

Theses of doctoral (PhD) dissertation

**INVESTIGATION OF METHICILLIN-
RESISTANT STAPHYLOCOCCUS AUREUS
INFECTION IN FARM ANIMALS AND FOOD
USING MALDI-TOF MS TECHNIQUE**

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1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL DISSERTATION

The use of antimicrobials has revolutionized medicine in several ways but their use has been accompanied for decades by the emergence of antibiotic-resistant bacteria that cause issues in global health. Multidrug-resistant forms of pathogens, which can spread from animals to humans through contact, the food chain, and the environment, are a growing animal health concern due to - among other things - high levels of antibiotics used in intensive animal husbandry. Therapeutic options for infections caused by multidrug-resistant pathogens are becoming increasingly limited, so unprofessional use of antibiotics must be reduced to maintain their effectiveness in medicine (WHO, 2017).

Among antibiotic-resistant pathogens, the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections varies in different countries, however, it is still one of the most common causes of severe bacterial infections in Europe. Today, resistant *Staphylococcus aureus* species no longer occur only in health care settings (HA-MRSA) and communities (CA-MRSA) but also in animal husbandry (LA-MRSA), thus, they can also be present in foods of animal origin. Molecular typing results for isolates indicated that many types of MRSA are present in the food chain in different countries, however, surveillance of livestock-related and food-borne MRSA strains is currently voluntary. However, epidemiological, and evolutionary monitoring of MRSA strains is of great importance both in terms of food safety and public health (EFSA, 2009; ECDC, 2019).

In Hungary, as in the European Union, the determination of the number of coagulase-positive *Staphylococcus* in certain foodstuffs is mandatory by law, however, the required standard does not allow for identification and does not cover the mapping of antibiotic resistance. The mass spectrometric method based on the coupling of a matrix-assisted laser desorption ion source and a time-of-flight mass analyser, the MALDI-TOF MS technique, offers a new diagnostic opportunity in microbiology. It provides information on the macromolecules and protein profile of the test sample, which is the basis for determining the taxonomic position on the one hand and the starting point for the isolation of MRSA and MSSA (methicillin-sensitive *Staphylococcus aureus*) isolates on the other hand.

1.1. Objectives

The Food Testing Microbiology Laboratory of WESSLING Hungary Kft. receives nearly 5,000 food samples per year to determine the number of coagulase-positive *Staphylococcus* species. However, the MSZ EN ISO 6888-1: 2008 standard specified for this purpose in the decree does not allow the identification and isolation of species and subspecies, as well as the determination of antibiotic resistance. The identification and segregation of species and subspecies of outstanding importance in both animal husbandry and veterinary medicine is not negligible due to the different degrees of virulence and pathogenicity, and the mapping of antibiotic resistance is essential from a public health point of view. In our work, we aimed to study protein-based and molecular biological studies of *S. aureus* species isolated from food and farm animals:

- Comparison and development of MALDI-TOF MS sample preparation methods to optimize the parameters of the obtained mass spectra.
- Investigation of the media and culture time used and their effects on the mass spectral parameters of the matrix solutions.
- Secure identification of *S. aureus* species and subspecies using MALDI Biotyper software.
- Identification of specific peaks suitable for the isolation of species of the “*Staphylococcus aureus* clonal complex”.
- Determination of methicillin resistance-specific peaks suitable for the isolation of MRSA and MSSA strains.
- Providing data for the detection of MRSA infection in foods of animal origin.
- Mapping of *Staphylococcus* enterotoxin genes in strains isolated from food.
- *Spa* and MLST typing of isolates for epidemiological significance of strains as well as for revision.
- Comparison of marker sets obtained during the MALDI-TOF MS technique and MRSA strains characterized by molecular biological typing.

2. MATERIAL AND METHOD

2.1. Identification of coagulase-positive *Staphylococcus* species

Coagulase-positive *Staphylococcus* species were isolated according to the methods of the standard MSZ EN ISO 6888-1: 2008, and the coagulase positive test was confirmed by performing a latex agglutination (PASTOREX™ STAPH-PLUS) rapid test.

2.2. *Staphylococcus aureus* strains tested

Reference Strains: 6 methicillin-sensitive *S. aureus* (ATCC 29213, ATCC 6538, ATCC 14458, ATCC 19095, ATCC 27664), 1 *S. argenteus* (DSM 28299^T), 1 *S. schweitzeri* (DSM 28300^T) and 2 methicillin-resistant *S. aureus* (ATCC 33591, ATCC 43300) strains were used to develop the sample preparation method for the MALDI-TOF MS study. In addition, the reference strain DSMZ 20231 *S. aureus* was used for sequence-based typing.

Human isolates: MRSA strains isolated from 6 nasal secretions and 2 wound exudates were also included in the studies which were provided to us from the home stock collection of MikroMikoMed Kft.

Strains isolated from farm animals: The MRSA strain isolated from the 13 farm animals included in the study was provided to us by the Department and Clinic of Equine Medicine of the University of Veterinary Medicine Budapest from its domestic strain collection.

Strains isolated from food: In the study, 58 coagulase-positive *Staphylococcus* strains were identified from 647 food samples, of which 47 isolates were found to be *S. aureus*.

2.3. MALDI-TOF MS assay

2.3.1. Sample preparation methods

Ethanol extraction (EtOH-FA): 300 µl of deionized water was pipetted into a 1.5 ml Eppendorf tube. A few tenths of a milligram of the sample was suspended in the deionized water by vigorous stirring, 900 µl of absolute ethanol was added. Following vortexing and centrifuging (13,000 rpm, 3 minutes), the supernatant was discarded and the residual solvent was allowed to evaporate. 20 µl of 70% formic acid was added to the sample and allowed to

stand for 2 minutes. Then, 20 µl of acetonitrile was added to the sample, mixed, and centrifuged (13,000 rpm, 3 minutes). 1 µl of the solution supernatant was added dropwise to one position of the sample plate. Immediately after evaporation of the solvent, 1 µl of matrix solution was added dropwise to the sample and the sample was allowed to dry out in a biosafety box (Bruker Daltonik GmbH, 2013a).

Direct sample transfer with formic acid (DT-FA): A few tenths of a milligram of biological sample was applied to one position on the prepared sample holder plate. 1 µl of 70% formic acid was added to the smeared samples and allowed to dry out in a biosafety box. 1 µl of matrix solution was then pipetted onto the samples (Bruker Daltonik GmbH, 2013a).

Direct sample transfer (DT): A few tenths of a milligram of biological sample was applied directly to one position on the prepared sample plate. 1 µl of matrix solution was dropped on the sample and the solvent was allowed to evaporate in a biosafety box, drying out the sample (Bruker Daltonik GmbH, 2013b).

2.3.2. Measurement parameters of MALDI-TOF MS technique

Test parameter settings such as activation of method files and calibration were performed using **FlexControl** software (Bruker Daltonik GmbH). Mass spectra were recorded in automatic spectrum acquisition mode, preferably from an average of 200-500 laser shots. After smoothing and baseline correction, calibration was performed based on the identification of reference mass numbers. During the calibration, the deviations of the mass numbers were not allowed to exceed 300 ppm. The method file was then saved and the measurement of samples was started in automatic spectrum recording mode in the mass range of 2-20 kDa.

2.3.3. Evaluation of mass spectra

Isolates were identified using **MALDI BioTyper 2.0** software (Bruker Daltonik, GmbH). The software compares the mass spectra obtained with the reference spectra in its library. In the case where the identification log score value was greater than 2.300, the identity between the test micro-organism and the micro-organism in the reference spectrum library was very likely, the species were considered as identified. In the case of a log score value between 2.000 and 2.299, the identity was less certain, therefore in this case the genus of the microorganism can only be considered as identified. Between 1.700 and 1.999 log scores,

even the identification of the genus can no longer be considered sufficiently certain. Between 0.000 and 1.699 log scores, the identification of the micro-organism failed.

Spectrum analysis was performed using **FlexAnalysis 3.4** software (Bruker Daltonik, GmbH), which allows calibration of the recorded spectra as well as their detailed analysis. During the study, the obtained mass spectra were smoothed with the Savitzky-Golay filter, and the baseline correction was performed with the TopHat algorithm.

FlexAnalysis 3.4 software made the export the mass numbers of the peaks and their parameters to **Microsoft Office Excel 2007 possible**, thereby providing the opportunity to analyze the recorded spectra using statistical methods.

The effect of sample preparations, matrix solutions, media used and culture time on the measurable parameters of the spectra as the average of the number of peaks in the spectrum and the average of the identification log score values was analysed in **SPSS 16.0** statistical program by a factor analysis of variance, Tukey's test, during which a significance level of 5% was used.

2.4. Antibiotic susceptibility testing of *S. aureus* strains

2.4.1. Culture based methods

In the **disk diffusion method**, antibiotic resistance of the strains was tested according to the specifications defined by CLSI (Clinical and Laboratory Standards Institute) (2019), with Cefoxitin 30 µg discs. According to the specifications for MRSA strains, the clearance zone was 6 to 19 mm.

CHROMagar MRSAII is a selective differential medium for the cultivation of methicillin-resistant *Staphylococcus aureus* species. Strains that formed colonies that were mauve-coloured and morphologically similar to *Staphylococcus* species were considered MRSA.

2.4.2. Study of the *mecA* gene complex

The study was performed according to a protocol issued by the National Food Institute (NFI) of Denmark in 2012. Genomic DNA was isolated from the bacterium then the *mecA* gene region was amplified by PCR. The size of the *mecA* gene segment obtained with the given primer pair is 527 bp in length (Table 1).

Table 1. PCR primers used to amplify the *mecA* gene region

Primer name	Sequence of primer
mecA_fwd	5'- GGGATCATAGCGTCATTATTC-3'
mecA_rev	5'- AACGATTGTGACACGATAGCC-3'

2.5. Sequence-based typing methods

2.5.1. *spa* typing

Genomic DNA was isolated from the bacterium and the *Staphylococcus aureus* protein A gene region (*spa*) was amplified by PCR. The procedure was performed according to the protocol issued by the National Food Institute (NFI) in 2012. The primer pair shown in Table 2 was used to amplify the *Staphylococcus aureus* protein A gene region.

Table 2. PCR primers used to amplify *Staphylococcus aureus* protein A gene region

Primer name	Sequence of primer
spa-1113F	5'-TAAAGACGATCCTTCGGTGAGC-3'
spa-1514R	5'-CAGCAGTAGTGCCGTTTGCTT-3'

DNA sequencing was also performed separately from both directions. Sequence results were evaluated and *spa* types were determined using **BioNumerics 7.6** software and **Ridom SpaServer** database. Sequence alignments were checked and then '*spa*' types identifiable by repeats were analysed.

2.5.2. Multilocus sequence typing

Genomic DNA was isolated from the investigated strains and then the 7 gene segments specific to *Staphylococcus aureus* were amplified by PCR. The nucleotide sequence of the PCR products was determined and sequence data evaluation was performed using *BioNumerics 7.6* software. The software identified the gene segment boundaries defined in the PubMLST database and assigned the sequence type and allele version numbers to the sample. The procedure was developed based on a study by THOMAS et al. (2007). The 7

household genes developed for *Staphylococcus aureus* strains and the sequences of the primers used to amplify the genes are shown in Table 3.

Table 3. Genes and primers used to amplify in the MLST method

(<https://pubmlst.org/saureus/>)

Gene name	Primer name	Sequence of primer (5'-3')
<i>Carbamate kinase (arcC)</i>	arc up_1	TTG ATT CAC CAG CGC GTA TTG TC
	arc dn_2	AGG TAT CTG CTT CAA TCA GCG
<i>Sikimic acid dehydrogenase (aroE)</i>	aro up_3	ATC GGA AAT CCT ATT TCA CAT TC
	aro dn_4	GGT GTT GTA TTA ATA ACG ATA TC
<i>Glycerol kinase (glpF)</i>	glp up_5	CTA GGA ACT GCA ATC TTA ATC C
	glp dn_6	TGG TAA AAT CGC ATG TCC AAT TC
<i>Guanylate kinase (gmk)</i>	gmk up_7	ATC GTT TTA TCG GGA CCA TC
	gmk dn_8	TCA TTA ACT ACA ACG TAA TCG TA
<i>Phosphate acetyltransferase (pta)</i>	pta up_9	GTT AAA ATC GTA TTA CCT GAA GG
	pta dn_10	GAC CCT TTT GTT GAA AAG CTT AA
<i>Triose phosphate isomerase (tpi)</i>	tpi up_11	TCG TTC ATT CTG AAC GTC GTG AA
	tpi dn_12	TTT GCA CCT TCT AAC AAT TGT AC
<i>Acetyl coenzyme A acetyltransferase (yqiL)</i>	yqi up_13	CAG CAT ACA GGA CAC CTA TTG GC
	yqi dn_14	CGT TGA GGA ATC GAT ACT GGA AC

2.6. Investigation of genes encoding enterotoxins

Following genomic DNA isolation, multiplex PCR protocols were performed to detect SE genes (*sea*, *seb*, *see*, *sed*, *sec*, *ser*, *seh*, *selj*, *seg*, *sei*, *selp*) in the strains using primer sets from the European Union Reference Laboratory for Coagulase-Positive Staphylococci (EU-RL CPS). Primers detecting the *seh* and *ser* genes were run as a third, separate reaction to avoid overlapping PCR products of similar size during gel electrophoresis. Amplicons were separated by agarose gel electrophoresis and the presence or absence of each gene was identified based on their estimated size.

3. RESULTS

3.1. Development of sample preparation methods for MALDI-TOF MS investigation

When comparing and selecting sample preparation methods, the most important factors considered were the number of peaks that make up the spectra, the intensity of the entire spectrum, and the minimum and maximum m/z peaks detected. Reference strains were grown on Columbia blood agar for 24 h and using α -HCCA matrix based on the results of previous studies (JOSTEN et al., 2013). Manual analyses of the mass spectra in the preliminary experiments indicated the need to develop a sample preparation procedure that produced spectra in similar resolution and intensity as with ethanol extraction, but in a much shorter time and in fewer steps.

A modified formic acid suspension procedure (M-FA) was developed which the first step was pipetting 20 μ l of 70% formic acid into a 1.5 ml Eppendorf tube. A few milligrams of biological samples were taken from the test culture with a sterile loop and suspended in 20 μ l of 70% formic acid for 45 seconds. Then 20 μ l of acetonitrile was added to the suspension and mixed well. Thereafter 1 μ l of the solution was dropped to one position of the prepared sample plate. For each sample, 6 parallel measurements were performed. 1 μ l of matrix solution was dropped to the dried sample and allowed to dry in the laminar box.

First, the effect of sample preparation procedures on parameters of spectra was examined. Out of the 4 methods, the fewest peaks were detected by direct transfer preparation (DT). When complemented by direct transfer preparation with formic acid (DT-FA), a more favorable number (78) of peaks appeared in the spectrum. The most number of peaks (119) were detected by ethanol extraction (EtOH), however, the number of peaks in spectra was also greater than 100 for the modified formic acid suspension (Table 4).

Table 4. Effect of sample preparation procedures on parameters of spectra

Tested parameters	Sample preparation methods			
	DT	DT-FA	EtOH	M-FA
Average number and deviation of detected peaks (pc)	67 (1.51)	78 (1.13)	119 (2.51)	104 (1.66)
Intensity of spectra (a.u.)	1×10^4	1×10^4	1×10^5	1×10^4
Mass number range (m/z)	1901- 15051	1901-15051	2090-15051	1901-19084

The intensities of spectra displayed similar behaviour with the different sample preparation methods. The intensity of the spectra of reference strains was 104 a.u. with DT, DT-FA, M-FA sample preparation methods. The highest spectra intensity was obtained by EtOH extraction, however, in this case, the smallest detectable m/z peak was larger (2090 m/z) than in the other 3 methods. The highest mass number range was achieved by the M-FA method, in that case, the smallest detectable peak was 1901 m/z, while the largest was 19084 m/z. Above 15.000 m/z peaks were detected only with this sample preparation.

Finally, the sample preparation methods were compared based on MALDI BioTyper results of identification of the reference *S. aureus* strains. The lowest identification means log score values were obtained by the DT method, furthermore, with the DT-FA method, the mean identification score of the strains was only 2.203. During the EtOH and M-FA sample preparation methods, the mean identification log score of the strains was greater than 2.300, so the identification of microorganisms was highly probable. There was no significant difference ($p=0.56$) between the mean identification log score values obtained with M-FA and EtOH methods. However, the M-FA sample preparation method is a faster method, which, similarly to the EtOH method, allows the safe identification of species and subspecies.

3.2. Comparison of matrix solution

After comparing and selecting the sample preparation method, the effect of the matrix solutions on the spectra was analyzed. The culture conditions were the same as described in the previous subsection (3.1). In addition to the α -HCCA matrix solution, the effect of 2 matrix solutions, SA and DHB, on the spectra was also investigated in the studies (Table 5).

Table 5. Effect of matrix solutions on parameters of spectra

Tested parameters	Matrix solution		
	SA	DHB	α -HCCA
Average number and deviation of detected peaks (db)	98 (1.87)	86 (2.78)	104 (1.66)
Intensity of spectra (a.u.)	3×10^3	1×10^4	1×10^4
Mass number range (m/z)	1827- 9627	1901-15051	1901-19084

SA matrix is used to detect higher mass proteins, however, based on the results, we were able to detect peaks in the smallest mass number range with this matrix solution. In this case, the largest detectable protein was the 9627 m/z peak. DHB matrix allowed the detection of peaks in the range of 1901–15051 m/z, but the average number of peaks appeared was below 100. In terms of intensity of spectra, the DHB and α -HCCA matrix solution met the expectations, however, using the SA matrix, the average intensity of spectra was only 3000.

3.3. Comparison of culture conditions

During the experiments, the spectra of reference strains grown on 4 different media were analyzed. In the spectra of strains grown on Baird-Parker (BP) selective medium, the average number of detectable peaks was 62, which was significantly less compared to the other 3 cases (Table 6). There was no significant difference between the mean number of detectable peaks of strains grown on Mueller-Hinton (MH) Chocolate (CS) and Columbia (C) blood agar.

Table 6. Effect of culture media on parameters of spectra

Tested parameters	Culture media			
	BP	MH	CS	C
Average number and deviation of detected peaks (pc)	62 (2.45)	98 (3.00)	102 (2.69)	104 (1.66)
Intensity of spectra (a.u.)	3×10^3	6×10^3	8×10^3	1×10^4
Mass number range (m/z)	1901-19084	1901-19084	1901-19084	1901-19084

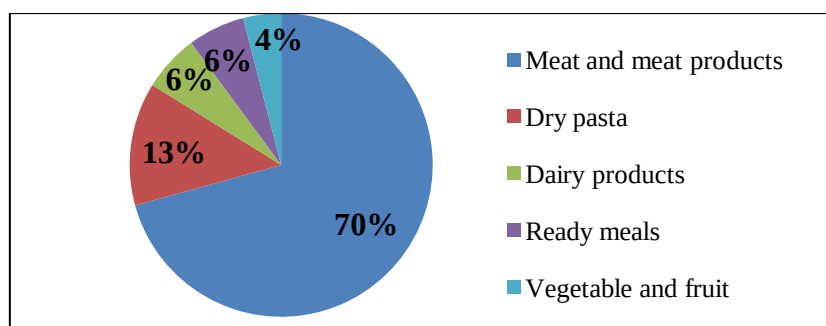
After selecting the media, the effects of the culture time on the spectra were investigated. Reference strains were incubated on Columbia blood agar at 37°C for 24, 48, and 72 h. There was no significant difference ($p=0.538$) in the number of detectable peaks after 24 h and 48 h of culture (Table 7). After a culture time of 72 h, the number of detectable peaks decreased, which was observed mainly between the range of 1900-4306 m/z and 9627-19084 m/z. Based on the results, the 24 h culture time was used for further measurements.

Table 7. Effect of culture time on parameters of spectra

Tested parameters	Culture time		
	24 h	48 h	72 h
Average number and deviation of detected peaks (pc)	104 (1.72)	102 (1.89)	76 (± 3) (1.81)
Intensity of spectra (a.u.)	1×10^4	1×10^4	1×10^4
Mass number range (m/z)	1901-19084	1901-15051	1901-19084

3.4. Identification of isolates with MALDI Biotyper software

Identification of coagulase-positive strains isolated from food and farm animals at the species and subspecies level was performed using MALDI Biotyper software. All isolates were identified above 2.300 log scores, 56% of which exceeded 2.400 log score values. During the study, *S. aureus* strains were identified from 5 different food groups, 70% of which were isolated from meat and meat products, and 13% from dry pasta (Figure 1).

**Figure 1.** Distribution of *S. aureus* strains isolated from the food by food group

S. aureus strains isolated from animals were also identified by MALDI Biotyper software. All isolates were identified above 2.400 log scores and 77% of the strains were showed great similarity with the mass spectrum of the reference strain of *Staphylococcus aureus* subsp. *aureus* DSM 799 (Table 8).

Based on the study of PÉREZ-SANCHO et al. (2018), the 3430 m/z and the 6861 m/z peaks are the subspecies-specific peaks for *S. aureus* subsp. *anaerobius*. Following the identification, the mass spectra of the isolates were also manually analyzed using FlexAnalysis 3.4 software, during which we could rule out the presence of two subspecies specific peaks.

Table 8. The result of the identification of strains isolated from husbandry animals

ID number	Species	Log score value	Best match
SA-127	cattle	2.453	1
SA-146	cattle	2.534	1
SA-297	horse	2.467	1
SA-136	swine	2.543	1
SA-237	cattle	2.465	1
SA-034	swine	2.467	1
SA-113	swine	2.474	1
SA-126	swine	2.408	3
SA-116	cattle	2.483	2
SA-121	cattle	2.526	2
SA-145	cattle	2.488	1
SA-329	horse	2.517	1
SA-065	swine	2.462	1

1: *Staphylococcus aureus* subsp *aureus* DSM 799; 2: *Staphylococcus aureus* subsp *aureus* DSM 20231T DSM;
3: *Staphylococcus aureus* ATCC 33862 THL

3.5. Identification of *S. aureus* complex species by MALDI-TOF MS technique

In previous studies, a number of specific peaks were identified that might be suitable for the identification and discrimination of *S. aureus*, *S. argenteus*, and *S. schweitzeri*. Within the scope of the study, 29 species-specific peaks determined by JOSTEN et al. (2013) and SCHUSTER et al. (2017) were tested on the reference strains (Table 9). Out of the 29 peaks, 8 peaks (6889 m/z, 10105 m/z, 5045 m/z, 5002 m/z, 6580 m/z, 2289 m/z, 6613 m/z, 3007 m/z) appeared in different species compared to the results of previous studies.

Table 9. Appearance of specific peaks in the mass spectra of *S. aureus* complex species

Species	m/z
<i>S. aureus</i> / <i>S. argenteus</i> / <i>S. schweitzeri</i>	2636, 3891, 4306, 4814, 6354, 6889*, 8091, 8891, 9627
<i>S. aureus</i> / <i>S. argenteus</i>	10105*, 3037
<i>S. aureus</i> / <i>S. schweitzeri</i>	5045*, 6613*
<i>S. argenteus</i>	5002*, 6580*
<i>S. aureus</i>	2201, 2289*, 2307, 2685, 3007*, 3875, 4485, 4497, 5507, 5525, 5539, 5551, 6423, 6552

* Peaks were appeared in different species compared to the results of previous studies.

Furthermore, 8 specific peaks were determined by detailed analysis of the mass spectra of the reference strains, that allow species to be discriminated and identified (Table 10). In the mass spectrum of strains isolated from food and husbandry animals *S. argenteus* and *S. schweitzeri* specific peaks were not detected.

Table 10. New specific peaks to discriminate and identify species of the *S. aureus* complex

Species	m/z
<i>S. argenteus</i> / <i>S. schweitzeri</i>	2641
<i>S. schweitzeri</i>	3453
<i>S. schweitzeri</i>	4911
<i>S. aureus</i> / <i>S. argenteus</i>	4942
<i>S. aureus</i>	5032
<i>S. argenteus</i> / <i>S. schweitzeri</i>	5286
<i>S. aureus</i> / <i>S. argenteus</i>	6818
<i>S. schweitzeri</i>	6908

3.6. Investigation of methicillin resistance by MALDI-TOF MS Technique

The determination of methicillin resistance-specific peaks as well as the testing of the peaks found in previous studies were first performed on the reference strains. During the study, 10 different MRSA-specific peaks identified in previous studies were tested on the reference MSSA and MRSA strains (Table 11).

Table 11. MRSA-specific peaks detectable in the reference strains

(m/z)	Reference strains						
	MRSA		MSSA				
	ATCC33591	ATCC43300	ATCC29213	ATCC6538	ATCC14458	ATCC19095	ATCC27664
2127	+	-	-	-	-	-	-
2414	+	+	-	-	-	-	-
3043	+	+	+	+	+	+	+
4607	-	-	-	-	-	-	-
5070	+	-	-	-	+	-	-
6576	+	+	-	+	+	-	+
6885	+	-	-	-	-	-	-
9216	-	-	-	-	-	-	-
9632	+	+	+	+	+	+	+
10152	-	+	-	-	-	-	-

Of the peaks tested, the peaks at 2414 m/z, 6885 m/z, and 10152 m/z were those detected only in MRSA strains and did not appear in MSSA strains.

The specific peaks we determined (Table 12, m / z values marked with *) by analysing the spectra of the reference strains, which were tested on the spectra of livestock animals and clinical MRSA isolates.

3.7. Investigation of methicillin resistance in *S. aureus* strains isolated from food

3.7.1. Investigation of MRSA-specific peaks in mass spectra of strains isolated from food

The presence or absence of MRSA-specific peaks was examined in the mass spectra of strains isolated from food. In the analysis of the spectra, a strain isolated from a pork rib and a goose liver proved to be methicillin resistant.

Table 12. Incidence of MRSA-specific peaks in the mass spectra of strains isolated from food

MRSA specific peaks (m/z)	Incidence (%)	
	MSSA strains isolated from food (n=45)	MRSA strains isolated from food (n=2)
2127	0	100
2414	0	100
3043	53	100
4607	0	0
5070	62	100
6576	71	100
6885	0	100
9216	0	0
9632	97	100
10152	0	50
2453*	0	100
4461*	46	100
2548*	80	50
2718*	0	100
3885*	0	0

* New methicillin resistance-specific peaks determined from reference strains

3.7.2. The result of disk diffusion method and MRSA CHROMagar selective differentiating media

For strains isolated from food, the presence of antibiotic resistance was also confirmed by the disk diffusion method and MRSA selective differentiation medium. Parallel measurements were performed for all isolated strains and the test was also performed on the reference MSSA and MRSA strains.

In the case of the disk diffusion method, the clearance zone of 45 strains fell between 23 and 29 mm. The clearance zone of the strain isolated from goose liver was 9 mm and the clearance zone of the strain isolated from pork rib was 17 mm, so both *S. aureus* strains proved to be methicillin-resistant by the disk diffusion method. Strains isolated from goose liver and pork ribs formed mauve colonies on MRSA CHROMagar medium, but no colonies were observed for reference MSSA strains and other food-isolated strains.

3.7.3. Result of *mecA* gene typing

MRSA selective, differentiation medium, disk diffusion method and MALDI-TOF MS studies showed that strains isolated from goose liver and pork rib showed methicillin resistance, therefore it was hypothesized that the *mecA* gene responsible for PBP2a synthesis could be detected in the strains. In the study, the ATCC 43300 MRSA strain was used as a positive control strain, while the ATCC 29213 MSSA strain was used as a negative control. Among the strains isolated from food, the *mecA* gene complex was detected in the strains isolated from goose liver and pork rib.

3.8. Investigation of enterotoxin producing in *S. aureus* strains isolated from food

SE genes were detected in 23 of the 47 *S. aureus* strains isolated from food in a multiplex PCR study. 91% of these strains carried only one *SE* gene, however, 2 genes were also detected in 2 strains. The *seh* gene was detected in 44% and the *seip* gene in 31% of the 23 strains producing enterotoxin. (Figure 2). *Seh* genes were detected in strains isolated from dry pasta and meat, and *seip* genes were detectable in some *S. aureus* strains isolated from dairy products, meat, and vegetables. 17% of enterotoxin-producing strains carried *sei* genes, which were mainly isolated from poultry meat.

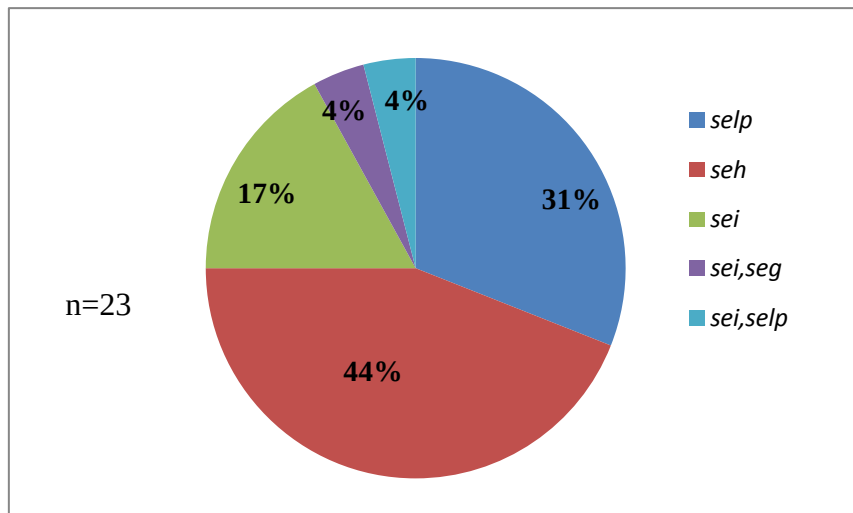


Figure 2. Distribution of SE genes in *S. aureus* strains -producing enterotoxin (n=23)

3.9. Results of sequence-based typing of *S. aureus* strains isolated from food and livestock animals

Strains isolated from food showed high variability as 20 different *spa* types were identified in the 47 isolates during typing. The most common *spa* type was the t084. 19% of the strains belonged to this type. Furthermore, there were two common *spa* types: t091 and t1491. The incidence was 13% for both types. For two pork isolates, a hitherto unknown combination of repeats has been identified in the sequences. The MRSA strain isolated from goose liver belonged to the t706 *spa* type and the MRSA strain isolated from the pork rib belonged to the t011 *spa* type.

Of the 20 *spa* types identified, 8 were able to assign the sequence type to that sample. The most common t084 *spa*-type strains belonged to the ST15 MLST type. *Spa* strains t091 were ST7, *spa* strains t213 were ST12, *spa* strains t127 were ST1, *spa* strain t002 was ST5 and *spa* strain t011 was ST398. types, respectively.

Of the 13 strains isolated from livestock animals, 4-4 strains belonged to *spa* types t011, t034, and t127, while 1 strain belonged to *spa* type t458. Based on the MLST study, *spa* type strains t011 and t034 belonged to type ST398 MLST. Sequence type ST1 was assigned to *spa* type t127 and ST97 to *spa* type t458.

3.9.1. Analysis of the correlations between the identified sequence types and the marker sets obtained by the MALDI-TOF MS technique

In the mass spectra of strains isolated from food and livestock animals (n = 60), 4 specific peaks were tested for 7 different ST types and 14 specific peaks were determined. One type had several isolates, so the frequency of specific peaks appearing was indicated (Table 13).

Table 13. ST-specific peaks appeared in the spectra of strains isolated from food and livestock animals

Specific peaks m/z	Előfordulási gyakoriság a szekvencia típusokban (%)							
	ST7 n=6	ST15 n=9	ST1 n=14	ST5 n=1	ST12 n=3	ST97 n=1	ST398 n=8	Ismeretlen n=18
3007 ^a	0	0	100	0	0	0	0	0
3891 ^b	0	0	0	100	0	0	0	0
5004 ^b	0	0	0	0	0	0	13	0
4511 ^c	0	0	0	0	0	0	100	0
3037	0	0	79	0	0	0	0	0
4860	100	100	0	0	0	0	0	0
4874	100	100	0	0	0	0	0	0
5598	0	0	36	0	0	0	0	0
5653	0	0	36	0	0	0	0	0
5800	0	0	0	0	100	0	0	0
6180	0	0	0	0	0	100	0	0
6453	0	0	21	0	0	0	0	0
6498	0	0	21	0	0	0	0	0
6536	0	0	21	0	0	0	0	0
6569	0	0	50	0	0	0	0	0
6575	100	0	0	100	100	0	100	78
6630	0	0	21	0	0	0	0	0
7143	0	0	0	0	0	100	0	0

^a JOSTEN és mtsai. (2014); ^b JOSTEN és mtsai. (2013); ØSTERGAARD és MOLLER (2018); ^c CAMOEZ és mtsai. (2015)

The 3 specific peaks identified in previous studies (3007 m/z, 5004 m/z, 4511 m/z) are used to isolate ST1 and ST398 type strains. The 3007 m/z peak appeared only in ST1-type strains, and the 5004 m/z and 4511 m/z peaks appeared only in ST398-type strains according to the results of previous studies. Of the 14 peaks identified in the study, the 4860 m/z, 6575 m/z, and 4874 m/z peaks were detectable in several different ST types, however, in combination with other specific peaks, unique marker sets were created.

4. NEW SCIENTIFIC RESULTS OF THE DISSERTATION

1. A new sample preparation method (modified formic acid suspension - M-FA) was allowed to identify isolates with significantly ($p < 0,05$) higher log score values by the M-FA method than by direct transfer (DT) and formic acid direct transfer (DT-FA). There was no significant difference between the identification log score values identified by M-FA and EtOH extraction, however, M-FA is a faster and more cost-effective procedure than EtOH extraction, making it easier to integrate into routine laboratory diagnostics.
2. To identification and discrimination “*S. aureus* complex” species, 8 specific peaks (2641 m/z, 3453 m/z, 5032 m/z, 5286 m/z, 4911 m/z, 4942 m/z, 6818 m/z, 6908 m/z) were identified.
3. To discriminate MRSA and MSSA, 3 methicillin resistance-specific peaks were determined (2453 m/z, 2718 m/z, 3885 m/z) that allowed rapid diagnosis of MRSA from *S. aureus* strains isolated from food.
4. Methicillin resistance was determined in 2 isolates from 47 *S. aureus* strains isolated from food. The MRSA strain isolated from pork rib is of type t011 *spa* and belongs to sequence type 398, which is the most common type of livestock-related MRSA in Europe.
5. Based on the sequence types, 7 specific marker sets were determined by MALDI-TOF MS method, which facilitated the identification and separation of ST7, ST15, ST1, ST5, ST12, ST97 and ST398 strains.

5. PRACTICAL APPLICABILITY OF THE RESULTS

1. Isolation and identification of coagulase-positive *Staphylococcus* species is essential in food microbiology as well as in human and veterinary medicine due to the different degrees of pathogenicity and host specificity of the species. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs provides for the standard MSZ EN ISO 6888-1: 2008 for the determination of the number of coagulase-positive *Staphylococcus* species, however, it does not allow the identification of species, nor does it cover the mapping of antibiotic resistance. The MALDI-TOF MS technique is a reliable, fast and cost-effective diagnostic method that is now widely used to identify microorganisms.
2. The newly developed modified (M-FA) sample preparation procedure allows the identification of *S. aureus* species with a high log score and detailed analysis of biomarkers. Furthermore, the *S. argenteus* and *S. schweitzeri* specific peaks allow the separation of “SAC” species for which classical microbiological methods are not suitable. The M-FA procedure is a simple, timesaving, low-chemical and low-resource procedure that facilitates integration into routine microbiological diagnostics.
3. Thanks to the specific peaks tested and determined, a single measurement can provide a wealth of information for the analysis of the mass spectra of *S. aureus* strains. Methicillin resistance-specific peaks facilitate rapid identification of MRSA, which is an essential part of successful therapy. Furthermore, the use of ST-specific biomarkers can facilitate the isolation and identification of individual ST types, thereby allowing epidemiological and evolutionary tracing of strains, which is of great importance for both food safety and public health.

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7. PUBLISHED OWN SCIENTIFIC PUBLICATIONS



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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (2)

1. **Horváth, B.**, Peles, F., Gasparik, J., Pocklán, E., Sipos, R., Erős, Á., Szabóné Petróczi, F. M., Szűcs, K. D., Albert, E., Micsinai, A.: Élelmiszerekből izolált *Staphylococcus* fajok antibiotikum rezisztencia vizsgálata.
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