INTRODUCTION

Staphylococcus aureus is one of the most important human pathogens. It is widespread all over the world, often colonising the anterior nares, skin, oropharynx, and intestinal tract of healthy individuals.

It causes various pyogenic and toxin mediated diseases with high mortality. Infections can be classified as superficial skin and soft tissue infections (SSTIs); deep seated, systemic and life-threatening infections, as endocarditis, osteomyelitis, pneumonia, meningitis, sepsis; toxicoses as food poisoning, staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS). Severity of the infection is dependent on the virulence of the particular strain, inoculum size and immunity of the infected host. For *S. aureus* bacteraemia (SAB), the best-described manifestation of the infection, population incidence ranges from 10 to 30 per 100,000 person-years. Case fatality rate (CFR) for SAB remained between 15-50% in the past decades.

S. aureus is well known for its capacity to produce an armada of virulence factors to overcome host defence systems and cause diseases. Among the many virulence factors, *S. aureus* toxins (haemolysins, Panton-Valentine leucocidin, exfoliative- and entrotoxins, toxic shock syndrome toxin), enzymes (coagulase, staphylokinase, aureolysin etc.) and immune-evasive surface factors (PIA, CNA, SPA) are the most important types.

S. aureus infections are notoriously difficult to treat due to its extensive resistance to antibiotics of various classes. *S. aureus* has the ability to become resistant to every antibiotic used in therapy. The most remarkable resistant type is methicillin resistant *S. aureus* (MRSA) that is resistant to almost all β-lactam antibiotics and often to antibiotics of other classes as well.

MRSA most importantly differ from methicillin sensitive *S. aureus* (MSSA) strains in their β-lactam resistance, however, this is not their only dissimilarity. MRSA strains carry the SCC*mec* genetic element, that most MSSA lack, and besides *mec* genes, several other antibiotic resistance and regulatory genes are encoded on this cassette. Because of the presence of SCC*mec* and other acquired resistance mechanisms, MRSA are often multi-resistant to antibiotics of different classes, whereas MSSA strains are generally more sensitive to non-β-lactam antibiotics as well. Virulence of the pathogen and outcome of infection are, however, difficult to compare between MRSA and MSSA isolates. Most studies report increased mortality rate in patients with MRSA infections. Some other

investigations debate this and suggest that MSSAs may cause more severe infections, supposed to be related to higher prevalence of virulence genes in MSSA or to the greater fitness cost associated with SCC*mec* cassette in MRSA.

Population structure of MSSA strains is diverse, whereas MRSA is highly clonal. A few successful MRSA clones dominate in a given area and time period and isolates of these major clones cause the vast majority of infections. Clones of MRSA may differ from each other in terms of level of resistance, virulence, and in types and outcome of the caused infections. Different clones compete with each other in a niche and more successful clones replace the previous clones in a given setting. The first international clone of hospital acquired (HA)-MRSA, ST5-II (New York–Japan clone) emerged and became widespread in the 1990s worldwide. In Hungary, during the 1990s the most frequent *S. aureus* clone was the ST239-III (Hungarian/Brazilian), later being replaced by the ST228-I (South-German) and the ST5-II (New York–Japan) clone from the beginning of the 2000s. Later, these clones started to decrease in prevalence and ST22-IV (EMRSA-15), an originally community-associated type started to become increasingly frequent. The phenomenon of the clonal replacement, i.e. the change in the dominant clones over time, is studied extensively to identify driving forces of the population dynamics of *S. aureus*.

Acquisition of antibiotic resistance is a necessary step for a bacterium to become widespread and successful under the selective pressure of antibiotics in hospital environment. However, maintaining resistant phenotype and carrying various resistance genes in the absence of selective pressure imposes a cost in the fitness (capability to survive and to reproduce) of the bacterium. Differences in antibiotic resistance and associated level of fitness may attribute to the varying success of different clones of MRSA.

OBJECTIVES

The objectives of our study were the following:

1. To examine the possible role of fluoroquinolone resistance in the varying fitness of different clones of MRSA, as a potential contributor to the emergence and success of new epidemic clones.
2. To compare the antibiotic susceptibility, virulence factors and associated mortality in MRSA and MSSA bloodstream infections (BSI).

3. To evaluate the clonal composition of MRSA isolates currently causing BSI at the Semmelweis University Clinics and to compare characteristics of isolates of different MRSA clones.

METHODS

1. Study of fitness cost associated with fluoroquinolone resistance in different MRSA clones

We examined 8 HA-MRSA strains belonging to the following major international clones: ST228, ST22, ST5, ST239; and 3 community-acquired (CA) MRSA isolates of the ST8, ST80 and ST30 clones, originating from the strains collection of the National Centre for Public Health. Fluoroquinolone resistance was induced in the CA-MRSA strains by exposure to increasing concentrations of ciprofloxacin. Changes in the fitness of the bacteria was compared in propagation assay by measuring the growth rate of the isogenic ciprofloxacin sensitive strains and their multiple ciprofloxacin resistant derivatives in monocultures. Growth rate was determined by measuring optical density (OD) values. Area under the curve (AUC) was used for the numerical comparison of the growth capacity of the strains, higher AUC values indicating faster replication rates. Identity of the wild type CA-MRSA strains with their ciprofloxacin resistant variants was confirmed by pulsed field gel electrophoresis (PFGE). Mutations in the genes encoding DNA gyrase (*gyrA, gyrB*) and topoisomerase IV (*grlA, grlB*) were detected by sequencing the respective genes.

2. Characterisation of MRSA and MSSA isolates of bloodstream infection (BSI)

All non-duplicated BSI MRSA strains isolated between January 2011 and December 2016 at the Institute of Laboratory Medicine, Semmelweis University, Budapest, Hungary, overall 153 MRSA isolates were included in the study. Each year, the same number of MSSA BSI isolates, representing the same gender and age distribution of population and hospital wards were enrolled (from a much larger pool) to be compared to the MRSA strains. In total, 306 *S. aureus* BSI isolates (153 MRSA and 153 MSSA strains) were analysed. From patient related factors, gender, age, comorbidities, current chemotherapy and steroid therapy was

registered. Charlson comorbidity index (CCI) and all-cause 30 days mortality were determined for each patient.
Identification of *S.aureus* strains was carried out by standard and MALDI-TOF MS analysis. Genotypic identification was based on the detection of *nucA, mecA* and *mecC* genes by PCR. Antibiotic susceptibility to oxacillin, erythromycin, clindamycin, gentamicin, tobramycin, amikacin, doxycyline, sulfamethoxazole-trimethoprim, rifampicin, linezolid and ciprofloxacin was tested by disc diffusion method and MRSA isolates were additionally tested for vancomycin and teicoplanin susceptibility by broth microdilution according to the European Committee of Antibiotic Susceptibility Testing (EUCAST) guidelines, during the routine identification procedure. Presence of virulence genes encoding cytotoxins (*hla, hlb, hlg, hlg-v, lukS-PV/lukF-PV),* adhesion factors *(spa, icaA, cna)* and superantigens *(sea, seb, sec, tst, eta, etb)* was detected by PCR. For the detection of *sea, eta* and *etb*, we used newly designed primers. For determination of the genetic relatedness of the strains PFGE was performed after *Sma*I digestion for 151 MRSA and 153 MSSA strains. SCC*mec* typing was performed for all MRSA isolates by PCR. Multi locus sequence typing (MLST) was carried out on a subset of representative isolates of the most prevalent PFGE pulsotypes and SCC*mec* types identified in our study and MLST sequence types were assigned through the MLST database.

RESULTS

1. Study of fitness cost associated with fluoroquinolone resistance in different MRSA clones

As the result of exposure to ciprofloxacin, the originally ciprofloxacin sensitive CA-MRSA strains gained mutations in the genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*grlA, grlB*). These mutations developed in a stepwise manner, more mutations resulted in higher ciprofloxacin MIC values. No genetic alteration in the *gyrB* gene could be detected.

Each ciprofloxacin resistant CA-MRSA strain variant grew much slower than their respective ciprofloxacin sensitive ancestor. However, development of ciprofloxacin resistance influenced the speed of replication to different extent in the different CA-MRSA clones. Derivatives of ST30-IV grew the slowest. The ST8-IV derivative with 256 mg/L MIC value suffered greater loss of speed than

the ST80-IV derivative with the same MIC (23.7 vs 33.5 AUC, respectively). The Ser80→Phe mutation in the *grlA* gene seemed to severely compromise the fitness of ciprofloxacin resistant derivatives of ST8-IV and ST80-IV isolates (Figure 1).

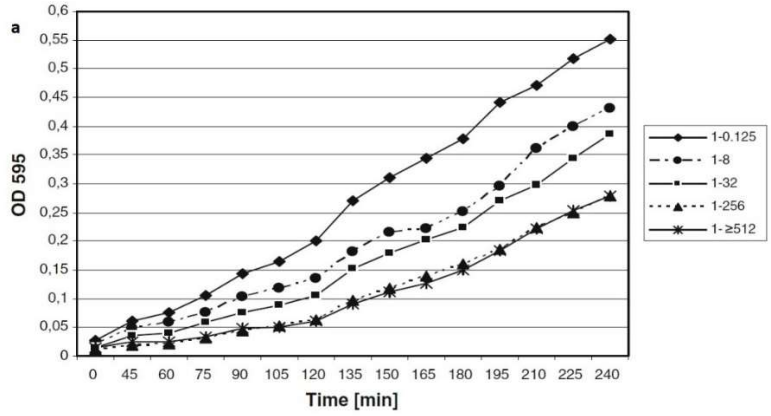

Figure 1. Growth curves of the ST8-IV CA-MRSA strain and its ciprofloxacin resistant derivatives.

When comparing the replication rate of ciprofloxacin resistant HA-MRSA strains representing international clones, we found that ST239-III and ST228-I isolates replicated much slower compared to strains from other clones. Overall ST22-IV isolates had the highest growth rate, and could combine the fastest replication with the highest ciprofloxacin MIC values (Figure 2).

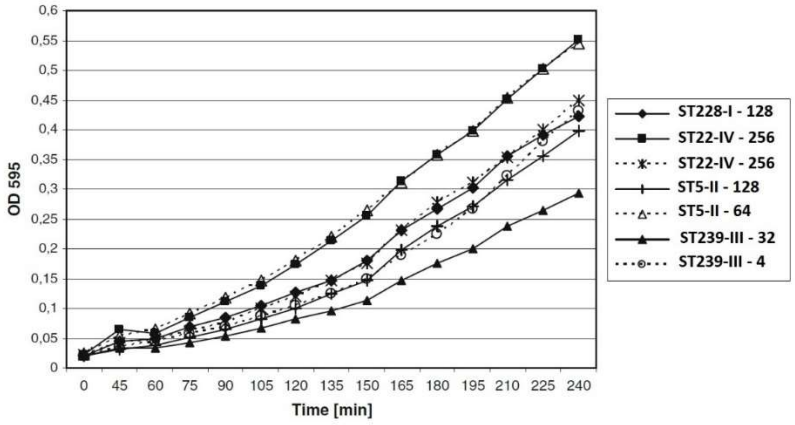

Figure 2. Growth curves of HA-MRSA strains representing the most prevalent MRSA clones (The figure legends show the clonal type and ciprofloxacin MIC value (mg/L))

2. Characterisation of MRSA and MSSA isolates of bloodstream infection (BSI)

Of the patients with MRSA BSI, significantly more were males than females (61.4% vs 38.6%, p=0.044), MSSA isolates were selected to match this ratio. CCI was significantly higher in female patients (4.92 vs 4.24 in males, p=0.0164). Chronic liver disease and chemotherapy was more frequent in MSSA patients, whereas more of MRSA patients had surgery in the previous 30 days or endocarditis, however, CCI did not differ significantly in the two groups.

MRSA isolates were more resistant to all antibiotics except for doxycycline. Multidrug resistance (MDR) was also significantly more prevalent in MRSA isolates, whereas 75.8% of MSSA isolates were susceptible to all tested antibiotics. All MRSA and MSSA isolates were sensitive to vancomycin, teicoplanin and linezolid (Figure 3).

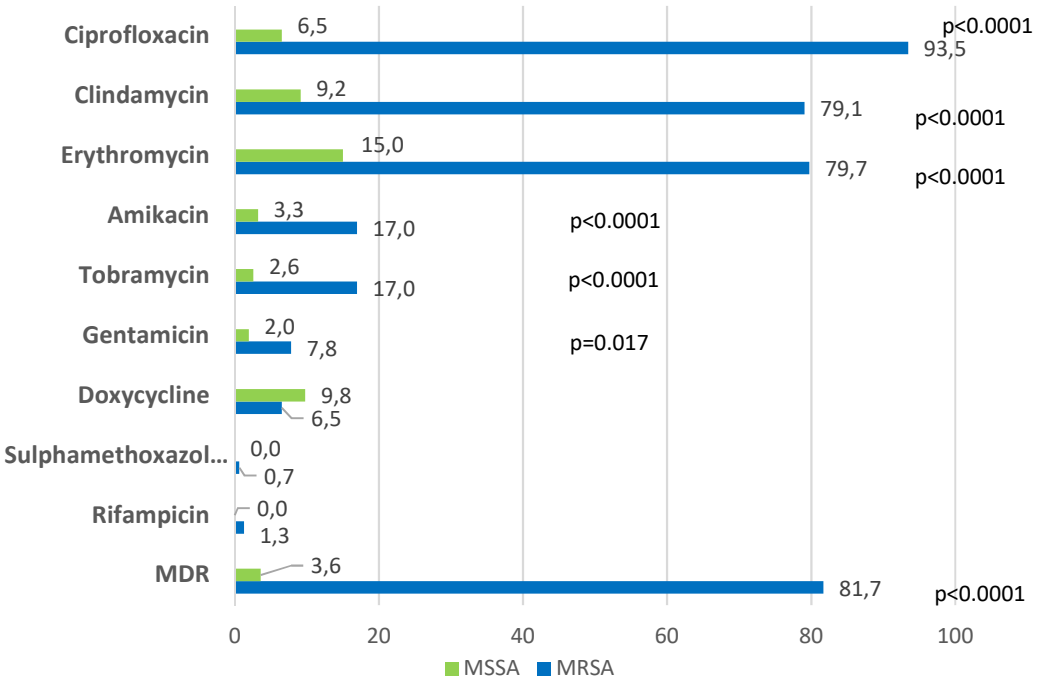

Figure 3. Antibiotic susceptibility and multidrug resistance of MRSA and MSSA isolates

MRSA strains carried a median of six virulence genes. The most frequent virulence type in MRSA carried genes encoding *hla, hlb, hlg, ica, spa, cna*, and *sea or seb* (11.1% and 14.4% of the isolates, respectively). Isolates were highly diverse; we identified 57 different virulence gene combinations in MRSA isolates.

MSSA strains carried less virulence factors (median of 5). The most frequent virulence type in MSSA was *hla, hlb, hlg, hlgv, ica, spa* positivity.

Cna, sea, ica and *hlb* were significantly more prevalent in MRSA, whereas *tst, eta, sec* and *hlg-v* were significantly more frequent in MSSA. Superantigens were more frequent in MSSA, whereas adhesins were more frequent in MRSA isolates. *LukS-PV/lukF-PV* positivity rate was 3.3% and 1.3% in MRSA vs MSSA, respectively. The prevalence of this gene changed significantly during the 6 years of the study: it was 13% in MRSA isolates in 2011, but never exceeded 4% in the later years.

Our MSSA strains showed high level of diversity based on the PFGE comparison, no dominant clone was identified. On the contrary, most of the MRSA isolates belong to 3 main PFGE pulsotypes. SCC*mec* typing of the MRSA isolates showed that the vast majority of our strains belonged to SCC*mec* type IV. SCC*mec* type III and VI isolates were not found, and only one of the isolates from 2013 had SCC*mec* type V. SCC*mec* IV isolates kept their dominance throughout the 6 years of the study. SCC*mec* type IV isolates were significantly more frequent in females (78.0% vs 59.6% in males, p=0.0188). MLST was carried out for 12 representative MRSA isolates from the most frequent PFGE pulsotypes and SCC*mec* types, representing all six years of the study. All eight tested SCC*mec* IV, PFGE type A isolates belonged to the ST22 clone. Three SCC*mec* II, PFGE type B isolates were typed: two belonged to ST5 and one to ST225, the latter being also part of CC5. Our representative SCC*mec* type I, pulsotype C isolate belonged to ST1.

Significant differences were found in the antibiotic resistance, virulence and associated mortality among MRSA isolates of different clones. SCC*mec* II isolates were associated with especially high resistance rates to gentamycin, amikacin, tobramycin and doxycycline. The highest vancomycin MIC values were observed in this group. SCC*mec* IV isolates were relatively less resistant to antibiotics compared to the other two groups, except for ciprofloxacin (Table 1).

Table 1. Antibiotic resistance and high vancomycin MIC values in MRSA isolates of different SCC*mec* types

	SCC*mec* I n=14		**SCC*mec* II n=36**		**SCC*mec* IV n=102**		**MRSA all n=153**	
	R	**%**	**R**	**%**	**R**	**%**	**R**	**%**
Erythromycin	13	**92.9**	33	**91.7**	75	73.5	122	79.7
Clindamycin	13	**92.9**	32	**88.9**	75	73.5	121	79.1
Gentamicin	6	**42.9**	3	8.3	3	2.9	12	7.8
Tobramycin	7	**50.0**	12	**33.3**	7	6.9	26	17.0
Amikacin	7	**50.0**	12	**33.3**	7	6.9	26	17.0
Ciprofloxacin	11	78.6	35	**97.2**	97	**95.1**	143	93.5
Sulfamethoxazole-trimethoprim	0	0.0	0	0.0	1	1.0	1	0.7
Doxycycline	3	**21.4**	1	2.8	5	4.9	10	6.5
Rifampicin	1	**7.1**	0	0.0	1	1.0	2	1.3
Vancomycin MIC=2mg/L	3	**21.4**	4	**11.1**	4	3.9	10	6.5

SCC*mec* II isolates carried the highest number of virulence factors. Panton-Valentine leukocidin gene was found exclusively in SCC*mec* I and II isolates. Our sole SCC*mec* V isolate did not carry any of the tested virulence factor genes (Table 2).

Table 2. Virulence factors in MRSA isolates of different SCC*mec* types

	SCC*mec* I n=14		**SCC*mec* II n=36**		**SCC*mec* IV n=102**		**MRSA all n=153**	
	R	%	R	%	R	%	R	%
tst	0	0.0	0	0	0	0	0	0%
eta	0	0.0	0	0	0	0	0	0%
etb	0	0.0	1	**2.8**	0	0	1	0.7%
sea	6	**42.9**	11	**30.6**	13	12.7	30	19.6%
seb	5	35.7	12	33.3	41	**40.2**	58	37.9%
sec	0	0.0	4	**11.1**	8	7.8	12	7.8%
lukS-PV/lukF-PV	2	**14.3**	3	**8.3**	0	0.0	5	3.3%
hla	10	71.4	29	**80.6**	72	70.6	111	72.5%
hlb	8	57.1	23	63.9	75	**73.5**	106	69.3%
hlg	5	35.7	22	**61.1**	62	**60.8**	89	58.2%
hlg-v	10	**71.4**	19	**52.8**	2	2.0	31	20.3%
icaA	11	78.6	28	77.8	83	**81.4**	122	79.7%
spa	14	**100.0**	36	**100.0**	100	98.0	150	98.0%
cna	5	35.7	12	33.3	93	**91.2**	110	71.9%
Median n of virulence genes	5.5		7		6		6	

Although CCI of the patients did not differ significantly, BSI caused by MRSA led to significantly higher mortality rates (39.9% vs 30.7% in MSSA BSI, respectively, $p<0.0001$). Overall 30-day mortality was 35.3%.

Mortality was significantly higher in females (38.7% vs 33.2% in females and males, respectively ($p<0.001$)), this could be attributed to higher CCI of the female patients in this study. Mortality increased with age: it was 20.0% in the age group 0-49y, 28.0% in 50-64y, 40.2% in 65-79y and 55.8% in patients older than 80 years.

Higher vancomycin MIC did not influence mortality rates. Although we have found only 10 isolates with teicoplanin MIC of 2 mg/L, mortality was 70% in this group.

Number of carried virulence genes, presence of specific virulence factors and antibiotic resistance to other drugs besides glycopeptides did not influence mortality.

Mortality rates differed in BSI caused by MRSA isolates of different clones (Table 3). Interestingly, patients infected with SCC*mec* IV isolates had higher mortality, than patients infected with SCC*mec* I or II MRSA strains (42.2% in SCC*mec* IV vs 28.6% and 36.1% in SCC*mec* I and II, respectively), however, these differences were not statistically significant. CCI of patients infected with MRSA isolates of SCC*mec* I and II group did not differ significantly when compared to CCI of SCC*mec* IV group.

Table 3. Mortality rates (%) and CCI of patients in BSI caused by different types of *S. aureus*

	MRSA-SCC*mec* I	**MRSA-SCC*mec* II**	**MRSA-SCC*mec* IV**	**MSSA**
Charlson comorbidity index	4.29	3.97	4.46	4.65
Mortality rate (%)	28.6	36.1	42.2	30.7

CONCLUSIONS

1. Fluoroquinolone antibiotics play a crucial role in the selection of international clones of *S. aureus*. Strains that are able to maintain high replication rate in parallel to developing high level of fluoroquinolone resistance have a great advantage over other clones in hospital settings, where fluoroquinolone antibiotics are frequently used. This observation may provide an explanation for the selection of the globally successful major clones of *S. aureus*.
2. CA-MRSA strains, which suffer high fitness lost upon acquisition of fluoroquinolone resistance will not be able to take ground in healthcare setting. In our study, we demonstrated this overwhelming decrease in fitness

by inducing fluoroquinolone resistance in ST8-IV, ST80-IV and ST30-IV CA-MRSA strains. Upon acquisition of mutations in the QRD regions of their appropriate genes, these strains suffered great loss of vitality, proving that they are capable of developing resistance, however, in their resistant form, they cannot compete with the higher replication rates of successful HA-MRSA clones.

3. Moreover, differences in fitness cost related to fluoroquinolone resistance shed light on the possible background of clonal replacement. According to our data, strains of ST5-II and particularly ST22-IV clones are able to sustain a high replication speed while exhibiting high fluoroquinolone MIC values. On the contrary, strains of ST239-III and ST228-I clones are less fit and replicate considerably slower. These data suggests that a dominant clone in an area may get replaced by another emerging one, if the new clone is able to withstand the effect of antibiotics in the setting, and is able to better maintain the speed of replication despite mutation in QRDR. Such new clones are able to outcompete and surpass previously dominant clones.
4. Most fluoroquinolone resistant clones, however, lose their superiority in fluoroquinolone-free environment, as most of them cannot compete with the replication speed of fluoroquinolone susceptible strains. ST22-IV MRSA, an internationally successful major clone is a remarkable exception, as it has comparable replication speed to fluoroquinolone susceptible MRSA clones.
5. Because of the decisive role of fluoroquinolone antibiotics in the selection and rise of successful *S. aureus* clones, more judicious use of these antibiotics could help in the regress of the major clones which is described to be associated with decline in the incidence of HA-MRSA.

6. Our study on the *S. aureus* isolates causing BSI over a 6-year time period revealed differences in antibiotic resistance, virulence factors and associated mortality in infections caused by different types of *S. aureus*. Outcome of the infection was much worse in patients with MRSA BSI. Mortality rates were also higher in females, older patients, and in patients who had BSI caused by SCC*mec* IV isolates, and by isolates with high teicoplanin MIC. MRSA isolates had much higher resistance rates, MDR was also more frequent among them. MRSA strains also carried more virulence genes, and different types compared to MSSA. We have found low prevalence of PVL and superantigens. Number and type of carried virulence factors was not associated with increased mortality rates.

7. Among MRSA isolates, we have found that similarly to other parts of the world, the vast majority of the strains belong to ST22-IV clone. Isolates of ST5-II, ST225-II and ST1-I were also found among our samples. MRSA isolates of different clones had different characteristics. SCC*mec* I and II isolates had high rates of antibiotic resistance in general, and particularly high glycopeptide MIC values. SCC*mec* II isolates had the highest number of virulence genes. Interestingly, PVL was found exclusively in SCC*mec* I and II isolates. Surprisingly, despite these advantages of SCC*mec* I and II isolates, mortality of BSI caused by SCC*mec* IV isolates was higher than that of SCC*mec* I and II MRSA infections. SCC*mec* IV isolates maintained their dominance in our samples during the six years of the study.

8. Based on our study regarding the impact of fluoroquinolone resistance on the fitness cost of various MRSA clones, we suggest that the success of ST22-IV clone, and its role in the replacement of the previously dominant clones by this type, moreover, the sustained dominance of this clone during our study and the high mortality rate of patients with BSI caused by the ST22 clone is likely the result of greater fitness and higher replication rate of this clone compared to other MRSA clones in our region.

LIST OF PUBLICATIONS RELATED TO THE THESIS

1. A Horváth; O Dobay; J Sahin-Tóth; E Juhász; J Pongrácz; M Iván; E Fazakas; K Kristóf:
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2. A Horváth: **Acceptance of varicella vaccination.** Hum Vacc Immunother, DOI: 10.1080/21645515.2020.1843337 Epub Ahead of Print; 2020 IF= 2,619
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