Arta Balode

Molecular Biological Characteristics of Methicillin-Resistant *Staphylococcus aureus* for Patients Treated at a Multi-Profile Hospital in Latvia

SUMMARY OF THE DOCTORATE THESIS

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Scientific supervisors:

Dr. habil. med., Prof. **Dace Gardovska** (Riga Stradins University)
Dr. biol. asoc., Prof. **Edvins Miklaševičs** (Riga Stradins University)

Approved reviewers:

Dr. med., Prof. **Juta Kroiča**
(Riga Stradins University)
Dr. habil. biol., Prof. **Aleksandrs Rapoports**
(University of Latvia)
Dr. med. **Jolanta Miciuleviciene** (Lithuania)

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Dr. habil.med., Prof. *Līga Aberberga - Augškalne*

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Abbreviations and Definitions

aArc C – *houskeeping* gene
aroE – *houskeeping* gene
BD – *Becton Dickinson*
AST – antimicrobial susceptibility test
CA-MRSA – community-acquired methicillin-resistant *Staphylococcus aureus*
CC – *Clindamycin*
ccr – cassette chromosome recombinase genes
CDC – Centers for Disease Control and Prevention
CIP – *Ciprofloxacin*
clfA – *S. aureus* marker
CLSI – *Clinical and Laboratory Standards Institute*
DNA – deoxyribonucleic acid
EARSS – European Antimicrobial Resistance Surveillance System
em – primer pair
ERY – *Erythromycin*
GEN – *Gentamicin*
glpF – *houskeeping* gene
gmk – *houskeeping* gene
HA-MRSA – hospital-acquired methicillin-resistant *Staphylococcus aureus*
luk-PV – PVL toxin gene
McFarland standard – *McFarland* standard
mecA – methicillin-resistance gene
MIC – Minimum inhibitory concentration
MLST – multilocus sequence typing
MRSA – methicillin-resistant *Staphylococcus aureus*
OX – *Oxacillin*
pta – *houskeeping* gene
PBP_2 – penicillin-binding protein
PFGE – pulsed field gel electrophoresis
PCR – polymerase chain reaction
PSKUS – Pauls Stradins Clinical University Hospital
PVL toxin – *Panton-Valentine leukocidin* toxin
RNA – ribonuclein acid
RIF – *Rifampin*

S. aureus – *Staphylococcus aureus*
SCC *mec* – staphylococcal chromosomal cassette
spa – staphylococcal protein A gene
ST – sequence type
STX – *Trimethoprim-sulfamethoxazole*
TET – *Tetracyclin*
tpi – *houskeeping* gene
tsst – toxic shock syndrome toxin-1 gene
VAN – *Vancomycin*
VISA – Vancomycin - intermediate *S.aureus*
VRSA – Vancomycin – resistant *S. aureus*
yqiL – *houskeeping* gene

Definitions

MRSA case: patient with an MRSA-induced infection's clinical symptoms, laboratorially confirmed MRSA and MRSA carriers.

Colonisation/MRSA carrying: laboratorially confirmed MRSA for a patient without MRSA-caused symptoms of infection.

MRSA bacteremia: MRSA infection's symptoms for a patient with laboratorially confirmed MRSA from the patient's blood sample.

Invasive MRSA isolate: MRSA isolated from a patient with MRSA symptoms.

MRSA isolate: a microorganism isolated from the pathological material.

MRSA strain: a microorganism complex with identical properties (antibipictogram, molecular biological characterisation), isolated from the pathological material.

Risk groups for MRSA infection

1. Patients transported from other medical institutions or patients who have been at the intensive care unit of another medical institution during the previous 30 days.

2. Patients who have been operated on during the previous six months.

3. Patients who have had contact with a patient, infected by MRSA.

4. If a patient has purulent wounds, atrophic ulcers, or long-term catheters at the time of hospitalisation.

Risk factors for MRSA infection

1. General risk factors (immunosuppression, sugar diabetes, chronic skin damage).

2. Risk factors connected with hospitalisation (long-term hospitalisation, catheters, surgical manipulations, artificial ventilation of the lungs, the severity of the patient's state of health).

3. Community risk factors (close contact with a large number of people, being present at medical institutions for prolonged periods of time, playing contact sports, etc.).

Nosocomial/intrahospital infection – infection that a patient has contracted during the time of being hospitalised and that has not been at its incubation period at the moment of being admitted to hospital.

MRSA screening – the microbiological test of a sample taken from the nose, the armpits, or the perineum. The sample is taken following epidemiological indications to duly identify MRSA carriers.

Adapted from the epidemiological bulletin, issued by the government agency 'Public Health Agency' in 2007.

Introduction

As an opportunistic pathogen, *S. aureus* may initiate a wide variety of infections in community conditions and medical institutions, thus increasing the cost of treatment and the risk of mortality. MRSA is considerably different from other multi-resistant microorganisms, because MRSA not only replaces MSSA as the infectious agent but also often gets attracted to patients, afflicted by a serious disease or influenced by the risk factors. Thereby, the incidence of diseases caused by *S. aureus* increases (*Muder et al*, 1991, *Stamm et al*, 1993, *Wyllie et al*, 2006, *Davis et al*, 2004, and *Dumpis et al*, 2007).

Initially, MRSA had spread as a dangerous agent of nosocomial infections at hospitals worldwide. Nowadays, however, it has become an important pathogen, acquired in community conditions. Contrary to HA-MRSA, CA-MRSA spreads by direct human contact. In the recent years, MRSA acquired in community conditions has become highly relevant due to its relation to cases of infection involving children and young adults who lack contact with medical institutions. The increasing number of cases when CA-MRSA strains are associated with necrotising pneumonia is alarming, for this condition is often lethal for the patient.

Well-timed and correct identification of MRSA is an important factor in order to stop the development of diseases caused by MRSA and their outbreaks. The main characteristics that differentiate the MRSA strains contracted at hospital environment and outside it are the nonexistence of nosocomial infection risk factors, the sensibility towards the main antimicrobials, excluding beta-lactam antibiotics, a chromosomal background unlike the most widespread intrahospital strain genotypes, type IV or type V SCC*mec*, which is rarely present in intrahospital MRSA strains, and genes, coding Panton-Valentine leukocidin – a PVL toxin. All of these characteristics can be determined by using methods of molecular biology.

After the first laboratorially confirmed cases of HA-MRSA and CA-MRSA in 2003, it was clear that these pathogens can be found in Latvia. Still, the MRSA strain genotypes, their distribution, diseases, and other risk factors were not known.

1. Significance of the issue

According to the CDC National Nosocomial Infections Surveillance System analysis results in the period from 1986 to 2003, it was concluded that the most often found intrahospital infection agents with a rising trend are gram-positive microorganisms, including MRSA (*Gaynes et al*, 2005).

The main and most significant difference between methicillin-sensitive and methicillin-resistant *S. aureus* is that the latter contains a gene cassette integrated in the chrosome (*Katayama et al*, 2000), also known as *SCCmec* (*staphylococcal chromosomal casette mec*). The main element of *SCCmec* is the *mecA* gene which ensures that the host is resistant to all of the known beta-lactams. Most of the *SCCmec* gene mecA types of MRSA strains, isolated in European countries, are found in five clonal complexes of *S. aureus*: CC5, CC8, CC22, CC30, and CC4 (*Deurenberg et al*, 2007). Up to this time, eight types of *SCCmec* with subtypes have been discovered and, depending on the type, they contain numerous genes associated with pathogenicity and resistance. Moreover, these resistance genes maintain the survival of *S. aureus* not only in the presence of antibiotics (such as gentamicin or erythromycin), but also in other adverse conditions (such as the presence of heavy metals).

In addition, the strains of *S. aureus* are able to produce at least a part of the numerous staphylococci toxins, e.g. PVL and TSST, where PVL together with type IV *SCCmec* are molecular markers of the MRSA acquired in community conditions (CA-MRSA).

In Latvia, vancomycin has been chosen to treat the infections initiated by MRSA. Glycopeptides are the antibiotics used in treating the infections initiated by MRSA in other countries as well (*Gemmel et al*, 2006). Unfortunately, the wide application of these antibiotics, including in Europe, is responsible for spreading VISA and VRSA strains. Therefore, employing antibiotics from this group in the treating of infections requires strictly determined indications (*Finch et al*, 2006, *and Appelbaum*, 2006). Furthermore, there is growing doubt over the efficiency of vancomycin regarding the increase of the MIC of staphylococci and the observations which confirm bad penetration of this antibiotic in the tissues (*Finch*, 2006). This is also why the efficiency of teicoplanin is decreasing, causing problems in the treatment of MRSA-induced infections (*Tenover et al*, 1998).

Information about the distribution of MRSA in Latvia in 2007 is available in just one report. However, a systematic research of MRSA

distribution and molecular epidemiology of MRSA strains have not been performed until 2007 and later (*Pujate et al*, 2008).

2. Aim of the study

The aim of the study is to determine the trends of MRSA distribution for the patients treated in a multi-profile medical institution and to describe the MRSA strains found in Latvia from 2004 to 2010, regarding their molecular biological structures compared to other European countries.

3. Tasks of the study

1. To determine the distribution of MRSA and the efficiency of MRSA risk group patients' screening at PSKUS.
2. To compare the situation in Latvia and other European countries according to the results of the first MRSA-positive blood samples.
3. To perform a phenotypic and genotypic study of the chosen HA-MRSA and CA-MRSA strains isolated from 2004 to 2010.
4. To analyse the possible epidemiology of MRSA strains in Latvia, comparing their molecular biological characteristics with the characteristics of MRSA strains in other European countries.

4. Research questions and hypothesis

1. How often is MRSA encountered at PSKUS and what are the trends of this pathogen at a multi-profile hospital?
2. Is there a correlation between carrying MRSA and a bacteremia, induced by MRSA, at PSKUS from 2004 to 2010?
3. Are the MRSA isolated from pathological materials from 2004 to 2010 phenotypically and genotypically identical or different and what are these differences?
4. What are the trends regarding the distribution of MRSA-induced bacteremias in Latvia, compared with other European countries, from 2004 to 2010?

5. What types of MRSA strains can be found in Latvian medical institutions? Their phenotypic and molecular genetic characterisation.

Hypothesis

In 2003, the first laboratorially confirmed MRSA cases are not coincidental and they can be found in Latvia. MRSA in Latvia is related to the strains common in its neighbouring countries.

5. Scientific Novelty

1. A collection of Latvian MRSA isolates and a database for the molecular biological analysis of Latvian MRSA strains have been created.
2. The incidence of MRSA cases has been determined at a multi-profile hospital from 2004 to 2010.
3. For the first time in Latvia, using technologies based on PCR and sequence determination, the clinically important factors of pathogenicity of MRSA (types of *SCCmec*, toxins, resistance genes of antibiotics) have been established as well as the methods of their determination.
4. The dominating HA-MRSA and CA-MRSA strains in Latvia have been described.
5. The molecular epidemiology of MRSA strains isolated in Latvia has been researched, connected with the studies performed in other European Union countries.
6. The research has provided evidence, suggestions, and methods for further scientifically justified control and surveillance of MRSA infections in Latvia.

6. Scope and structure of the study

The thesis are written in latvian language and contain thirteen sections: introduction, significance of the issue, aim of the thesis, objectives of the thesis, research questions and hypothesis, scientific novelty, methodology of the theses, applied methods, results, discussion, conclusion, as well as list of abbreviations, definitions, references and appendixes. The volume of the thesis is 170 pages, 31 tables, 20 figures, 9 appendixes and 249 references.

7. Methodology of the study

The research has been divided into four subsections.

7.1. Study of MRSA cases at PSKUS from 2004 to 2010

Period of time: 2004 – 2010.

Object of study: patients with laboratorial confirmed first MRSA.

Study population: hospitalised patients.

Analysed indicators:

1. MRSA intensive indicators from 2004 to 2010, using the number of patient-days;
2. The distribution of MRSA cases at different units of the hospital;
3. The proportion of MRSA carriers.

7.2. Analysis of MRSA isolated from blood at PSKUS

Period of time: 2004 – 2010.

Object of study: first MRSA-positive blood sample from a patient hospitalised at PSKUS.

Study population: hospitalised patients.

Analysed indicator: the proportion of MRSA against all the *S. aureus* isolated from blood.

7.3. Distribution of all the MRSA isolated from blood in Latvia from 2004 to 2009, compared to the MRSA distribution of the European Antimicrobial Resistance Surveillance System member states

Period of time: 2004 – 2009.
Places of carrying out the research:

- Pauls Stradins Clinical University Hospital, Central Laboratory;
- Children Clinical University Hospital, Laboratory of Microbiology;
- Riga Eastern Clinical University Hospital, Laboratory of Microbiology;
- State agency 'Infectology Center of Latvia', Laboratory of Microbiology;
- State agency 'Infectology Center of Latvia', Clinic of Tuberculosis and Lung Diseases, Laboratory of Microbiology;
- Riga City Hospital No.1, Laboratory of Microbiology;
- Rīgas Maternity Hospital, Laboratory of Microbiology;
- 'Centrala laboratorija', Ltd.;
- Vidzeme Hospital, Laboratory of Microbiology;
- Daugavpils Regional Hospital, Laboratory of Microbiology;
- North Kurzeme Regional Hospital, Laboratory of Microbiology;
- Liepaja Regional Hospital, Laboratory of Microbiology.

Object of study: first *S. aureus* microorganism cultures from blood.

Study population: Patients who have been in contact with a medical institution.

Analysed indicators:

1. Information about the Surveillance System member laboratories and hospitals, in accordance with the EARSS methodology (Appendix 1);

2. Testing results of the *S. aureus* isolates, in accordance with the EARSS test protocol;

3. MRSA selection for an analysis at the European Antimicrobial Resistance Surveillance System:

In accordance with a united EARSS protocol, all the first *S. aureus* and MRSA isolated from blood from 2004 to 2009. During the selection procedure, the *S. aureus* and MRSA isolated in 2010 were not included, because centralised data processing takes place at the second half of the next year.

7.4. Phenotypic and genotypic analysis of MRSA isolates

Period of time: 2004 – 2010.

Place of carrying out the research: PSKUS Central Laboratory.

Object of study: MRSA isolates chosen by random sampling from the PSKUS Central Laboratory collection of isolates.

Analysed indicators:

Phenotypic and genotypic characteristics of MRSA and *S. aureus* isolates.

Selection of HA-MRSA isolates for phenotypic and genotypic analysis:

First isolated MRSA from the PSKUS Central Laboratory collection were selected, proportionally representing different months from 2004 to 2010.

Selection of CA-MRSA isolates for phenotypic and genotypic analysis: First phenotypically sensitive but oxacillin-resistant MRSA strains, isolated from clinical materials and received from Latvian microbiology laboratories in the period from 2004 to 2007, were selected. From 2007 onwards, phenotypically sensitive but oxacillin-resistant MRSA strains were not received from Latvian microbiology laboratories.

Selection of *S. aureus* isolates to determine the *spa* type

The ten first isolated *S. aureus* strains received from the European Antimicrobial Resistance Surveillance System member laboratories, including up to five MRSA isolates, from September 2006 to February 2007, in accordance with the European *spa* typing protocol.

Member laboratories:

- Pauls Stradins Clinical University Hospital, Central Laboratory;
- Children Clinical University Hospital, Laboratory of Microbiology;
- Riga Eastern Clinical University Hospital, Laboratory of Microbiology;
- State agency 'Infectology Center of Latvia', Laboratory of Microbiology;
- State agency 'Infectology Center of Latvia', Clinic of Tuberculosis and Lung Diseases, Laboratory of Microbiology;

- Riga City Hospital No.1, Laboratory of Microbiology;
- Rīgas Maternity Hospital, Laboratory of Microbiology;
- Vidzeme Hospital, Laboratory of Microbiology;
- Daugavpils Regional Hospital, Laboratory of Microbiology;
- North Kurzeme Regional Hospital, Laboratory of Microbiology;
- Liepaja Regional Hospital, Laboratory of Microbiology.

Criteria of exclusion from the research:
Repeated MRSA and *S. aureus* isolates from the patient**.**

8. Materials and methods

8.1.Creation of a collection and database of MRSA isolates

Preparation of the first isolated MRSA strain stock cultures and their placing into a freezer at –70 °C for long-lasting storage.

The entry of a patient's identification and demographic data into an electronic database file.

8.2. Isolating the *S. aureus* strains from pathological material

The clinical specimen was plated on Columbia blood agar and mannitol salt agar with an inoculating loop. The Columbia blood agar plates were incubated for 18–24 hours in an incubator at a temperature of 36 ^{0}C ± 1 ^{0}C and the mannitol salt agar plates for 24–48 hours at the same temperature. Belonging to the *S. aureus* species was confirmed by coagulation test. If there was suspicion of sepsis caused by staphylococci, 5–10 ml of the patient's blood were put into an aerobic BACTC (BD) blood culture bottle and tested further as described above.

8.3. Determination of the *S. aureus* antimicrobial sensitivity with the agar diffusion test

From *S. aureus* colonies on the Columbia blood agar, a 0.5 McFarland standard suspension was prepared in normal saline and plated on Mueller-

Hinton agar. The chosen antibiotics disks (BD) were put on and the plates were incubated at a temperature of 36 ^{0}C ± 1 ^{0}C for 18-24 hours in the incubator. The results were read after the zone of growth delay.

8.4. Determination of the minimum inhibitory concentration by using the Etest®

The MIC for oxacillin and vancomycin was determined by using the commercial Etest® (Bio Merieux), according to the instructions of the manufacturer and assessed by the CLSI standards.

8.5. Determination of the *S. aureus* antimicrobial sensitivity and assessment of the results in the SIR system with the automated VITEK 2, using the AST 292 kit

The MIC of *S. aureus* was determined by using the automated VITEK 2 for all the antimicrobial substances included in the AST 292 kit. The assessment of sensitivity was determined automatically, using the VITEK 2 software.

8.6. Molecular genetic analysis of MRSA isolates

8.6.1. Molecular verification of MRSA

The molecular verification of methicillin-resistant *Staphylococcus aureus* was performed by using the multiplex polymerase chain reaction, which is based on the specific amplification of particular genes: *mecA* and *clfA* (*S. aureus* marker). Simultaneously the 16S RNA region, typical only to staphylococci, was amplified as well.

8.6.2. Determination of the *Staphylococcus aureus* PVL genes

The *luk-PV gene of Staphylococcus aureus* was determined by PCR, using the specific primer pairs of em 117 and em 118.

8.6.3. Determination of the *SCCmec* type (*Oliveira et al, 2002*)

To determine the SCC*mec* type, the multiplex PCR method with 17 primers was used. These primers are specific to eight loci of different type cassettes and the *mecA* gene as an internal positive control.

8.6.4. Determination of the *mecA* gene class (*Okuma et al,* 2002)

The *mecA* gene class was determined by PCR, using three pairs of primers.

8.6.5. Determination of the ccr gene type (*Okuma et al, 2002*)

The type of the *ccr* gene was determined by four PCR reactions, using five specific primers. One of the primers was common for all the reactions, and it was paired with one of the remaining four primers for each reaction.

8.6.6. Determination of the *Staphylococcus aureus spa* type

To determine the *Staphylococcus aureus spa* type, initially the specific sequence of the *spa* gene was amplified, using PCR. Then, the obtained product of PCR was purified and sequenced.

8.6.7. Determination of the MRSA ST

MLST was performed with a polymerase chain reaction that is based on the specific amplification of seven particular *S. aureus* housekeeping genes: *arcC, aroE, glpF, gmk, pta, tpi, yqiL*. The obtained PCR products were purified and sequenced. The data acquired from sequencing were compared with the ones available in the *SeqNet* database and the isolate sequence type (ST) was determined.

8.7. Statistical data processing

The incidence confidence intervals of MRSA cases and the MRSA isolated from blood were calculated, using the SPSS 16 software. The incidence of MRSA was calculated per 100 000 patient-days, using the official statistical data of the hospital.

9. Results

9.1. Research of the MRSA cases at PSKUS from 2004 to 2010

In 2003, two phenotypically different MRSA were isolated from the pathological materials of patients at the PSKUS Central Laboratory. To prove that these types of *S. aureus* belong to MRSA and to study the distribution of the different MRSA strains at multi-profile hospitals, molecular typing methods were devised at the PSKUS Central Laboratory. In addition, a collection of MRSA strains and a database of the MRSA isolates received from Latvian microbiology laboratories were created and molecular research and typing of the isolated strains was performed. The obtained results were compared with the results in other countries of the EU (Table 10.4.3).

In the period from 2004 to 2010, first MRSA was isolated and registered at the Infection Control Unit as a case of MRSA for 689 patients treated at PSKUS. Analysing each of the cases, 41.2% (n=284) were assessed as MRSA carriers while 58.8% (n=405) were patients with clinical characteristics of MRSA infection. The incidence of MRSA-induced infections at PSKUS is shown in table 9.1.1.

Table 9.1.1.

Number and characterisation of MRSA cases at PSKUS from 2004 to 2010 (n=689)

Year	2004	2005	2006	2007	2008	2009	2010
Patient-days	282198	287813	298077	298973	311022	243777	264126
Number of MRSA cases	136	82	131	83	95	70	92
MRSA incidence per 100 000 patient-days	48.2	28.5	43.9	27.8	31.8	28.7	34.8
95% confidence interval	40.8–57.0	22.6–35.4	37.0–52.1	22.4–43.4	26.0–38.9	22.7–36.2	28.4–42.7
Proportion of MRSA carriers against the total number of MRSA cases (%)	40.4	36.6	32.1	47	48.4	52.9	56.5

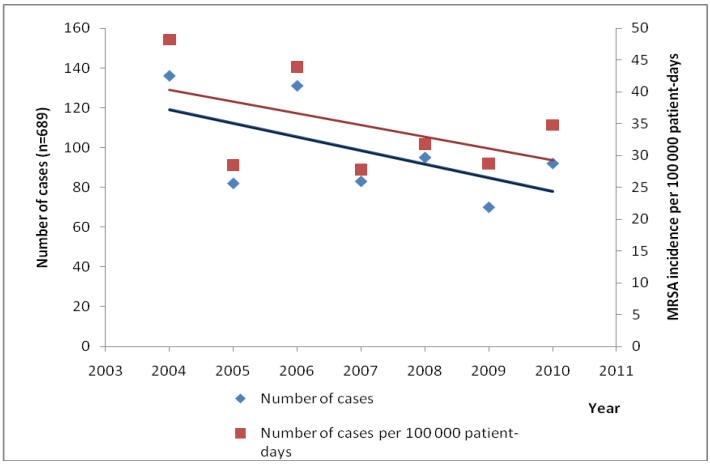

Figure 9.1.1. Correlation diagram of MRSA cases and MRSA incidence indicators from 2004 to 2010. **Number of MRSA cases; R= –0.43, p = >0.05 MRSA incidence; R= –21, p = >0.05**

As seen in figure 9.1.1, although there is a tendency for the number of MRSA cases and MRSA incidence per 100 000 patient-days to shrink, the obtained indicators are not statistically significant, because the observation period is too short. The changes in the proportion of MRSA carriers against all the MRSA cases at PSKUS are shown in figure 9.1.2.

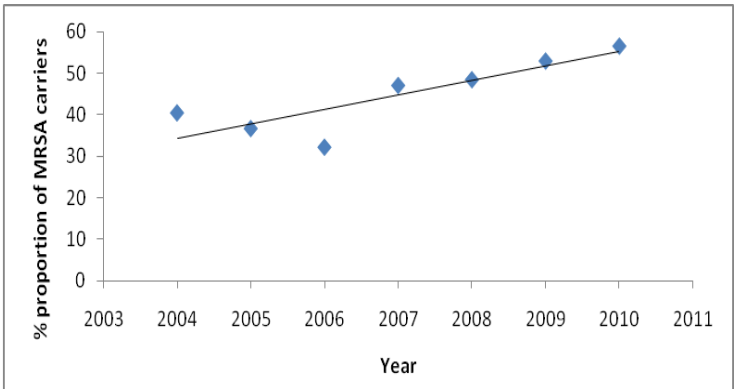

Figure 9.1.2. Correlation diagram of MRSA carriers from 2004 to 2010 (*S. aureus* n=689, MRSA carriers n=284). Proportion of MRSA carriers against all the MRSA cases (%); **R = 0.86, p = <0.05**

At the time of observation, there is a tendency for the proportion of MRSA carriers to rise. This could indicate active screening of MRSA patients at PSKUS, and the increase is statistically significant.

All the MRSA cases were analysed in relation to the patient being located in the units of PSKUS. The distribution of cases at the intensive care, surgical, therapeutic and all other units is shown in figure 9.1.3.

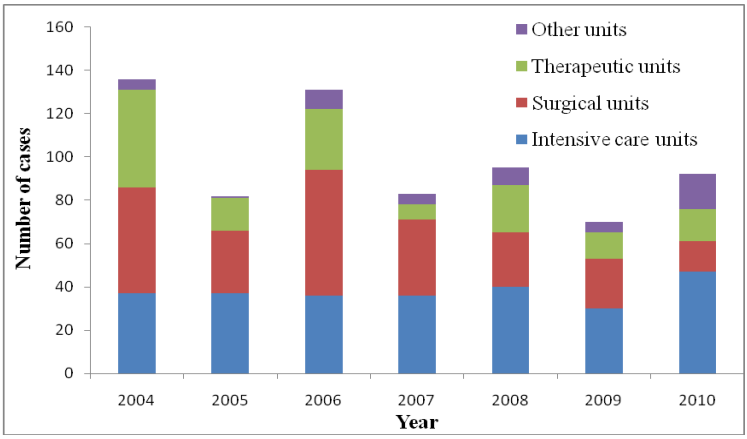

Figure 9.1.3. Distribution of MRSA at different units of the hospital (%) (number of MRSA cases n=689)

To compare the results at PSKUS with other Latvian hospitals and the European database, the MRSA isolated from blood are analysed separately in table 9.1.2.

Table 9.1.2

Analysis of MRSA isolated from blood at PSKUS from 2004 to 2010 (number of blood cultures n=18914, MRSA n=38)

Year	2004	2005	2006	2007	2008	2009	2010
Number of blood cultures	2389	2605	2695	2704	2723	2530	3268
S. aureus	46	45	38	31	56	55	56
MRSA from total number of *S. aureus*, %	0	17.8	28.9	12.9	5.4	1,8	19.6
MRSA incidence in blood per 100 000 patient-days	0	2.8	3.7	1.3	1	0.4	6.4
Confidence interval	0	1.2-5.4	1.8-6.6	0.4-3.4	0.2-2.8	0.01-2.3	4.62-10.31

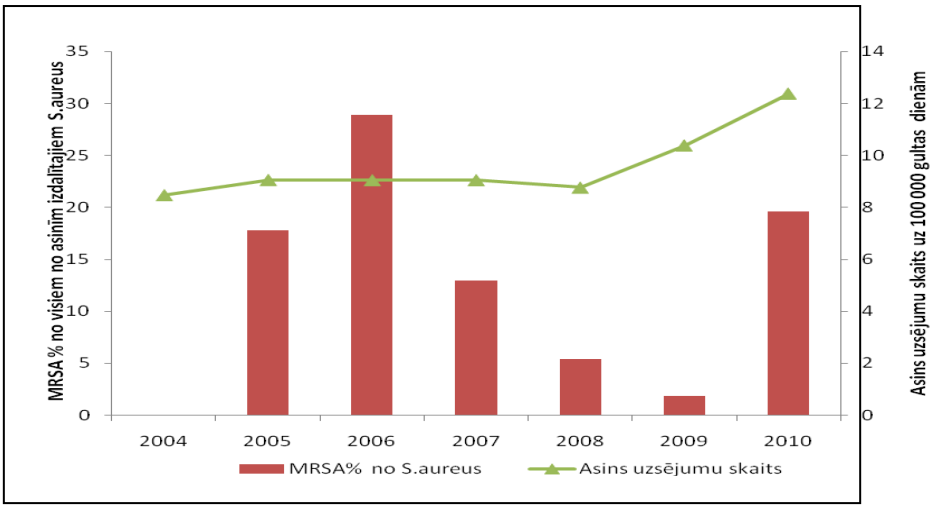

Figure 9.1.4. Proportion of MRSA isolated from blood at PSKUS (%) (number of blood cultures n=18914, MRSA of all the isolated *S. aureus* in percent = 11.6%)

In the period from 2004 to 2010, 18 914 blood samples were examined at the PSKUS Central Laboratory. The first positive *S. aureus* specimen for each patient has been included in the further analysis. Despite the shrinking trend in the percentage of the proportion of all the isolated *S. aureus* found in blood, except the year 2006 (n=28.9%), this trend is not statistically significant to be associated with the changes in the number of examined blood samples from 2004 to 2010.

9.2. Distribution of MRSA isolated from blood in Latvia from 2004 to 2009, compared with the member states of the European Antimicrobial Resistance Surveillance System

Seven laboratories and the hospitals they serviced got involved in the Antimicrobial Resistance Surveillance System from 2004 to 2005. From 2006 to 2009 five additional laboratories along the hospitals they serviced joined the Antimicrobial Resistance Surveillance System. The results of 2010 have not been included for further analysis, because the processing of results takes place at the second half of the next year.

Figure 9.2.1. Geographical placement of the regions and laboratories, participating in the Antimicrobial Resistance Surveillance System

Table 9.2.1

Data of the laboratories and hospitals included in the European and National Antibacterial Resistance Surveillance System from 2005 to 2009

Year	2005	2006	2007	2008	2009
Laboratories providing data	11	12	12	12	7
Hospitals providing data	11	12	12	12	9
Number of inhabitants*	2 306 534	2 294 590	2 281 305	2 270 894	2 261 294
Number of blood culture sets	9395	15 409	15 019	14 584	9717
Number of hospital beds	4603	6129	5969	6329	5508

Year	2005	2006	2007	2008	2009
Patient-days	1 276 513	1 651 925	1 609 778	1 746 084	1 304 118
Average occupancy rate (%)	79	77	75	76	82
Median length of stay (days)	8	7	7	7	7.3
Estimated catchment population **	1 801 593	1 076 400	1 580 323	1 710 000	1 697 000
% total population covered, **	78	47	70	77	75

*Central Statistical Bureau of Latvia

**The absolute and relative population covered is subjective information of the member laboratories. Although the indicators regarding the population covered in regional hospitals are based on the number of inhabitants in the respective region, the most inaccurate information has been received about Riga. This is because Riga is somewhat serviced by two universities and multiple specialised hospitals, which are used not only by the inhabitants of Riga, but also the inhabitants of other regions. Therefore, the data about the population covered were not used for further analysis.

In 2004, the information shown in table 9.2.1 was not collected.

Table 9.2.2

Proportion of *S. aureus* and MRSA isolated from blood (%) (*S. aureus*: n=907, MRSA: n=129).

S. aureus isolates	Year						Total
	2004	2005	2006	2007	2008	2009	
Number of *S. aureus* isolated from blood	87	127	172	169	164	188	907
MRSA (%)	26.4	19.7	18.6	7.7	11.6	9	14.2

As seen in table 9.2.2, the proportion of the MRSA strains isolated from blood against all the isolated *S. aureus* from 2004 to 2009 decreased accordingly by 26.4% (n=23), 19.7% (n=25), 18.6% (n=32), 7.7% (n=13), 11.6% (n=19), and 9% (n=17), but the proportion of the MSSA strains isolated from blood against all the isolated *S. aureus* increased from 73.6% (n=64) in 2004 to 91% (n=171) in 2009.

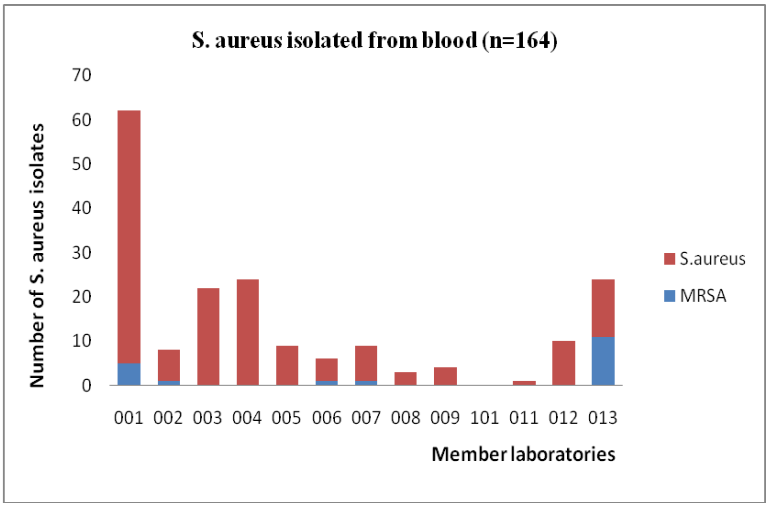

Figure 9.2.2. Distribution of MRSA isolated from blood in the EARSS member laboratories in 2008

It should be noted that in 2008 the increase in the number of MRSA isolated from blood is made up mainly by the results of one (013) of the member laboratories (see figure 9.2.2).

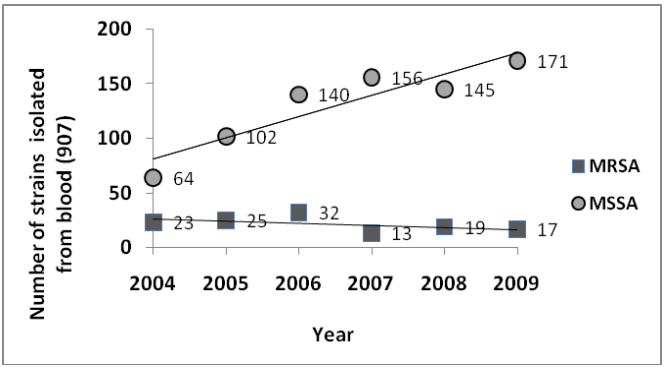

Figure 9.2.3. Correlation diagram of the *S. aureus* and MRSA isolated from blood from 2004 to 2009 (*S. aureus* r=0.94, p<0.05, MRSA r = –0.6; p=0.2)*

*Using Spearman's rank correlation coefficient, because there is not even distribution of data.

In the period from 2004 to 2009, there was an increase in the number of *S. aureus* isolated from blood ($p<0.05$), which indicates a rise in the number of bacteremias initiated by this microorganism. Although there is a shrinking trend of the MRSA isolated from blood, the period of observation is too small to gain statistically significant results ($p>0.05$). The obtained results were also confirmed by the incidence of *S. aureus* isolated from blood per 100 000 patient-days in Latvia (table 9.2.3).

Table 9.2.3.

Incidence of *S .aureus* and MRSA isolated from blood per 100 000 patient-days in Latvia

	Year				
	2005	2006	2007	2008	2009
Incidence of *S. aureus* isolated from blood	8.0	8.5	9.7	8.3	13.1
Confidence interval (CI)	6.6-9.7	7.2–10.0	7.1–9.8	7.1–9.8	13.1–15.2
Incidence of MRSA isolated from blood	1.97	1.94	0.81	1.09	0.81
Confidence interval (CI)	1.33-2.89	1.37-2.79	0.47-1.38	0.70-1.70	1.30 – 2.08

9.3. Phenotypic and genotypic characterisation of MRSA isolates

Regardless of the antimicrobial sensitivity determination method (the agar diffusion test, determination of the minimum inhibitory concentration by using the Etest or the VITEK 2 *Bio Merieux* analyser), two types of MRSA strains were observed for the isolated MRSA, according to their phenotypic characteristics:

a) with multiple resistance to macrolides, tetracyclines, quinolones, and with varying results to aminoglycosides and folate pathway inhibitors, as well as sensitivity to rifampicin and vancomycin that, as already mentioned in the literature review, is characteristic to HA-MRSA;

b) resistant only to oxacillin and sensitive to other antibiotics that is characteristic to CA-MRSA.

9.3.1. Phenotyping of isolates characteristic to HA-MRSA

From the PSKUS Central Laboratory collection of isolates, the profile of antimicrobial resistance was determined with regard to the MIC by using 53 MRSA isolates, suspected to be HA-MRSA, chosen by the VITEK®2 Systems analyser and proportionally representing the period from 2004 to 2010. The obtained results are summarised in table 9.3.1.1.

Table 9.3.1.1.

Antimicrobial sensitivity of MRSA isolates, expressed in mg/l (n=53)

	OXA		GEN		GIP		ERY		CC		TET		VAN		STX		RIF	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
2004.																		
Number of isolates (n=9)	0	9	0	9	0	9	0	9	2	7	0	9	9	0	2	7	0	9
Sensitivity results, mg/l		≥4		≥16		≥8		≥8	≤0,25,-0.5	ind.rez		≥16	≤0,5-1,5		≤10-40	80 - >320		≤0,5
2005.																		
Number of isolates (n=8)	0	8	2	6	0	8	0	8	0	8	0	8	8	0	4	4	8	0
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8		ind.rez.		≥16	≤0,5-1		≤10 - 40	80-160	≤0,5	
2006.																		
Number of isolates (n=8)	0	8	2	6	0	8	0	8	0	8	0	8	8	0	3	5	8	0
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8		ind.rez. / ≥8		≥16	1		20	≥320	≤0,5	
2007.																		
Number of isolates (n=6)	0	6	1	5	0	6	0	6	0	6	0	6	6	0	3	3	6	0
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8		ind.rez/ ≥8		≥16			≤10-20	≥320	≤0,5	
2008.																		
Number of isolates (n=5)	0	5	2	3	0	5	0	5		5	0	5	5		3	2	5	0
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8		ind.rez.		≥16	≤0,5-1		≤10-20	≥320	≤0,5	
2009.																		
Number of isolates (n=5)	0	5	1	4	0	5	0	5	0	5	0	5	5	0	2	3	5	0
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8		4,0		≥16	1,0		≤10-40	80	1,0	
2010.																		
Number of isolates (n=12)	0	12	2	10	0	12	0	12	1	11	0	12	12	0	12	0	11	1
Sensitivity results, mg/l		≥4	≤0,5	≥16		≥8		≥8	≤0,25	4 -≥8		≥16	1		≤10-40		≤0,5	≥32

All the examined *S. aureus* isolates were resistant to oxacillin. The MIC of oxacillin was determined by using the Etest to differentiate between the intrahospital and community-acquired MRSA isolates on a phenotypic level. The resistance of oxacillin of all the examined MRSA strains was >256 mg/l, corresponding to the highest concentration possible to be determined by using the E test All the MRSA isolates were resistant to ciprofloxacin, erythromycin and tetracycline. For 50/53 of the MRSA strains clindamycin-induced resistance was found; 12/53 were sensitive to gentamicin in a concentration of ≤0.5mg/l; 29/53 were sensitive to sulfamethoxazolum/trimethoprimum in a concentration of up to 2/38 mg/l (40 mg/l). All the MRSA isolates were sensitive to vancomycin in the range of 0.5–1.0 mg/l and rifampicin in the range of ≤0.5–1.0 mg/l, except for one strain. This type of phenotypic resistance profile corresponds with HA-MRSA strains, according to literature.

9.3.2. Genotyping of isolates characteristic to HA-MRSA

After the molecular verification of the *mecA* gene which determines the methicillin-resistance of the MRSA (n=53) chosen for the study, genetic typing of these MRSA was performed, using PCR:

a) determining the *SCCmec* type, using multiplex PCR;

For all the MRSA strains chosen for the study, one type III *SCCmec* was defined.

b) MLST for the chosen MRSA;

c) spa type.

Table 9.3.2.1

HA-MRSA sequence types (n=53)

Year	Number of isolates	Gene							ST
		arcC	*aroE*	*glpF*	*Gmk*	*Pta*	*tpi*	*yqiL*	
2004	9	2	3	1	1	4	65	3	368
2005	8	2	3	1	1	4	65	3	368
2006	8	2	3	1	1	4	65	3	368
2007	6	2	3	1	1	4	65	3	368
2008	5	2	3	1	1	4	65	3	368
2009	5	2	3	1	1	4	65	3	368
2010	12	2	3	1	1	4	65	3	368

Table9.3.2.2

spa types detected in HA-MRSA (n=53)

Year	Number of isolates	*spa* repeats	*spa* type
2004	9	15-12-16-02-25-17-25	t425
2005	8	15-12-16-02-25-17-25	t425
2006	8	15-12-16-02-25-17-25	t425
2007	6	15-12-16-02-25-17-25	t425
2008	5	15-12-16-02-25-17-25	t425
2009	5	15-12-16-02-25-17-25	t425
2010	12	15-12-16-02-25-17-25	t425

The obtained results show that there is one endemic HA-MRSA strain common in Latvia – ST368-MRSA-III-(t425).

9.3.3. Phenotyping of isolates characteristic to CA-MRSA

In a separate group, from the PSKUS Central Laboratory collection of MRSA isolates, the antimicrobial resistance profile was determined, using the agar diffusion test for all the phenotypically different MRSA suspected of being CA-MRSA (n=9). Resistance to oxacillin was confirmed with the Etest and expressed in mg/l to determine the MIC (see table 10.3.3.1.).

Table 9.3.3.1.

Antimicrobial sensitivity of CA-MRSA isolates (n=9), assessed in the SIR system

MRSA isolates	Year	OXA*	GEN	CIP	ERY	TET	CC	VAN	STX	RIF
S-5408	2003	2,0	S	S	S	S	S	S	S	S
Amb 7	2004	2,0	S	S	S	S	S	S	S	S
M 228	2004	2,0	S	S	S	S	S	S	S	S
A-2185	2004	0,19	S	S	S	S	S	S	S	S
A-0439	2005	0,38	S	S	S	S	S	S	S	S
20050317-708	2005	0,19	S	S	S	S	S	S	S	S
3256	2005	0,09	S	S	S	R	S	S	S	S
200601100524	2006	0,19	S	S	S	S	S	S	S	S

* Oxacillin MIC mg/l, S – susceptible, R – resistant

9.3.4. Genotyping of isolates characteristic to CA-MRSA

Before continuing the research, all the *S. aureus* strains with different phenotypic characteristics were verified as MRSA by using PCR. To characterise and confirm the difference between these isolates and other MRSA strains in the database and to study their possible affiliation with CA-MRSA, screening was done using PCR to detect the PVL toxin gene called luk-PV, which is one of the main characteristics of CA-MRSA strains.

Although the production of the PVL toxin is an important sign of CA-MRSA identification, intrahospital strains can be producers of the PVL toxin as well (*Naimi et al*, 2003, *Holmes et al*, 2005). Therefore, the first isolate in Latvia suspected of being CA-MRSA was compared to the MRSA strains in Sweden that were genetically similar.

Then, the isolated CA-MRSA PVL-positive MRSA were classified according to their SCC*mec*, sequence type (ST), and *spa* complex. For all the CA-MRSA, one type IV SCC*mec* was found. Regarding the sequence type (ST), the isolated CA-MRSA belonged to two different sequence types: ST30 and ST1.

Table 9.3.4.1.

CA-MRSA sequence types (n=9)

Isolate No.	Gene							ST
	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*	
S-5408-03	2	2	2	2	6	3	2	30
Amb-7	2	2	2	2	6	3	2	30
A-2185-04	2	2	2	2	6	3	2	30
M228	2	2	2	2	6	3	2	30
A-0439-05	2	2	2	2	6	3	2	30
20050317-708	2	2	2	2	6	3	2	30
20050511-676	1	1	1	1	1	1	1	1
3256	1	1	1	1	1	1	1	1
200601100524	2	2	2	2	6	3	2	1

Table 9.3.4.2

CA-MRSA *spa* types

Strain	***spa* sequence**	***spa* type**	ST	***SCC mec***
S-5408-03	08-16-02-16-02-25-17-24	t019	30	IV
Amb-7	08-16-02-16-02-25-17-24	t019	30	IV
A-2185-04	08-12-02-43-34-16-02-16	t1496	30	IV
M228	08-16-02-16-02-25-17-24	t019	30	IV
A-0439-05	15-12-16-02-16-02-25-17-24	t021	30	IV
20050317-708	15-12-16-02-16-02-25-17-24-24	t012	30	IV
20050511-676	07-23-21-22-16-34-33-13	t1497	1	IV
3256	07-23-21-16-34-34-33-13	t098	1	IV
200601100-524	07-23-21-16-34-33-13	t127	1	IV

According to the *spa* type, two new CA-MRSA were found with the *spa* types t1496 and t1497 that had not been known before and were described for the first time.

9.4. Characterisation of *S. aureus* isolated from blood, according to their *spa* type, in Latvia in comparison with the *S. aureus* found in Europe, including MRSA strains

To find out the dominant *S. aureus* and MRSA strains in Latvia, to compare them with the *S. aureus* and MRSA strains common in Europe, and to determine the possible MRSA distribution in Latvia, the PSKUS Central Laboratory took part in a collaborative research, encompassing 26 European countries, 450 hospitals, and 357 laboratories.

38 *S. aureus* and 5 MRSA isolates, received from 11 Latvian laboratories and 12 hospitals, comprised altogether 21 different *S. aureus spa* types (see table 10.4). Out of them, the most common *spa* types were t435 (18.6%) and t015 (11.63%).

Table 9.4.1.

S. aureus spa types (n=43) isolated from blood from 01/09/2006 to 01/03/2007

	spa types								
	MSSA	MSSA	MRSA	MSSA	MSSA	MSSA	MSSA	MSSA	MSSA
	t084	t435	t425	t015	t331	t693	t1255	t1877	t164
	t091	t435	t425	t015	t331	t693	t1255	t1877	t164
	t189	t435	t425	t015	t331	t693			
	t002	t435	t425	t015					
	t160	t435	t425	t015					
	t2928	t435							
	t2934	t435							
	t127	t435							
	t267								
	t779								
	t056								
	t2497								
	t700								
Amount%	30,23	18,6	11,63	11,63	6,98	6,98	4,65	4,65	4,65

For all the analysed MRSA isolates that were received from three laboratories, one t425 *spa* complex was observed, which confirmed the earlier stated supposition about the endemic distribution of this MRSA strain in Latvia. The results coincide with those of studies done in other European countries, thus confirming the hypothesis that *S. aureus* and MRSA strains mostly spread as geographically defined complexes with varying distribution radii.

LV001 *spa* Type Summary

No. isolates	*spa* Type	Pattern	other laboratories (no. isolates elsewhere with same spa Type)	Action
1	t189	r07r23r12r21r17r34	7(7)	click to view on map
1	t1877	r07r23r12r34r12r12r23	3(3)	click to view on map
1	t084	r07r23r12r34r34r12r12r23r02r12r23	71(89)	click to view on map
1	t015	r08r16r02r16r34r13r17r34r16r34	65(92)	click to view on map
1	t002	r26r23r17r34r17r20r17r12r17r16	93(154)	click to view on map

Figure 9.4.1. Europe-wide distribution of *spa* types of *S. aureus* isolated in Latvia (n=43)

10. Discussion

In the cases described in literature, the indicators of MRSA prevalence vary considerably, depending on the profile of the hospital, its units, the number of beds, a country with either high or low distribution of MRSA, among other factors. Although it has not been proven that MRSA strains are more virulent than *S. aureus* strains, it is likely that some *S. aureus* strains, compared with others, may trigger an epidemic of staphylococcal infections (*Shanson* 1981, *Cookson*, *Phillips*).

There is a shrinking trend of MRSA cases at PSKUS in the period from 2004 to 2010; however, this trend is not statistically significant, for the

distribution of MRSA at the hospital is influenced by many factors that have not been studied and there are too few years of surveillance in this case. According to the results, the increase in the proportion of MRSA carriers from all the registered cases at PSKUS is statistically significant. This is likely to be connected with better screening for MRSA carriers, detection of MRSA infections, and early laboratory diagnosis of MRSA, thus influencing the number of bacteremias at the hospital.

To determine the distribution of MRSA in Latvia and in comparison with other European countries, all the *S. aureus*, including the positive MRSA blood cultures, were examined, registered, and analysed, according to the EARS-Net protocol. In Latvia, from 2004 to 2009 there was an overall shrinking trend regarding the MRSA isolated from blood, as shown in figure 10.1.

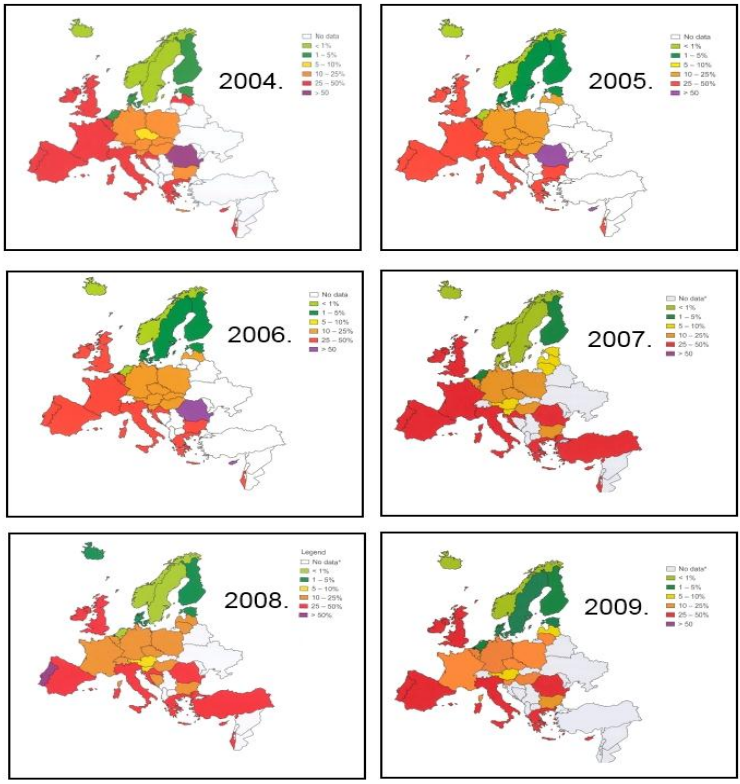

Figure 10.1.Distribution of MRSA in Europe, expressed in percent, and its falling trend in Latvia (EARSS 2004 – 2009)

Clonal origin of the obtained virulence and resistance genes is characteristic to *S. aureus*, because it obtains these genes through horizontal DNA transfer (*Enright et al*, 2002). Therefore, it is possible to find clones of various MRSA strains with specific determinants of virulence and resistance, using methods of genetic typing (*Feil et al;* 2003). On the basis of diversity or homogeneity of the identified *S. aureus* clones, a relatively precise distribution control of strains important for the health of society is possible, e.g. the evolutionary and epidemiological surveillance of CA-MRSA and HA-MRSA.

It was not possible to examine all the isolates at the PSKUS collection by using genetic typing methods due to financial pressure. Thus, only some of the isolated MRSA strains from the PSKUS Central Laboratory collection of isolates were used for the research. According to the SCC mecA type, the ST and the *spa* type, all the MRSA isolates from 2003 to 2010 were genetically identical – ST368-MRSA-III – except the first S-4105 MRSA strain. An MLST analysis showed that the ST368-MRSA-III isolated from the blood of Latvian patients belongs to the clonal complex CC8.

Furthermore, starting from the year 2003, the MRSA strains isolated at PSKUS and received from other hospitals for a confirmation and an in-depth study were researched. The typing method used was determining the *spa* type of all the examined isolates and entering all the obtained data in the central *Rindom SpaServer* server (www.spaserver.rindom.de) for centralised data processing. As seen from the results of the research at the member states of the European Union, MRSA clones mostly spread as geographically defined complexes with varying distribution radii. Hypothetically, one may suppose that the *S. aureus* strains common in Latvia have spread from the nearby European countries, such as Poland, Germany, and Croatia. In these countries, the *spa* complexes characteristic to Latvia can be found, although the dominant types are different over there (http://www.spatialepidemiology.net/SRL-Maps).

Although first it was common just in hospitals, MRSA has now become an important pathogen possible to acquire in community conditions.

The first PVL-positive CA-MRSA strain in Latvia was isolated soon after the first discovered HA-MRSA strains at the beginning of 2003. Isolating the PVL-positive ST30-MRSA-IV strain was an important discovery, confirming the hypothesis about the distribution of CA-MRSA in Latvia as well. According to the results of gene sequencing, the isolated CA-MRSA belonged to two different sequence types: ST30 and ST1, and various *spa* types.

Although further analysis of the molecular epidemiologic information of MRSA infections is required, the performed research has given evidence for the distribution of MRSA in Latvia and has suggested directions for a scientifically justified MRSA infection control and epidemiology research in cases of MRSA infections, and new methods of determining the clonal affiliation of the isolated MRSA have been devised.

11. Conclusions

1. The total number of MRSA cases at PSKUS from 2004 to 2010 is without significant changes. Over the period of the study there was statistical valid number of increase of newly identified MRSA carriers, however, the proportion of MRSA bacteremias decreased, which indicate efficient surveillance measures.
2. During the research, similarly other European countries - Austria, Bulgaria, France, Greece, Ireland, Romania, Great Britain, the proportion of MRSA bacteremias shrank throughout the country from 26,6% (2004) to 9% (2009).
3. There is one endemically common HA - MRSA strain in Latvia - ST368-MRSA-III belonging to the *spa* type *t425*. Besides that two CA-MRSA strains have been found: ST30-MRSA-IV and ST1-MRSA-IV. Their distribution in Latvia, however, is not known.

4. The ST368-MRSA-III MRSA strain is not common for the MRSA isolates in other European countries confirming the endemic distribution of this strain in Latvia. There are known MSSA strains that belong to this sequence type. ST30-MRSA-IV and ST1-MRSA-IV are identical CA – MRSA strains found in other European countries.

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