Streptococcus agalactiae screening of pregnant women and detection of methicillin-resistant *Staphylococcus aureus* using MALDI-TOF mass spectrometry: application and improvement of the method

PhD Dissertation

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LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Publications in referred journals

I. **Ábrók M,** Tigyi P, Kostrzewa M, Burián K, Deák J (2020) Evaluation of the results of group B streptococcus screening by MALDI-TOF MS among pregnant women in a Hungarian hospital. *PATHOGENES* 9: 1. IF: 3.405

II. **Ábrók M,** Lázár A, Szécsényi M, Deák J, Urbán E (2018) Combination of MALDI-TOF MS and PBP2′ latex agglutination assay for rapid MRSA detection. *JOURNAL OF MICROBIOLOGICAL METHODS* 144: 122-124. IF: 1.701

III. **Ábrók M,** Arcson Á, Lázár A, Urbán E, Deák J (2015) Combination of selective enrichment and MALDI-TOF MS for rapid detection of *Streptococcus agalactiae* colonisation of pregnant women. *JOURNAL OF MICROBIOLOGICAL METHODS* 114: 23-25. IF: 1.857

IV. Virók DP, **Ábrók M,** Szél B, Tajti Zs, Mader K, Urbán E, Tálosi Gy (2014) *Chryseobacterium gleum* - a novel bacterium species detected in neonatal respiratory tract infections. *JOURNAL OF MATERNAL-FETAL & NEONATAL MEDICINE* 27: 1926-1929. IF: 1.367

V. Nagy E, **Ábrók M,** Bartha N, Bereczki L, Juhász E, Kardos G, Kristóf K, Miszti C, Urbán E (2014) Mátrix-asszisztált lézer deszorpciós, ionizációs, repülési idő mérésen alapuló tömegspektrometria speciális alkalmazása a klinikai mikrobiológiai diagnosztika területen *ORVOSI HETILAP* 155: 1495-1503. (*in Hungarian*)

Book chapter

I. Nagy E, **Ábrók M,** Urbán E, Veloo ACM, Winkelhoff AJ, Dekio I, Gharbia SE, Shah HN (2017) Transformation of Anaerobic Microbiology since the Arrival of MALDI-TOF Mass Spectrometry In: Shah HN, Garbia SE (eds.) MALDI-TOF and Tandem MS for Clinical Microbiology. New York: Wiley, pp. 123-146. (ISBN:9781118960257)

Conference abstracts

I. **Ábrók M,** Tigyi P, Kostrzewa M, Lange C, Lázár A, Urbán E, Deák J (2018) Prevalence of *Streptococcus agalactiae* ST-17 and ST-1 clones detected by MALDI-TOF MS in pregnant women in a Hungarian hospital. In: ESCMID 28th European Congress of

Clinical Microbiology and Infectious Diseases, Paper P0808. (Madrid, Spain: 21.04.2018-24.04.2018)

II. **Ábrók M,** Lázár A, Szécsényi M, Urbán Edit (2016) Application of MALDI-TOF MS combined with PBP2' latex agglutination test for MRSA screening In: ESCMID 2016: 26th European Congress of Clinical Microbiology and Infectious Diseases, Paper EV0526. (Amsterdam, The Netherlands: 09.04.2016-12.04.2016)

III. **Ábrók M,** Kostrzewa M, Lange C, Lázár A, Urbán E, Deák J (2016) Magas virulenciájú Streptococcus agalactiae ST-17 és ST-1 klónok kimutatása MALDI-TOF MS alkalmazásával várandós nők szűrése során. In: A Magyar STI Társaság XXI. Nagygyűlése, X. Venerológiai Továbbképző Tanfolyama és 30th IUSTI Europe Conference: Program and Abstract. p. 40. (Budapest, Hungary: 14.09.2016-17.09.2016) (*in Hungarian*)

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V. **Ábrók M,** Kostrzewa M, Lange C, Lázár A, Urbán E, Deák J (2016) Application of MALDI-TOF MS for detection of highly virulent *Streptococcus agalactiae* ST-17 and ST-1 clones in Group B Streptococcus screening of pregnant women *CLINICAL CHEMISTRY AND LABORATORY MEDICINE* 54:(10) eA194. (Szeged, Hungary: 25.08.2016-27.08.2016)

VI. **Ábrók M,** Arcson Á, Lázár A, Urbán E, Deák J (2014) MALDI-TOF MS in *Streptococcus agalactiae* screening of pregnant women. In: Drancourt M, Raoult D (eds.) 24th European Congress of Clinical Microbiology and Infectious Diseases Paper eP482. (Barcelona, Spain: 10.05.2014-13.05.2014)

VII. **Ábrók M,** Urbán E, Lázár A, Deák J (2011) Screening of pregnant women for *Streptococcus agalactiae* between 2008 and 2010. In: Várkonyi V, Tisza T (eds.) A Magyar STD Társaság XVI. Nagygyűlése, V. Venerológiai Továbbképző Tanfolyama, XVII. Alpok-Duna-Adria STD és Genitalis Dermatológiai Konferencia. pp. 79-80. (Budapest, Hungary: 17.11.2011-19.11.2011) (*in Hungarian*)

CONTENTS

ABBREVIATIONS

BORSA	borderline oxacillin resistant *S. aureus*
CAMP test	Christie-Atkins-Munch-Petersen test
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
EOD	early-onset disease
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GBS	Group B *Streptococcus*
IAP	intrapartum antibiotic prophylaxis
LOD	late-onset disease
MALDI	matrix-assisted laser desorption ionization
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
mecA	PBP2a gene (methicillin resistance gene)
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MODSA	modified *S. aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MSSA	methicillin-sensitive *Staphylococcus aureus*
PBP	penicillin-binding protein
PBP2a	penicillin-binding protein 2a
SCCmec	staphylococcal chromosome cassette encoding methicillin resistance
ST	sequence type
TOF	time of flight
TSB	trypticase soy broth
PFGE	pulsed-field gel electrophoresis
mecC	PBP2a gene variant (methicillin resistance gene)
spa	*S. aureus* protein A

1. INTRODUCTION

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is a powerful and reliable technique to identify bacteria and it has become an important part of the routine diagnostic arsenal of the clinical laboratories. Equipment, validated methods and databases are available for routine species level identification. Besides the permanent update and curation of the mass spectrum databases, currently, there are two main fields of efforts to improve the MALDI-TOF MS identification of bacteria. These are (i) studies to reduce the identification time and (ii) development of typing methods based on the MALDI-TOF MS profiles. As MALDI TOF MS basically uses pure cultures, identification time can be shortened by optimization of the sample preparation allowing the identification directly from the clinical specimen or the enrichment culture. Strain typing uses the analysis of the profiles to identify peaks specific to certain strains and types, especially with antibiotic resistance or high virulence.

1.1 Principles of the MALDI-TOF MS

Mass spectrometry (MS) is an analytical method, which separates the different ions formed by the ionization of the studied chemical compounds based on their mass-to-charge (m/z) ratio. Thus, MS analysis can be used to determine the mass spectrum (i.e. the masses of the different compounds/ions) of a sample where each peak corresponds to an ionized molecule and the height of the peaks is proportional with the amount of the compounds. The potential of MS to discern the samples based on their specific mass spectra can be exploited in the identification of microorganisms. For this purpose, the most frequently used method is the matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS (Bizzini and Greub, 2010). This approach allows the proteomic analysis of whole cells/organisms. During matrix-assisted laser desorption ionization (MALDI), the sample is mixed with a matrix (e.g. 3,5-dimethoxy-4-hydroxycinnamic acid, α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid), which absorbs the laser energy and ionizes and vaporizes the sample (Siuzdak, 1994; Lewis *et al.*, 2000; Singhal *et al.*, 2015). This method can form ions from large molecules without significant fragmentation and thus, it can be used to examine the MS spectra of biomolecules, such as proteins, peptides and others (Hillenkamp *et al.*, 1991; Croxatto *et al.*, 2012). Determination of the mass-to-charge ratio is carried out by the measurement of the time of flight (TOF) of the ions (i.e. the time, which is required for the ions to travel in the drift space

until they reach the detector) by the TOF mass spectrometer (Singhal *et al.*, 2015). In this case, charged molecules are accelerated by an electric field, which results in that the ions with the same charge have the same kinetic energy and thus, the velocity of the ions depends on their mass-to-charge ratio (Figure 1). This means that lighter ions of the same charge (ions with smaller *m*/*z* values) and more highly charged ions move faster than the heavier or less charged ions. As microorganisms have characteristic protein profiles, the mass spectra revealed by the MALDI-TOF MS analysis of their proteins can be specific for the distinct species or genera and can be used for their identification (Bizzini and Greub, 2010; Wieser *et al.*, 2012; Singhal *et al.*, 2015).

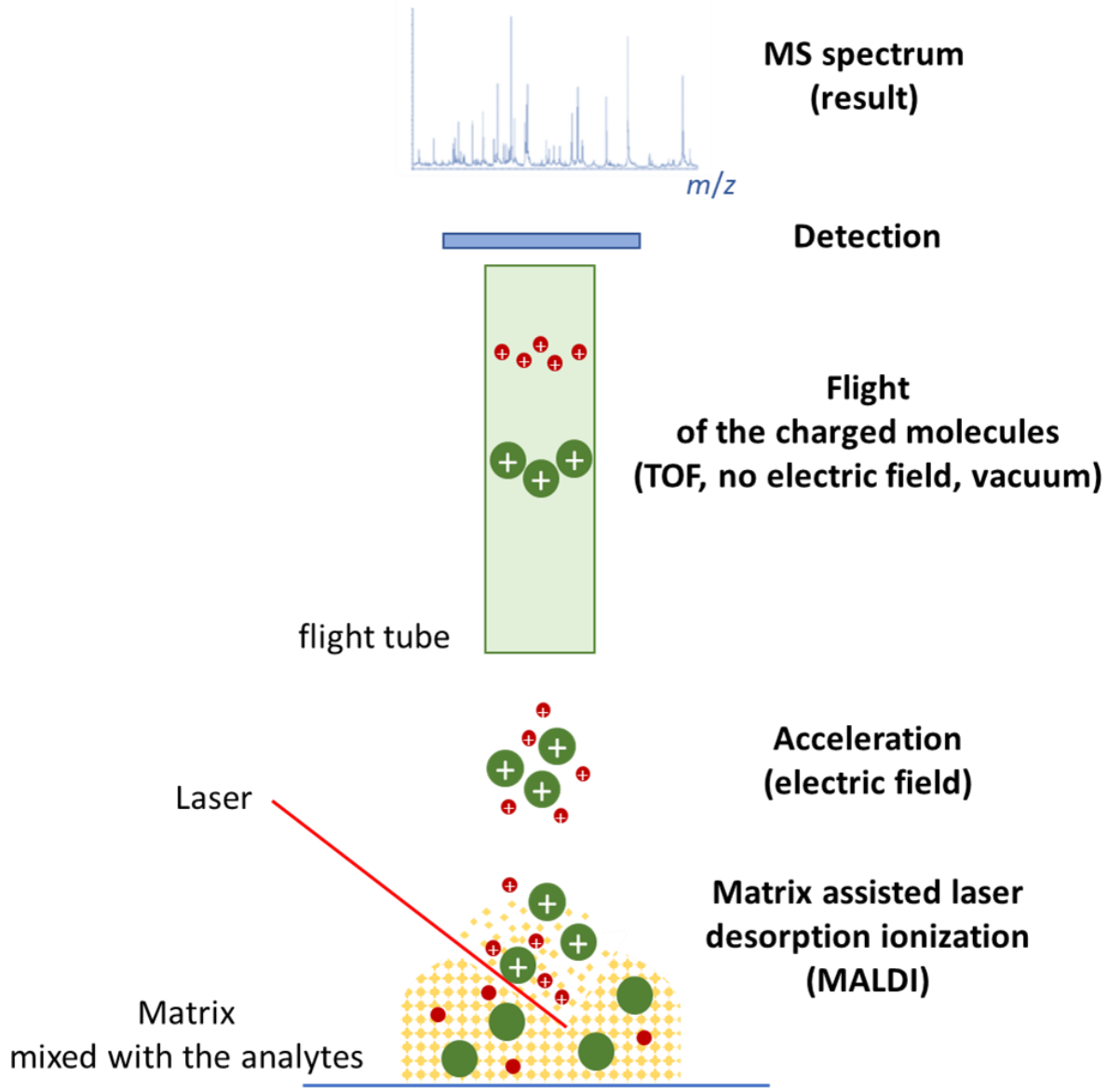

Figure 1 Basic principle of the MALDI-TOF MS analysis based on Croxatto *et al.* (2011). The technical description of the MALDI-TOF MS is presented in Figure 1.

The sample is applied onto the conductive target plate and mixed with the matrix. After crystallization, the mixture of the matrix and the sample is irradiated with short laser pulses, which vaporize the matrix-sample mixture (desorption). Analytes (i.e. the molecules to be examined) will be ionized by protonation or deprotonation with the help of the hot gas of the laser energy absorbing matrix. Ionized molecules are then accelerated in an electric field and are let fly in a metal tube being under vacuum. The easier and more charged ions travel faster than the heavier and less charged ions towards the detector in this tube. Therefore, the analytes will be separated by their individual TOF determined by their charge and mass and the result of the measurement will be a mass spectrum consisting of *m*/*z* peaks with various intensities.

As mentioned above, MALDI-TOF MS can be used to generate protein profiles or fingerprints, which are characteristic to the different microorganisms (Figure 2).

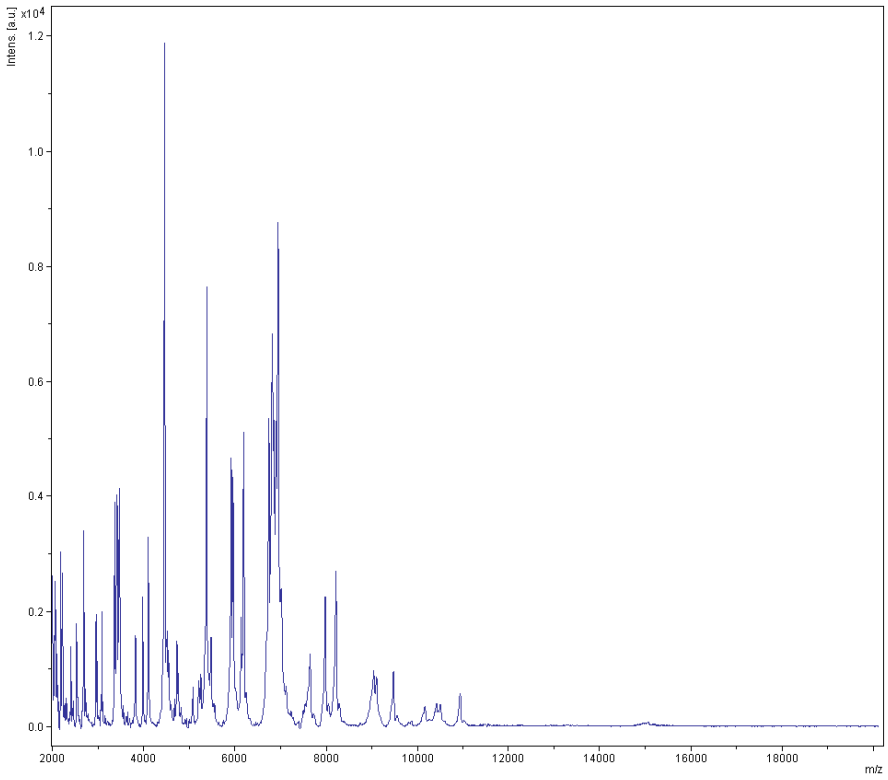

Figure 2 Protein MS spectrum of *Streptococcus agalactiae* recorded by the MALDI-TOF MS method.

The identification is done by comparing the recorded protein fingerprints of the unknown organisms with those of a previously build reference database (Wieser *et al.*, 2012; Singhal *et al.*, 2015). This is carried out automatically by the software of the MALDI-TOF MS instrument (Figure 3).

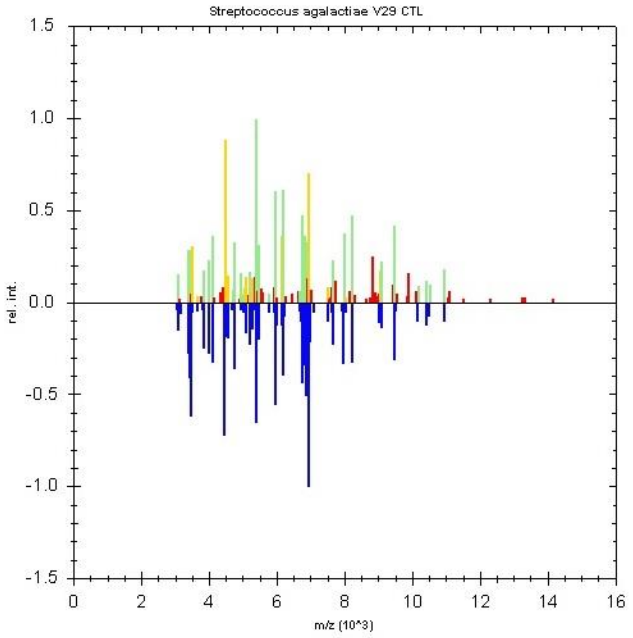

Figure 3 Identification of *Streptococcus agalactiae* by MALDI-TOF MS: comparison of the MS spectrum of the unknown strain (*upper colored peaks*) with the *S. agalactiae* reference spectrum (*lower blue peaks*).

Reference databases are available for medically relevant bacteria and fungi (i.e. yeasts and some filamentous fungi). As our study focuses on the identification of bacterial isolates, I will henceforth review only the questions and achievements of this field.

Currently, two types of MALDI-TOF MS systems differing in the sample preparation, the interpretation of the mass spectra and the reference databases are used in the routine clinical diagnostics. These are the MALDI Biotyper (Bruker Daltonik) equipped with microflex/autoflex MS and the MALDI Biotyper databases and the Vitek MS System (bioMérieux), which applies the Shimadzu Axima MS and the SARAMIS database (Croxatto *et al.*, 2011; Clark *et al.*, 2013). Applicability and reliability of these equipments and their databases has been evaluated by many laboratories and both are used in the routine clinical diagnostics worldwide (Kostrzewa *et al.*, 2013; Patel, 2013). At our laboratory, the Bruker Daltonik's MALDI Biotyper has been gone into service and this equipment was applied in the present study, as well.

1.2 MALDI-TOF MS identification of bacteria in the clinical practice

MALDI-TOF MS has proven to be an efficient tool for the rapid identification of bacteria. Therefore, it has been a routinely used tool to identify bacteria in many clinical microbiology laboratories in the past decade (Kostrzewa *et al.*, 2013). Currently, the above-

mentioned MS databases contain the spectra of the majority of the clinically relevant aerobic bacteria and a lot of environmental species (Wieser *et al.*, 2012).

1.2.1 Species-level identification

To generate protein mass fingerprints for species level identification of bacteria, constantly expressed, highly abundant proteins in a size range between 2 and 20 kDa are used (Maier *et al.*, 2006) (Figure 2). Application of proteins in this size range, where the majority belongs to the ribosomal proteins (Ryzhov and Fenselau, 2001), provides stable and reliable spectra, which are marginally affected by the conditions of the culture (Wieser *et al.*, 2012).

At the same time, the recorded spectrum always contains some noise and is never completely identical with that in the reference database. Therefore, the software analyzing the MS data gives a score value (log score) to the spectrum of the sample based on its similarity to the reference spectrum. Practically, these score values are taking into account during the identification. Generally, species level identification is accepted if the log score is higher than 2.0 while the values between 1.7 and 2.0 indicate reliable results rather at the genus level (Clark *et al.*, 2013, Patel, 2013). The number of the identifiable bacteria depends on the number of the reference spectra in the applied databases. Therefore, the extension and the improvement of the spectrum libraries are intensively studied fields. The method is highly sensitive as an amount of only 10^4-10^6 CFU is enough for a successful identification and high throughput as one measurement gives the result within a few minutes and the whole analysis of a target plate with 96 samples takes about 1 h from the start of the sample loading (Wieser *et al.*, 2012).

Generally, the sample, which is used for MALDI-TOF MS identification, is a cultured microbe. It is most frequently a small portion of the pure culture of an isolated bacterium. The bacterial colony can be examined by the MS directly after it was spotted or smeared onto the target plate and overlaid with the matrix solution. This method is called as “direct cell profiling” (Singhal *et al.*, 2015) or “direct transfer method” (Schulthess *et al.*, 2014). In some cases (such as for the identification of many Gram-positive bacteria), direct transfer method should be extended with a rapid on-target extraction using 70% formic acid to increase the efficiency of the identification (“preparatory extraction” or “direct transfer-formic acid preparation”) (Alatoom *et al.*, 2011; Saffert *et al.*, 2011; Theel *et al.*, 2012; Schulthess *et al.*, 2014; Singhal *et al.*, 2015). This method applies 1 µl of 70% formic acid added to the bacterial spot and allowed to air-dry before addition of the matrix solution. Alternatively, an on-target ethanol-formic acid extraction can be used to improve the effectiveness of the identification of some

Gram-positive aerobic and Gram-negative bacteria (such as *Klebsiella* and *Pseudomonas* spp. with biofilm formation or capsule), certain anaerobic bacteria or yeasts (such as *Candida* spp.), etc. (Farfour *et al.*, 2012; Schulthess *et al.*, 2014). Because of their complex cell wall structure, certain bacteria, such as aerobic actinomycetes, *Nocardia* or mycobacteria cannot be lysed directly on the target plate efficiently (Singhal *et al.*, 2015). Instead, bacteria are lysed and sample preparation including several protein extraction steps using formic acid or trifluoroacetic acid and acetonitrile are carried out before the sample is subjected onto the target plate (Verroken *et al.*, 2010; El Khechine *et al.*, 2011). Before the analysis, samples should let to dry on the surface of the target plate and then, it should be overlaid with the matrix solution (Bizzini and Greub, 2010). Usage of commercially available standard protein mixtures can serve as a control for the measurements.

Several large-scale, comparative studies have demonstrated that MALDI TOF MS is a rapid, reliable and accurate method for the routine clinical microbiological diagnostics of bacteria especially, compared to the traditional biochemical methods (Benagli *et al.*, 2011; Cherkaoui *et al.*, 2011; Singhal *et al.*, 2015; Patel, 2015). In these studies, MALDI TOF MS gave correct species-level identification in 84-95% of the cases (Croxatto *et al.*, 2011; Wieser *et al.*, 2012; Patel, 2015). In this respect, the completeness of the reference spectrum databases (such as the MALDI Biotyper or the SARAMIS) has a determining significance and therefore, they are regularly updated and continuously increased (Singhal *et al.*, 2015).

As mentioned before, MALDI TOF MS identification generally takes 1 h or less. However, this time refers only the sample preparation and the MS analysis and does not include the culturing prior to them (Wieser *et al.*, 2012; Kostrzewa *et al.*, 2013; Singhal *et al.*, 2015). In most cases, an accurate result can be obtained only by the application of pure cultures as the sample material and thus, culturing steps before the MS analysis cannot generally be omitted (Kostrzewa *et al.*, 2019). This means that we should count with at least 18, 24 or 48 h (or more) additional culturing time depending on the growth ability of the bacterium, the nature of the clinical sample or the need of an enrichment. As mentioned before, thus, there is a urgent need to develop methods to perform the identification directly from clinical samples (such as respiratory and urinary tract infections, cerebrospinal fluids and stool samples) or to shorten the incubation time (such as identification from blood cultures and the selective and enrichment broths) and such studies represent an intensively studied field of clinical microbiology (Singhal *et al*., 2015). In some studies, pre-treatment of body fluids with formic acid (Christner *et al*., 2010) and ammonium chloride (Prod'hom *et al*., 2010) improved the applicability of such direct

samples. Another possibility is the reduction of the cultivation time applying a short incubation in an appropriate enrichment medium to increase the amount of the bacteria to be detected (Sparbier *et al*., 2012; Idelevich *et al*., 2018; Chew *et al*., 2019). For example, 4-6 h pre-culture on chocolate agar plates before the MALDI-TOF MS analysis proved to be useful for rapid identification of pathogenic bacteria from positive blood cultures (Kohlmann *et al*., 2015; Chew *et al*., 2019; Rassolie *et al*., 2019). Sparbier *et al.* (2012) optimized a method to identify *Salmonella* sp. from stool samples combining MALDI-TOF MS analysis and culturing in selenite enrichment broth.

1.2.2 Identification of special intraspecific groups – strain typing

In the last decade, intensive efforts have been made to extend the identification capacity of MALDI TOF MS towards the intraspecies level and develop strain typing methods, which are able to discern, for example, different serotypes, toxin producing or highly virulent clones and strains displaying antibiotic resistance of the same species. Such methods build on the accurate analysis of the MS spectra to find single peaks or peak patterns, which are specific to a given strain type (Lartigue *et al*., 2011; Bader, 2013; Kostrzewa *et al*., 2013; Christner *et al*., 2014). Finding specific peaks or peak patterns involves statistical analyses of the MS profiles of a large number of well characterized strains. These analyses are frequently performed using sophisticated data processing workflows and extensive bioinformatic procedures, such as machine learning algorithms, like support vector machine and others (Kostrzewa *et al*., 2013; Christner *et al*., 2014; Sogawa *et al*., 2017; Tang *et al*., 2019) Results always should be compared with those obtained by validated cultivation based, serological and molecular typing methods (Rothen *et al.*, 2019; Savas *et al*., 2019; Welker *et al.*, 2019).

In many cases, reproducibility of MALDI TOF MS typing has remained problematic. For instance, a MALDI-TOF MS method based on genomic sequence types could identify vancomycin-resistant enterococci; however, the results were found to be valid under that actual clinical environment (Holzknecht *et al*., 2018; Welker *et al.*, 2019). Extended spectrum β-lactamase producing strains of *E. coli* also could be discerned using the MS profiles and these results well corresponded to those of amplification fragment length polymorphism (AFLP) analysis, although certain MALDI TOF MS types belonged to more than one AFLP types and application of strict experimental conditions was needed for reproductivity (Veenemans *et al*., 2016).

Several studies demonstrated the value of MALDI TOF MS in distinguishing bacterial strains, especially for those with different antibiotic susceptibility and resistance. However, majority of these studies are in a research phase and several difficulties need to be resolved until the revealed idea or primary method reach the routine application. Thus, currently, majority of the newly developed typing methods are used primarily in epidemiological studies and their usage in routine diagnostics is expected in the near future (Welker *et al*., 2019).

1.3 Identification of *Streptococcus agalactiae*

1.3.1 General characteristics and clinical significance

Streptococcus agalactiae or group B *Streptococcus* (GBS) is a Gram-positive β-haemolytic bacterium, which is a major cause of serious infections in newborns or pregnant and postpartum women. GBS is one of the most frequent agents of invasive diseases in neonates (Artz *et al*., 2003; Furfaro *et al*., 2018) and it is among the leading causes of neonatal sepsis and meningitis in the United States and Europe (Brimil *et al*., 2006; Jones *et al*., 2000; Melin, 2011). In neonates, GBS infections can be classified as an early-onset disease (EOD) occurring in the first week of life or a late-onset disease (LOD), which develops between the 7th day of birth and the end of the 3rd month of life (Oddie *et al*., 2002; Lin *et al.*, 2003). In case of EOD, GBS is mainly transmitted during the birth from the mother who is colonized by the bacterium (Berardi *et al.,* 2014). At the same time, GBS LOD is thought to be a result of a nosocomial or community acquired infection besides the vertical transmission from the mother to the neonate (Furfaro *et al.,* 2018; Shabayek and Spellerberg, 2018).

In women, clinical manifestations of GBS infection may include urinary tract infections, chorioamnionitis, endometritis or puerperal sepsis and fever (Baker, 2000; Melin, 2011; Schrag *et al.,* 2000). GBS can be present as a commensal of the gastrointestinal and genitourinary tracts of humans and about 10-30% of pregnant women are colonized by GBS without any symptoms (Artz *et al.,* 2003; Caliot *et al.,* 2012; Centers for Disease Control and Prevention, 2010; Furfaro *et al.,* 2018). However, these women are considered to be at increased risk of premature delivery and stillbirth (Melin, 2011).

1.3.2 Intraspecific groups of S. agalactiae

Until to date, ten GBS serotypes designated as Ia, Ib and II-IX have been described based on the serological characteristics of the polysaccharide capsule (Le Doare *et al.,* 2014; Teatero *et al.,* 2017). These serotypes have various distribution and frequency and these features

can be varied over time too (Shabayek and Spellerberg, 2018). In general, serotypes Ia, Ib, II, III, and V are frequent colonizers in Europe and the United States (Melin and Efstratiou, 2013; Florindo *et al.,* 2014; Fabbrini *et al.,* 2016), serotypes VI and VIII predominate in Japan (Matsubara *et al.,* 2002; Shabayek and Spellerberg, 2018) and serotypes IV and V are prevalent in North Africa and the Arabian peninsula (Shabayek *et al.,* 2014; Shabayek and Spellerberg, 2018). Regarding the pathogenicity, serotype III is considered as the predominant invasive type, which is responsible for most of the meningitis LOD cases (Florindo *et al.,* 2014; Alhhazmi *et al.,* 2016). At the same time, serotypes Ia and V are the main causative agents of invasive infections in non-pregnant persons (Alhhazmi *et al.,* 2016; Shabayek and Spellerberg, 2018). Recently, serotype IV strains, which proved to be invasive in neonates and adults, were also reported (Shabayek and Spellerberg, 2018).

Besides the serological characteristics, various sequence types (STs) corresponding to clonal complexes could be discerned by multilocus sequence typing (MLST) within the *S. agalactiae* species (Lartigue *et al.,* 2009). MLST is performed by sequencing of fragments of various housekeeping genes and is generally applied for large scale epidemiological and surveillance studies (Maiden *et al.,* 1998; Urwin and Maiden, 2003; Jolley *et al.,* 2004). MLST studies have been extensively carried out during the past decades and the resulting STs and sequences are available in online databases, which can be accessed over the *Streptococcus agalactiae* MLST website (https://pubmlst.org/sagalactiae/; Jolley *et al.,* 2004). Until to date, more than 750 STs have been discerned (Teatero *et al.,* 2017) but majority of the STs from human origin can be grouped only into 6 clonal complexes (Da Cunha *et al.,* 2014). Among them, certain STs have higher ability to cause invasive diseases than others, which mainly comprise strains with only colonizing potential (Lartigue *et al.,* 2011; Shabayek and Spellerberg, 2018). For example, ST-17 is well known as a clone, which is strongly associated with neonatal early-onset meningitis and GBS LOD (Manning *et al.,* 2009; Lartigue *et al.,* 2011; Kang *et al.,* 2017; Furfaro *et al.,* 2018). It belongs to serotype III and its isolates are referred as "highly virulent" strains (Lartigue *et al.,* 2011; Shabayek and Spellerberg, 2018). Several studies have highlighted the need of the rapid identification of strains belonging to the ST-17 lineage if the GBS screening is positive (Lamy *et al.,* 2006; Manning *et al.,* 2009; Lartigue *et al.,* 2011). However, molecular genotyping methods have not yet been well adapted for routine diagnostics. As the causative agent of neonatal invasive diseases, another important clone is ST-1, which mostly belongs to the serotype V (Salloum *et al.,* 2011). Both ST-17 and ST-1 are also isolated from diseases developed in adults also (Lartigue *et al.,* 2011; Shabayek and

Spellerberg, 2018). The recently emerged serotype IV isolates, which has also isolated from invasive neonatal infections, mainly belong to the ST-196 (Shabayek and Spellerberg, 2018). Other major STs, such as ST-8, ST19, ST-23 have mainly reported from adult infections (Lartigue *et al.,* 2011).

1.3.3 GBS screening of pregnant women

Because of the risk of transmission from the mother, GBS screening of pregnant women has been implemented in many countries (Di Renzo *et al.,* 2015; Furfaro *et al.,* 2018). If the screening detects vaginal or rectal GBS colonization, an intrapartum antibiotic prophylaxis (IAP) is administered to prevent the infection of the neonates (Di Renzo *et al.,* 2015; Furfaro *et al.,* 2018).

GBS screening is recommended to carry out at the 35-37 weeks of gestation (Centers for Disease Control and Prevention, 2010). The standard method to detect the colonization is a culturing method, which involves firstly a selective enrichment in a broth medium (for example in modified Todd Hewitt broth supplemented with nalidixic acid and colistin) for 18-24 h and then, a sub-culturing step on an appropriate medium (for example on Columbia agar plates containing 5% sheep blood) for another 24-48 h (Altaie and Dryja, 1994; Centers for Disease Control and Prevention 2010, Philipson *et al.,* 1995; Platt *et al.,* 1995). Inoculation directly on agar plates without an enrichment step often gives false negative results because of the low concentration of the colonizing bacteria in the clinical sample. Occasionally, a further 24-h culturing is required if the previous selective culturing gave ambiguous results. Therefore, the whole culture-based identification process can be altogether two or three days long. Traditional methods to identify presumptive GBS colonies include examination of the colony morphology, β-hemolysis, bacitracin resistance and CAMP tests, as well as Lancefield-group B detection by latex agglutination test.

As mentioned earlier, epidemiology of *S. agalactiae* is based on serotyping and/or MLST using PCR assays. Serotyping is traditionally performed by latex agglutination tests and various kits are available in the market (Andrade *et al.,* 2017). However, latex agglutination method sometimes gives erroneous results, or several strains can remain untyped (Dutra *et al.,* 2014; Brigtsen *et al.,* 2015; Andrade *et al.,* 2017). Several PCR-based methods targeting the capsular genes have also been developed to determine the serological groups (Yao *et al.,* 2013). Among them, the multiplex PCR assay developed by Imperi *et al.* (2010) has the capacity to discern all the 10 currently known serotypes in a single PCR reaction. A major drawback of the

PCR-based techniques, besides some technical difficulties, however, is that they can detect only the presence of the capsular polysaccharide biosynthesis genes but their expression as an existing capsule remains unknown (Brigtsen *et al.*, 2015).

MALDI-TOF MS has proven to be an efficient tool for the rapid and reliable identification of *S. agalactiae* and β-hemolytic streptococci and it is routinely used in the clinical diagnostics (Lartigue *et al.*, 2009; Cherkaoui *et al.*, 2011). However, as this method uses pure cultures, the above-mentioned culturing steps should be performed prior to the analysis (Benagli *et al.*, 2011; Cherkaoui *et al.*, 2011; Lartigue *et al.*, 2009). Thus, the whole identification also requires at least 48-h culturing after collection of the samples.

MALDI-TOF MS also proved to be applicable to detect certain MLSTs of *S. agalactiae*. Lartigue *et al.* (2011) developed a method, which is able to discern the most invasive ST-17 and ST-1 clones based on the characteristic peak-shifts present in their protein MS spectra (Lartigue *et al.*, 2011). These peaks correspond to the 6250- and 7625-Da proteins, which are characteristic to the ST-1 and ST-17 clones, respectively (Lartigue *et al.*, 2011). At the same time, similar protein peaks, which would be useful as potential biomarkers, could not be identified for other STs, such as for ST-8, ST-19, and ST-23 (Lartigue *et al.*, 2011). Recently, Rothen *et al.* (2019) determined 62 ribosomal subunit protein-based MS profiles, to which MLST clonal complexes, capsular serotypes and pilus variants could be linked.

1.3.4 Antibiotic susceptibility testing of S. agalactiae

Since *S. agalactiae* is considered to be susceptible to β-lactams, penicillins (including penicillin G, ampicillin and amoxicillin) are the first line antibiotics of choice for IAP and to treat GBS infections (Di Renzo *et al.*, 2015). In penicillin-allergic women, application of clindamycin is recommended and in case of the detection of clindamycin resistant GBS isolates, vancomycin treatment is used (Di Renzo *et al.*, 2015). However, an increasing resistance to antibiotics (primarily to macrolides and lincosamides) used as alternatives of β-lactams has been detected (Leclercq, 2002; Nakamura *et al.*, 2011; Capanna *et al.*, 2013; Di Renzo *et al.*, 2015). Therefore, susceptibility testing of strains isolated from antenatal GBS screening is important and applied in the routine diagnostics. In most of the European countries, antimicrobial susceptibility tests are performed according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/; Matuschek *et al.*, 2014). Besides, there are some laboratories where instructions of the Clinical

and Laboratory Standards Institute (CLSI) are used for antimicrobial susceptibility testing (https://clsi.org/; Clinical and Laboratory Standards Institute, 2020).

1.4 Identification of methicillin-resistant *Staphylococcus aureus* (MRSA)

1.4.1 General characteristics and clinical significance

Staphylococcus aureus is a Gram-positive bacterium, which can be both a commensal and a human pathogenic organism. It is a major cause of bacteremia and infective endocarditis but also known as the agent of osteoarticular, skin, pleuropulmonary and various device-related infections (Tong *et al.,* 2015). Methicillin-resistant *S. aureus* (MRSA) is notorious about causing nosocomial and community acquired infections worldwide (Marlowe and Bankowski, 2011).

Penicillin-binding protein (PBP) participating in the bacterial cell wall biosynthesis is the target of the β-lactam antibiotics. Generally, methicillin resistance of the MRSA strains is a consequence of the presence of an alternative penicillin-binding protein (PBP2a), for which β-lactams have low affinity; thus, they cannot block the cell wall synthesis (Peacock and Paterson, 2015). PBP2a is not an altered version of PBP encoded in the same locus and cannot be found in the MSSA strains. It is encoded by an additionally acquired gene (*mecA*) located on a mobile genetic element, the so-called staphylococcal chromosome cassette (SCCmec), which is present only in MRSA bacteria (Katayama *et al.,* 2000). Although vast majority of MRSA strains contain the *mecA* gene, a new variant of the *mec*, named as *mecC*, was also reported in 2011 (Dupieux *et al.,* 2017). MRSA strains with *mecC* gene are relatively rare and have been sporadically reported in European human and animal samples (Peacock and Paterson, 2015; Dupieux *et al.,* 2017).

Some other, less common *S. aureus* strains can possess other resistance mechanisms (Bhutia *et al.,* 2012). For example, borderline oxacillin resistant *S. aureus* (BORSA) strains over-produce a β-lactamase enzyme (Montanari *et al.,* 1990), while modified *S. aureus* (MODSA) strains have an altered version of the native PBP (Tomasz *et al.,* 1989).

MRSA infections most frequently include endocarditis, pneumonia, as well as skin and life-threatening blood stream infections (Maltezou and Giamarellou, 2006; Gordon and Lowy, 2008). Such infections are generally associated with higher morbidity and mortality rates than those caused by methicillin-sensitive *Staphylococcus aureus* (MSSA) (Wolk *et al.,* 2009b). Methicillin resistance also means resistance practically to all β-lactam antibiotics (except the latest generation cephalosporins) (Peacock and Paterson, 2015). Furthermore, MRSA isolates

can become resistant to other antibiotics, including vancomycin, which is considered as one of the treatment options for MRSA, especially in case of severe infections (Kos *et al.,* 2012). Thus, MRSA infections are frequently difficult to treat; however, a delay in the appropriate antibiotic therapy may highly and negatively affect the clinical outcome (Romero-Gómez *et al.,* 2012). Therefore, the rapid and accurate detection and identification are important prerequisites for an efficient infection control.

1.4.2 MRSA identification

Direct plating onto blood agar or a selective agar generally has low selectivity and sensitivity for MRSA (Wolk *et al.,* 2009b). Therefore, the standard MRSA surveillance uses a culture-based screening strategy consisting of a selective enrichment followed by a sub-culturing on an MRSA selective medium (Safdar *et al.,* 2003; Marlowe and Bankowski, 2011). Selective enrichment is performed in a culture broth, most frequently in trypticase soy broth (TSB), for 24 h. The subsequent sub-culturing can be carried out on a solid selective medium, for example on oxacillin containing mannitol salt agar. In this way, the whole identification process lasts about 48 h. If methicillin resistance should be determined, it requires further 24 h.

EUCAST guideline recommends the performance of cefoxitin susceptibility test to detect methicillin resistance. Cefoxitin susceptibility can be determined by the disk diffusion method or MIC determination. Disk diffusion test has to be performed with 30-µg cefoxitin disks on Mueller-Hinton agar using a 0.5-McFarland inoculum (EUCAST, 2020a). The test plates should be incubated on 35±1 ^{o}C for 18±2 h. To interpret the results of the antimicrobial susceptibility testing, EUCAST determined clinical breakpoints, which are the followings for MRSA testing using cefoxitin: higher MIC value than 4 mg/L indicates resistance; a diameter zone of ≥22 mm indicates sensitivity, while that of <22 mm indicates resistance (EUCAST, 2020b).

Various molecular typing methods including *S. aureus* protein A (spa) typing, MLST or pulsed-field gel electrophoresis (PFGE), have also been adapted to identify MRSA but these techniques are rather time consuming and rarely used in the diagnostic practice (Østergaard *et al.,* 2015). Several PCR assays, generally based on the amplification of the *mecA* and *mecC* genes or the SCCmec cassette, have also been developed for MRSA detection (Wolk *et al.,* 2009a; Luteijn *et al.,* 2011; Peacock and Paterson, 2015; Dupieux *et al.,* 2017). Because *mecA* and *mecC* show only a 70% of sequence homology, *mecA* PCR assays fail to detect *mecC*, for which new assays had to be developed (Peacock and Paterson, 2015).

Serological detection of the *mecA*-encoded PBP2a is also possible as this protein is located in the cell membrane of MRSA. Evaluated, rapid assays, such as latex agglutination tests and immunochromatographic membrane assays are commercially available and commonly used (Marlowe and Bankowski, 2011; Hong *et al.*, 2012; Romero-Gómez *et al.*, 2012; Alipour *et al.*, 2014). Although currently available PBP2a-based immunological assays proved to be not applicable for the direct detection of *mecC*-positive MRSA, the PBP2a Culture Colony Test (Alere Ltd.), which is an immunochromatographic membrane assay, could identify the *mecC*-positive clones after a 18-h induction by cefoxitin (Dupieux *et al.*, 2017).

MALDI-TOF MS is an efficient tool for *S. aureus* identification (Figure 4) (Wieser *et al.*, 2012; Østergaard *et al.*, 2015). However, as this method uses pure cultures, the enrichment and sub-culturing steps should be performed prior to the analysis.

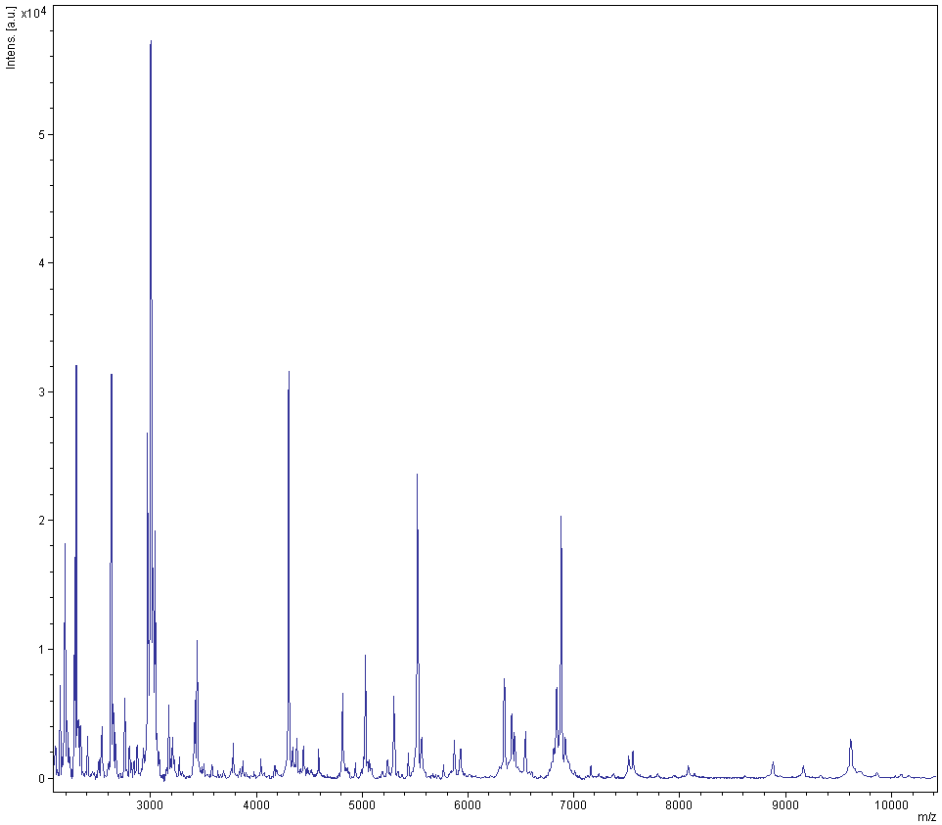

Figure 4 Protein MS spectrum of a methicillin-resistant *Staphylococcus aureus* recorded by the MALDI-TOF MS method.

Development of a MALDI-TOF MS-based method to detect MRSA directly from intact cells has been remained a great challenge during the last decade (Kostrzewa *et al.*, 2013). Josten *et al.* (2014) had reported a peak at m/z 2415, which indicates methicillin-resistance but later,

it was found that the absence of this peak does not indicate susceptibility (Schuster *et al.*, 2018). Recently, extensive bioinformatic analyses of *S. aureus* MS spectra have been used to propose sets of reference peaks characteristic to MRSA and MSSA strains and able to discern them (Sogawa *et al.*, 2017; Tang *et al.*, 2019). Although these methods seem to be appropriately sensitive and specific, their introduction in the clinical practice and evaluation using a large-scale sample have to be done and they are mainly used for epidemiological purposes (Josten *et al.*, 2014).

2. AIMS

Aims of the study involved the improvement of the MALDI-TOF MS-based species-level identification of certain bacteria and the application of this technique for the detection of some clinically relevant intraspecific groups of them.

Accordingly, our objectives were the followings:

1. Development of a MALDI-TOF MS method for the rapid identification of *S. agalactiae* useful for GBS screening of pregnant women.

This objective includes the optimization of the sample preparation to reduce the time of the identification process.

2. Comparative analysis of the results of the GBS screening among pregnant women and antibiotic susceptibility testing performed at our institution in the past years (i.e. between 2012 and 2018).

3. Application and testing of the MALDI-TOF MS-based method of Lartigue *et al.* (2011) to identify the highly virulent ST-1 and ST-17 *S. agalactiae* clones in samples from antenatal GBS screening.

4. Development of a MALDI-TOF MS method for the rapid identification of methicillin-resistant *S. aureus* strains.

This objective includes the optimization of the sample preparation prior to the MALDI-TOF MS analysis to reduce the identification time and a combination of the species-level MALDI-TOF MS-based identification with a latex agglutination test detecting the penicillin-binding protein 2a (PBP2a), which is present in the cell membrane of the MRSA strains.

3. MATERIALS AND METHODS

3.1 Strains and clinical samples

3.1.1 Strains and samples for GBS screening of pregnant women

For testing and optimization of the MALDI-TOF MS sample preparation methods, the following strains were used: *S. agalactiae* ATCC 13813, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 14053.

To test the optimized method for MALDI-TOF MS directly from the selective enrichment broth, 100 vaginal samples from GBS screening of pregnant women collected at the Albert Szent-Györgyi Clinical Center of the University of Szeged (Szeged, Hungary) in 2013 and 2014 were involved.

GBS screenings presented in this study were performed on vaginal or cervical samples of pregnant women who attended at the clinical center to detect their GBS colonization status from January 2012 to July 2018 (n = 19267). In case of the GBS positive samples, antibiotic susceptibility of the isolates (n = 3554) was tested. From those isolated in 2017 and 2018, 260 isolates were randomly selected for MALDI-TOF MS-based detection of ST-1 and ST-17 GBS types.

3.1.2 Clinical samples for MRSA screening

Two hundred fifty-five samples collected from September 2015 to June 2017 at the Albert Szent-Györgyi Clinical Center of the University of Szeged were involved in the MRSA screening with the following distribution: 92 nasal, 85 throat, 34 axillary, 32 inguinal and 12 other samples.

3.2 Standard culturing-based identification methods

3.2.1 Screening of the GBS samples

All GBS samples were screened by the standard culturing-based method according to the recommendations of CDC 2010 guideline (Centers for Disease Control and Prevention, 2010).

Samples were inoculated onto Columbia agar plates containing 5% sheep blood (bioMérieux, France) and incubated at 35-37 °C in 5% CO_2. Simultaneously, a selective

enrichment was also performed in modified Todd Hewitt broth (OXOID, England) supplemented with nalidixic acid (0.015 g/L) and colistin (0.010 g/L).

After enrichment for 18-24 h, a sub-culturing step was performed on CHROMagar StrepB plates (CHROMagar, France) at 37 °C under aerobic conditions for 18, 24 or 48 h. After incubation, Columbia agar or CHROMagar StrepB plates were inspected to detect suspicious colonies. Presumptive GBS colonies were identified by colony morphology, β-hemolysis, bacitracin resistance, CAMP test and Lancefield-group B detection by latex agglutination test with Pastorex Strep Kit (Bio-Rad, France).

3.2.2 MRSA screening

For MRSA detection, all samples were screened by the standard culturing-based method (Wolk *et al.*, 2009a; Safdar *et al*., 2003) as follows.

Selective enrichment was performed in tryptic soy broth (TSB; Oxoid, England) supplemented with NaCl (65 g/L) for 24 h at 35 °C. After that selective sub-culturing was carried out on mannitol salt agar (Oxoid, England) supplemented with 5% v/v egg yolk emulsion (Oxoid, England) for another 24 h at 35 °C. Incubation time of this sub-culturing could be prolonged if bacteria were found to develop small colony variant types.

3.3 Antibiotic susceptibility testing

3.3.1 Antibiotic susceptibility testing of the GBS isolates

Antimicrobial susceptibility of the isolates was tested to penicillin, cefuroxime, vancomycin, trimethoprim-sulfamethoxazole, erythromycin and clindamycin. Testing was performed by the disk diffusion method on Mueller-Hinton agar supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (BioMérieux) according to the EUCAST recommendations (http://www.eucast.org/; Matuschek *et al.*, 2014). The agar plates were incubated at 35 °C in 4-6% CO_2 for 18 h. After incubation, the plates were read. Inhibition zones were measured, and the results were interpreted according to the actual EUCAST Breakpoint Tables (EUCAST, 2020b).

In the present study, the following antimicrobial disks were used: benzylpenicillin (1 Unit), cefuroxime (30 µg), erythromycin (15 µg) clindamycin (2 µg), vancomycin (5 µg) and trimethoprim-sulfamethoxazole (1.25-23.75 µg).

3.3.2 Antibiotic susceptibility testing of the MRSA isolates

Methicillin resistance of the identified *S. aureus* strains was tested by the disk diffusion method according to the EUCAST recommendations (http://www.eucast.org/; Matuschek *et al.*, 2014). The test was performed on Mueller-Hinton agar (BioRad) incubated at 35 °C in air for 18 h. Disks containing cefoxitin (30 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), tigecycline (15 μg), norfloxacin (to screen for fluoroquinolone susceptibility; 10 μg), trimethoprim-sulfamethoxazole (1.25-23.75 μg), gentamicin (10 μg), tobramycin (10 μg) and amikacin (30 μg) were used for antimicrobial susceptibility testing. Besides, MICs for vancomycin and teicoplanin was determined by the agar dilution method according to the EUCAST recommendations (http://www.eucast.org/; Matuschek *et al.*, 2014).

3.4 PBP2' latex agglutination assay

To detect the *mecA*-encoded PBP2a protein producing *S. aureus* strains, the PBP2' latex agglutination assay (Oxoid, England) was used according the recommendations of the manufacturer.

The test was carried out with a portion of the pelleted bacterial sample prepared to the MALDI-TOF MS analysis (see in the chapter 3.5 MALDI-TOF MS analysis). Approximately 1.5×10^9 (3-5 μl) cells was subjected to the assay. To prepare the sample, the bacterial pellet was suspended in four drops of Extraction Reagent 1 (part of the kit) in a microcentrifuge tube and heated at >95 °C for 3 min. After cooling, one drop Extraction Reagent 2 (part of the kit) was added to the sample and mixed well. Finally, the sample was centrifuged at $1.500 \times g$ for 5 min and the resulting supernatant was used for the test.

As the negative and the positive controls of the test, the methicillin-sensitive *S. aureus* ATCC 29213 strain and the methicillin-resistant *S. aureus* ATCC 43300 strain were used, respectively.

3.5 Sample preparation for MALDI-TOF MS

For the routine species level identification (i.e. for both GBS and MRSA), sample preparation was performed according to the direct transfer method with on-target formic acid treatment (Schulthess *et al.*, 2014).

A small amount of the bacterial sample (i.e. approx. 10^4 to 10^6 CFU) (Wieser *et al.*, 2012) was smeared as a thin film using a wooden toothpick onto a spot of the MALDI target

plate and let to dry. The dried samples were then treated with 1 µl of 70% formic acid and allowed to dry again. Before the analysis, 1 µl matrix solution (i.e. a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was added onto the samples treated with formic acid and dried.

3.6 MALDI-TOF MS analysis

MALDI-TOF MS identification was performed according to Wieser *et al.* (2012). using a Microflex MALDI Biotyper mass spectrometer (Bruker Daltonics, Germany).

The measurement was carried out in positive linear mode across the *m/z* range of 2 to 20 kDa. For each spectrum, 240 laser shots at 60 Hz in groups of 40 shots per sampling area were collected. The resulting spectra were analyzed by a MALDI Biotyper RTC 3.1 software (Bruker Daltonics, Germany) using the MALDI Biotyper Library 3.1 (Bruker Daltonics, Germany). Log score values were interpreted as follows: log scores of ≥ 2.000 indicated an identification at the species level, log scores of 1.700 - 1.999 indicated an identification at the genus level while log scores ≤ 1.700 were not accepted as an identification.

3.7 MALDI-TOF MS-based detection of ST-1 and ST-17

To detect the ST-1 and ST-17 sequence types, the MS profiles of the selected GBS strains were analyzed as described by Lartigue *et al.*, (2011). The presence of the 6250-Da protein specific to ST-1 or that of the 7625-Da protein specific to ST-17 were recorded. The analysis was performed by visual inspection of the profiles using the flexAnalysis 3.4 software (Bruker Daltonics, Germany).

4. RESULTS

4.1 Development of a MALDI-TOF MS method for rapid detection of *S. agalactiae* colonization of pregnant women (Ábrók *et al.*, 2015)

To detect GBS colonization in pregnant women, a culture-based screening strategy is accepted as the standard method. To shorten the time of the GBS screening process, a sample preparation method directly from the selective broth was optimized and tested.

4.1.1 Optimization of the sample preparation from the GBS enrichment broth for MALDI TOF MS

This study was carried out using the *S. agalactiae* ATCC 13813 strain. Sample preparation procedures were tested on cultures incubated for 18 h in Todd-Hewitt broth (as the selective enrichment broth). During the optimization process, known strains of *S. agalactiae* and other bacteria were inoculated in the enrichment broth and, after the incubation period, MALDI-TOF MS analysis was performed to identify the inoculated strains. For all tested bacteria, 100 µl 0.5 McFarland inoculum was applied.

In the first experiment, three different preparation methods were tested, which were set up as follows, (1) after enrichment, 1 µl broth was directly subjected onto the spots of the MALDI target plate, (2) 1 µl broth was subjected onto the target plate, dried and treated with 70% formic acid, and (3) 1 ml aliquot of the enrichment broth was centrifuged in a table-top microfuge and a small amount of the pellet was smeared onto the target plate. After drying, 1 µl matrix was added to each sample. Before the analysis, treated samples were allowed to dry at room temperature again.

The first two methods (i.e. method 1 and 2), when samples were applied directly from the enrichment broth, did not give any reliable result, even if the sample contained only GBS and 70% formic acid-extraction was applied (Table 1).

Besides the possibly insufficient number of bacteria in the unconcentrated samples, high protein content of the enrichment broth disturbing the analysis could be in the background of this result. Using the method 3, GBS was detected from each sample with reliable score values indicating that centrifuging the enrichment broth is crucial to obtain a cell mass enough to receive reliable results.

Table 1 Result of the first experiment to optimize MALDI-TOF MS identification directly from the selective enrichment broth.

Strains inoculated in the selective enrichment broth	Species identified by MALDI-TOF MS			
	Method 1	Method 2	Method 3	Score
Streptococcus agalactiae ATCC 13813	-	-	GBS	2.481
Streptococcus agalactiae ATCC 13813	-	-	GBS	2.246
Streptococcus agalactiae ATCC 13813, *Escherichia coli* ATCC 35218	-	-	GBS	2.153
Streptococcus agalactiae ATCC 13813, *Staphylococcus aureus* ATCC 25923	-	-	GBS	2.253
Streptococcus agalactiae ATCC 13813, *Enterococcus faecalis* ATCC 29212	-	-	GBS	1.947

Method 1: 1 µl enrichment broth was directly subjected onto the spots of the MALDI target plate. Method 2: 1 µl broth was subjected onto the target plate, dried and treated with 70% formic acid. Method 3: 1 ml aliquot of the enrichment broth was centrifuged, and a small amount of the pellet was smeared onto the target plate. Score values are given for the identifications from the samples prepared by method 3.

As only method 3 gave reliable result in the first experiment, this method was tested on further samples, i.e. on 9 bacterial and yeast suspensions with known composition (Table 2). Besides, the enrichment broth of three clinical samples from GBS screening, from which GBS had been detected by the traditional GBS screening method, were also involved (after 18 h of enrichment). Inoculum size, incubation times and conditions, as well as the MALDI-TOF MS method was the same as in the previous experiment.

Although all samples, which contained one strain, were correctly identified, traditional culture-based method and MALDI-TOF identification gave different results for certain mixed samples. Therefore, a whole extraction procedure prior to subjecting the samples to the target plate was tested in the next optimization test. The centrifuging step after the incubation in the enrichment broth was kept but an ethanol-water purification step on the pellet was included to dispose of the disturbing components of the culture medium. After that, the sample was centrifuged again, and the pellet was treated with formic acid and acetonitrile to extract the proteins. After a final centrifugation, the supernatant was subjected onto the spots of the target plate. Applying this method, the 12 samples used in the previous experiment (Table 2) was tested again (Table 3). In this experiment, all conditions were the same as in the previous tests (except the sample preparation method).

Table 2 Result of the second experiment to optimize MALDI-TOF MS identification directly from the selective enrichment broth. Samples were prepared by method 3 centrifuging an aliquot of the enrichment broth.

Strains inoculated in the selective enrichment broth	Species identified by the conventional culture-based method	Species identified by MALDI-TOF MS	Score
Streptococcus agalactiae ATCC 13813	GBS	GBS	2.197
Escherichia coli ATCC 35218	*E. coli*	*E. coli*	2.286
Enterococcus faecalis ATCC 29212	*E. faecalis*	*E. faecalis*	1.741
Staphylococcus aureus ATCC 25923	*S. aureus*	*S. aureus*	2.200
Candida albicans ATCC 14053	*C. albicans*	*C. albicans*	2.032
Streptococcus agalactiae ATCC 13813, *Escherichia coli* ATCC 35218	GBS	*E. coli*	2.169
Streptococcus agalactiae ATCC 13813, *Enterococcus faecalis* ATCC 29212	GBS	GBS	1.830
Streptococcus agalactiae ATCC 13813, *Staphylococcus aureus* ATCC 25923	GBS	*S. aureus*	2.108
Streptococcus agalactiae ATCC 13813, *Candida albicans* ATCC 14053	GBS	GBS	2.351
Clinical sample	GBS	GBS	2.190
Clinical sample	GBS	GBS	2.082
Clinical sample	GBS	GBS	2.009

Table 3 Result of the second experiment to optimize MALDI-TOF MS identification directly from the selective enrichment broth. Samples were prepared by method 3 centrifuging an aliquot of the enrichment broth.

Strains inoculated in the selective enrichment broth	Species identified by the conventional culture-based method	Species identified by MALDI-TOF MS	Score
Streptococcus agalactiae ATCC 13813	GBS	GBS	2.201
Escherichia coli ATCC 35218	*E. coli*	*E. coli*	2.175
Enterococcus faecalis ATCC 29212	*E. faecalis*	*E. faecalis*	1.890
Staphylococcus aureus ATCC 25923	*S. aureus*	*S. aureus*	2.098
Candida albicans ATCC 14053	*C. albicans*	*C. albicans*	2.002
Streptococcus agalactiae ATCC 13813, *Escherichia coli* ATCC 35218	GBS	GBS	2.200
Streptococcus agalactiae ATCC 13813, *Enterococcus faecalis* ATCC 29212	GBS	GBS	2.038
Streptococcus agalactiae ATCC 13813, *Staphylococcus aureus* ATCC 25923	GBS	GBS	2.014
Streptococcus agalactiae ATCC 13813, *Candida albicans* ATCC 14053	GBS	GBS	2.235
Clinical sample	GBS	GBS	2.334
Clinical sample	GBS	GBS	2.197
Clinical sample	GBS	GBS	2.256

In this experiment, MALDI-TOF MS identified GBS with good score values from all samples, in which GBS were also detected by the conventional method. This result indicated that the extraction procedure can be applicable to identify GBS from clinical samples.

Based on the above described tests, a procedure for sample preparation directly from the Todd-Hewitt enrichment broth was proposed (Figure 5) as follows:

Firstly, 1 ml of broth is centrifuged at 15.500 × *g* for 2 min at 20 °C to collect the bacteria. The bacterial pellet is resuspended in 300 µl of distilled ultra-pure water. Nine hundred µl absolute ethanol is added to the bacterial suspension, mixed well and centrifuged again at 15.500 × *g* for 2 min at 20 °C. After discarding the supernatant, 25 µl formic acid is added to the pellet and mixed well. After that, 25 µl acetonitrile is added to the mixture and centrifuged at 15.500 × *g* for 2 min at 20 °C. One µl of the supernatant is transferred onto the spots of the

MALDI target plate to form a thin film layer and allowed to dry at room temperature. Finally, the sample is overlaid with 1 µl matrix solution, let to dry again and MALDI-TOF MS can be performed.

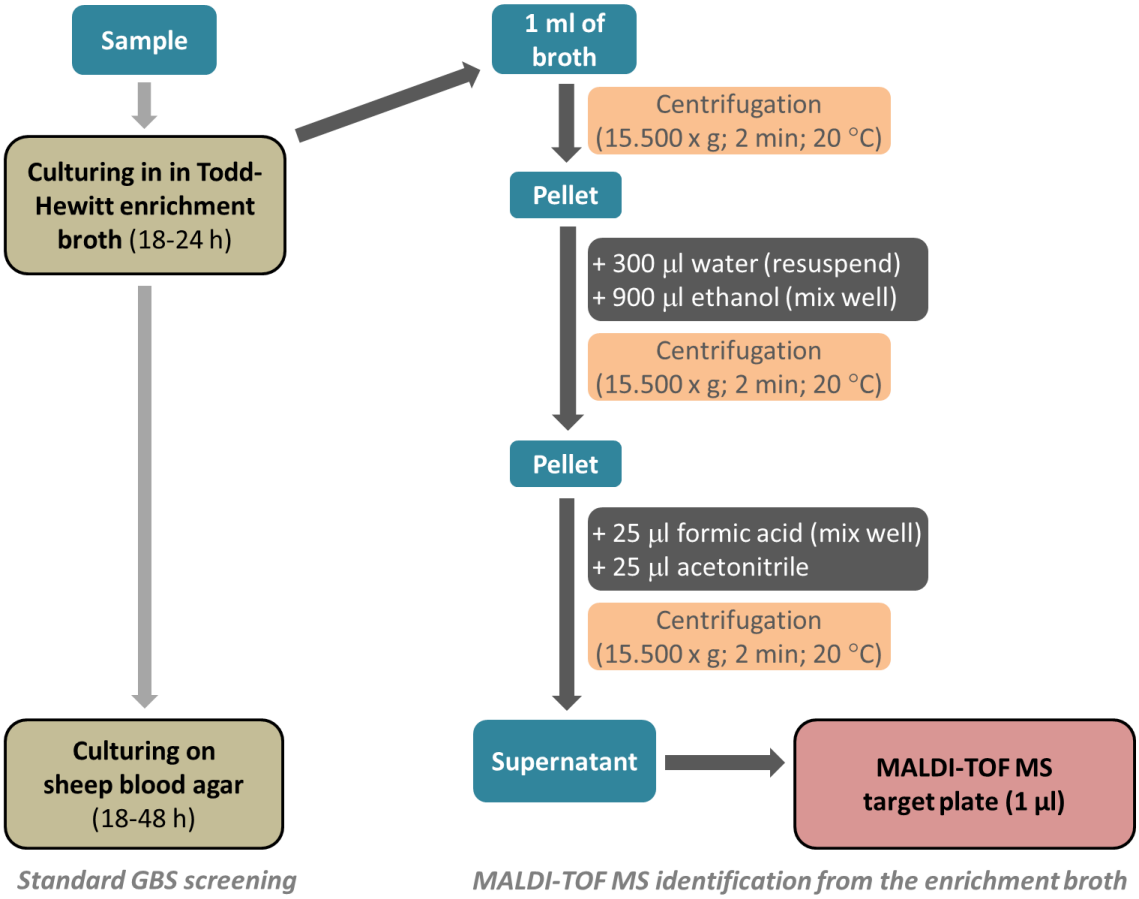

Figure 5 Scheme graph of the sample preparation process optimized for rapid GBS identification.

4.1.2 Testing of the optimized sample preparation on clinical samples

The above described method was tested on 100 vaginal samples and compared to the results of the standard GBS screening, i.e. subculturing on Columbia blood agar after enrichment (Table 4). The standard culture-based method proved the *S. agalactiae* colonization for 27 samples, among which MALDI-TOF MS from the enrichment broth also found 20 samples to be GBS positive (log scores: 1.711-2.322).

Table 4 MALDI-TOF MS identification directly from the selective enrichment broth in case of 27 vaginal samples of pregnant women, which proved to be GBS positive by the standard culture method.

Sample ID[a]	Species identified by MALDI-TOF MS	Score
22093	***S. agalactiae***	1.986
22564	***S. agalactiae***	2.258
24550	***S. agalactiae***	2.037
53198	***S. agalactiae***	1.810
54807	***S. agalactiae***	1.902
56309	***S. agalactiae***	1.880
60121	***S. agalactiae***	1.722
60171	***S. agalactiae***	1.749
63552	***S. agalactiae***	1.711
63565	***S. agalactiae***	1.726
63779	***S. agalactiae***	1.915
63977	***S. agalactiae***	1.911
63978	***S. agalactiae***	2.049
64610	***S. agalactiae***	1.737
22287	***S. agalactiae***	2.322
26541	***S. agalactiae***	2.054
30912	***S. agalactiae***	1.824
33015	***S. agalactiae***	2.067
35809	***S. agalactiae***	1.970
36032	***S. agalactiae***	1.815
22294	*E. faecalis*	2.051
52748	*E. faecalis*	2.039
56318	*E. faecalis*	2.070
59376	*E. faecalis*	1.982
59979	*S. aureus*	1.636
65308	*E. faecalis*	1.975
30901	*E. faecalis*	1.797

[a] Sample ID used by the Albert Szent-Györgyi Clinical Center of the University of Szeged (Szeged, Hungary). Conventional culture based GBS screening was performed according to the recommendations of CDC 2010 (Centers for Disease Control and Prevention, 2010) using selective enrichment performed in modified Todd Hewitt broth containing nalidixic acid (0.015 g/L) and colistin (0.010 g/L) and then subculturing on Columbia agar plates containing 5% sheep blood.

For the seven further samples identified as GBS positive by the standard method, our MALDI-TOF MS method detected *Enterococcus faecalis* in six cases (log scores: 1.797-2.070) and *Staphylococcus aureus* in one case (log score: 1.636) (Table 4). It should be mentioned that *E. faecalis* and *S. aureus* were the dominant bacteria in the enrichment broths of these seven samples and they were also detected by the conventional culture-based method.

All the 73 samples proved to be GBS negative by the standard method were also found to be negative by our method. For these samples, MALDI-TOF MS identified *E. faecalis* in 39 cases, *Lactobacillus* sp. in 12 cases, alfa-haemolytic *Streptococcus* in 6 cases, *Staphylococcus* sp. in 6 cases, *Bacillus* sp. in 1 case, *Candida* sp. in 1 case and Gram-negative bacteria in 3 cases; identification proved to be not reliable in 3 cases and no peaks were found in 2 cases (log scores: 1.443-2.422). This means that 93% of the rapid MALDI-TOF MS tests gave the same results as the standard GBS screening. In cases, where two methods led to different results, further bacteria (i.e. other than GBS) were also present in the samples. According to these data, the positive and negative predictive values of the developed MALDI-TOF MS-based method are 100 and 91%, respectively, and its specificity and sensitivity are 100 and 74.1%, respectively.

4.2 Comparative analysis of the results of GBS screening by MALDI-TOF MS among pregnant women at the Albert Szent-Györgyi Clinical Center (University of Szeged, Hungary) in the past years (Ábrók *et al*., 2020)

In this study, results of the GBS screening among pregnant women and antibiotic susceptibility testing performed between 2012 and 2018 were evaluated.

4.2.1 Colonization rate in the examined period

From January 2012 to July 2018, vaginal or cervical samples of 19267 pregnant women were screened for *S. agalactiae* harboring by MALDI-TOF MS identification after conventional enrichment and culturing. Out of them, 3554 samples were detected as GBS positive (Table 5).

Only slight changes could be observed in the colonization rates during the examined years: their values fluctuated between 17.4 and 19.8%. The mean value was 18.4%.

The colonization rate was higher than 10% in each age group. It is worth to mention that, although much less samples (3566) were tested in case of the age group 26-30 years than in the age group 31-35 years (6510), the colonization rate was found to be the same in both

groups (19%), and this value represented the highest rate compared to the other age groups (Table 5).

Table 5 Number of screened and the GBS positive samples and the colonization rates measured in the different age groups during the examined period.

Age groups (years old)		<21	21-25	26-30	31-35	36-40	41-45	>45	Total
Year									
2012	No. of screened samples	4	73	468	1037	823	357	114	2876
	No. of GBS positive samples	0	10	80	196	150	53	17	506
	(colonization rate; %)	(0)	(13.7)	(17.1)	(18.9)	(18.2)	(14.8)	(14.9)	(17.6)
2013	No. of screened samples	6	88	538	966	867	329	116	2910
	No. of GBS positive samples	1	24	96	176	142	52	17	508
	(colonization rate; %)	(16.7)	(27.3)	(17.8)	(18.2)	(16.4)	(15.8)	(14.7)	(17.5)
2014	No. of screened samples	10	94	539	1015	890	344	115	3007
	No. of GBS positive samples	2	15	106	212	168	69	23	595
	(colonization rate; %)	(20.0)	(16.0)	(19.7)	(20.9)	(18.9)	(20.1)	(20.0)	(19.8)
2015	No. of screened samples	3	103	557	992	884	352	89	2980
	No. of GBS positive samples	0	16	101	198	177	67	17	576
	(colonization rate; %)	(0)	(15.5)	(18.1)	(20.0)	(20.0)	(19.0)	(19.1)	(19.3)
2016	No. of screened samples	9	92	568	1001	866	341	89	2966
	No. of GBS positive samples	2	17	123	193	160	57	18	570
	(colonization rate; %)	(22.2)	(18.5)	(21.7)	(19.3)	(18.5)	(16.7)	(20.2)	(19.2)
2017	No. of screened samples	7	108	584	951	839	331	60	2880
	No. of GBS positive samples	1	14	111	158	147	62	9	502
	(colonization rate; %)	(14.3)	(13.0)	(19.0)	(16.6)	(17.5)	(18.7)	(15.0)	(17.4)
2018	No. of screened samples	3	75	312	548	480	197	33	1648
	No. of GBS positive samples	0	13	59	109	85	27	4	297
	(colonization rate; %)	(0)	(17.3)	(18.9)	(19.9)	(17.7)	(13.7)	(12.0)	(18.0)
Total	No. of screened samples	42	633	3566	6510	5649	2251	616	19267
	No. of GBS positive samples	6	109	676	1242	1029	387	105	3554
	(colonization rate; %)	(14.3)	(17.2)	(19.0)	(19.0)	(18.2)	(17.2)	(17.1)	(18.5)

4.2.2 Antimicrobial susceptibility of the GBS positive samples

Antibiotic susceptibility of the strains isolated from the GBS positive samples (n = 3554) to penicillin, cefuroxime, vancomycin, erythromycin, clindamycin and trimethoprim-sulfamethoxazole was also tested.

All tested isolates proved to be susceptible to penicillin, cefuroxime, vancomycin and trimethoprim-sulfamethoxazole.

The overall rate of the erythromycin and clindamycin resistant isolates were 34.9 and 34.6%, respectively. Majority of the erythromycin and clindamycin resistant isolates also proved to be co-resistant to these antibiotics and this co-resistance was detected in 33.2% of the GBS positive samples (Table 6). Only a slight portion of the isolates was found to be resistant to either erythromycin or clindamycin alone (1.9 and 1.4%, respectively). Majority of the detected clindamycin resistances proved to be constitutive and frequency of isolates with inducible clindamycin resistance was only 6.5% (Table 6).

Table 6 Number and frequency of the erythromycin and clindamycin resistant strains isolated from the GBS positive samples.

Year	GBS positive samples	Erythromycin resistant strains (%)	Clindamycin resistant strains (%)	Strains resistant to erythromycin alone (%)	Strains resistant to clindamycin alone (%)	Erythromycin - clindamycin co-resistant strains (%)
2012	506	148 (29.2)	153 (30.2)	6 (1.2)	11 (2.2)	142 (28)
2013	508	172 (33.9)	170 (33.5)	6 (1.2)	4 (0.8)	166 (32.8)
2014	595	190 (31.9)	182 (30.6)	17 (2.9)	9 (1.5)	173 (29.1)
2015	576	222 (38.5)	221 (38.4)	11 (1.9)	10 (1.7)	211 (36.6)
2016	570	196 (34.4)	182 (31.9)	14 (2.5)	0 (0)	182 (31.9)
2017	502	206 (41.0)	206 (41.0)	6 (1.2)	6 (1.2)	200 (39.8)
2018	297	118 (39.7)	115 (38.7)	12 (4.0)	9 (3.0)	106 (35.7)
Total	3554	1239 (34.9)	1230 (34.6)	68 (1.9)	49 (1.4)	1181 (33.2)

It should be emphasized that a clear increasing tendency can be observed in the frequency of resistant strains over the examined years. Between 2012 and 2018, proportion of the erythromycin and clindamycin resistant GBS strains increased from 29.2 and 30.2% to 39.7 and 38.7%, respectively, while the rate of the co-resistant strains increased from 28 to 35.7% (Table 6).

4.3 Application of a MALDI-TOF MS-based method to identify the highly virulent ST-1 and ST-17 clones of *S. agalactiae*

Using the method of Lartigue *et al.* (2011), MS spectra of 260 randomly selected GBS strains isolated from antenatal GBS screening in 2017 and 2018 were investigated for the presence of the protein peaks characteristic to the highly virulent invasive ST-1 and ST-17 *S. agalactiae* clones (Figure 6).

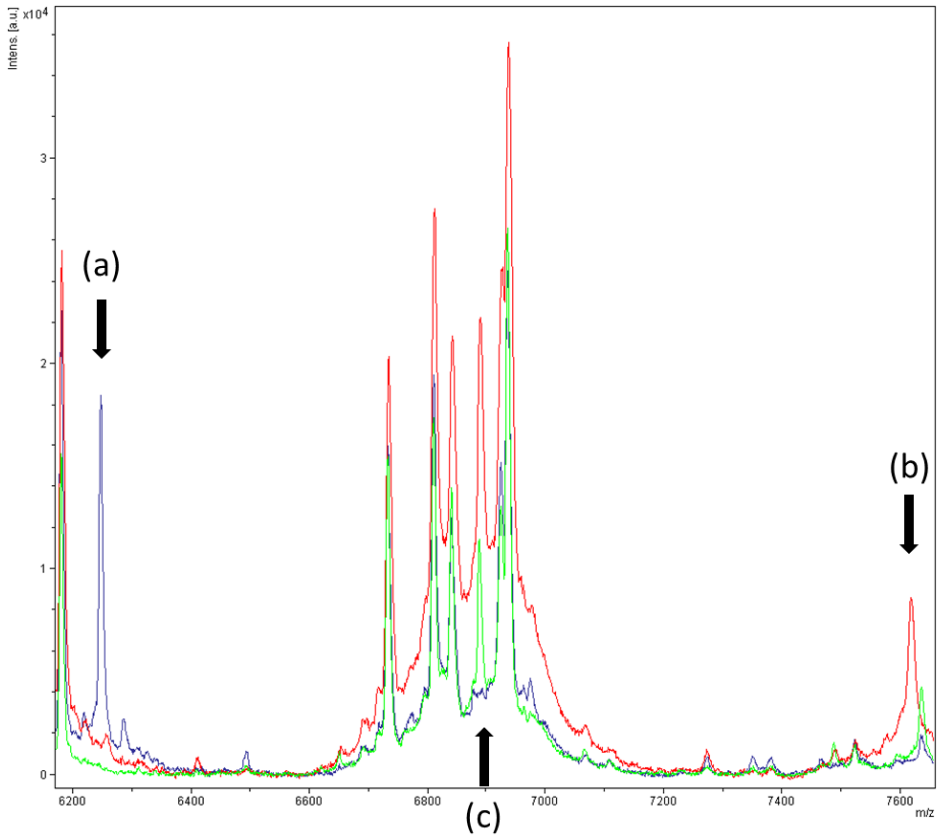

Figure 6 MS spectra of three *Streptococcus agalactiae* isolates representing an ST-1 (*blue*), an ST-17 (*red*) and a non-ST-1, non-ST-17 strain (*green*). Arrows show the discriminating mass peaks of GBS strains analyzed by MALDI-TOF MS, which were a peak at 6250 Da (**a**) for the ST-1 type and a peak at 7625 Da (**b**) for the ST-17 type. No mass peak at 6888 Da (**c**) was also characteristic to the ST-1 strains as described by Lartigue *et al.* (2011).

Out of the tested 260 isolates, 71 (27.3%) and 50 strains (19.2%) displayed the protein patterns characteristic to the ST-1 and the ST-17 types, respectively (Table 7).

Table 7 Number and rate of the ST-1 and ST-17 strains detected by the analysis of the MALDI TOF MS spectra and the number and rate of the erythromycin and clindamycin resistant strains among them.

Sequence type	No. of strains detected (%)	Erythromycin resistant strains (%)	Clindamycin resistant strains (%)	Strains resistant to clindamycin alone (%)	Erythromycin – clindamycin co-resistant strains (%)
ST-1	71 (27.3)	37 (52.1)	37 (52.1)	0 (0)	37 (52.1)
ST-17	50 (19.2)	16 (32.0)	17 (34.0)	1 (2.0)	16 (32.0)
Other (non-ST-1, non-ST-17)	139 (53.5)	43 (30.9)	47 (33.8)	4 (2.9)	43 (30.9)

Erythromycin and clindamycin resistance of the examined strains were also tested. Frequency of erythromycin and clindamycin co-resistance among the ST-17 strains (34%) did not show significant difference to that among the non-ST-1 and non-ST-17 strains (30.9%). However, more than half of the ST-1 isolates (52.1%) were resistant to these antibiotics (Table 7). While all the erythromycin and clindamycin resistant ST-1 isolates proved to be co-resistant, 2 and 2.9% of the ST-17 and the non-ST-1 and non-ST-17 strains were resistant only to clindamycin alone. Frequency of induced clindamycin resistance was found to be low in each group of isolates as it was detected in 4, 5.6 and 7.9% of the ST-17, ST-1 and the non-ST-1 and non-ST-17 strains, respectively. Erythromycin resistance alone without clindamycin resistance was not found in any of the tested 260 isolates.

4.4 Development of a MALDI-TOF MS method for the rapid identification of methicillin-resistant *S. aureus* strains (Ábrók *et al.*, 2018)

4.4.1 Optimization of MRSA identification from the enrichment broth by MALDI TOF MS combined with a PBP2' latex agglutination assay

To shorten the time of the culturing-based MRSA screening, MALDI-TOF MS identification directly from the selective broth was optimized and combined with the PBP2' latex agglutination assay.

While PBP2' latex agglutination assay is routinely used in the laboratory and its implementation requires a sufficient number of bacterial cells (Nakatomi and Sugiyama, 1998), MRSA selective enrichment broth was centrifuged, and the bacterial pellet was used in the

study. From the clinical samples sent for MRSA screening, 1.5 ml of the selective enrichment broth incubated previously for 18-24 h was centrifuged (15.500 × *g* for 2 min at 20 °C) and the bacterial pellet was resuspended in 50 µl ultrapure distilled water and centrifuged again (15.500 × *g* for 2 min at 20 °C). This pellet was used for MALDI-TOF MS identification by smearing a small amount of cells onto the target plate. For sample preparation, the on-target formic acid extraction was tested. If *S. aureus* was detected by the MALDI-TOF MS analysis, the PBP2' latex agglutination assay was performed from the rest of the pellet (approx. 1.5×10^9 cells, i.e. 3-5 µl, are necessary for the PBP2' test).

Figure 7 summarizes the proposed detection procedure. Standard MRSA screening and MALDI-TOF MS combined with PBP'2 latex test should be performed parallelly. The key elements of the rapid detection method are the two centrifugation steps to concentrate the cells for the analysis and the formic acid treatment before the MALDI TOF MS measurement.

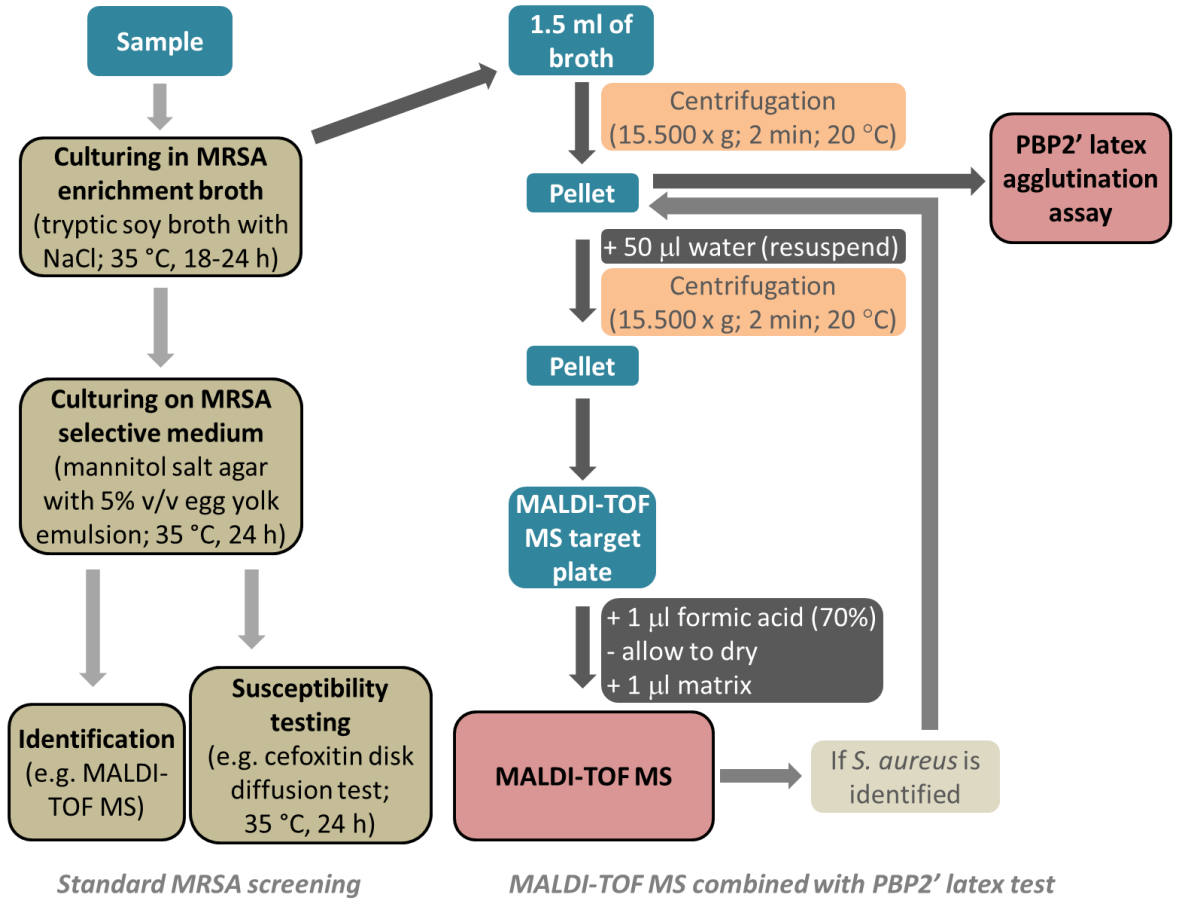

Figure 7 Scheme graph of the sample preparation and screening process optimized for rapid MRSA detection using MALDI-TOF MS combined with the PBP2' latex agglutination assay.

4.4.2 Evaluation of the optimized MRSA identification method using clinical samples

To test the method, 255 samples were collected from MRSA screening performed from 2015 to 2017 at our institution. For the rapid MALDI-TOF MS identification, sample preparation was performed directly from the sediments of the *S. aureus* selective enrichment broth after incubation for 18-24 h. If MALDI-TOF MS analysis detected *S. aureus*, the PBP2' latex agglutination assay (Oxoid, England) was performed from the rest of the sediments according to the recommendations of the manufacturer (Figure 7). As a control, all involved sample were also identified at the species level using the standard culturing-based method (Wolk *et al.*, 2009a) followed by a MALDI-TOF MS analysis of the pure culture. Antibiotic susceptibility testing used to prove the methicillin resistance of the isolated strains (Figure 7).

Both the standard method (i.e. enrichment, selective sub-culturing and MALDI-TOF MS analysis of the resulting pure cultures) and our optimized MALDI-TOF MS analysis of the pellets of the selective enrichment broth (log scores: 1.238-2.318) identified *S. aureus* in the same 49 samples (Table 8). Identifications were accepted if the log scores were higher than 2.0 (Wieser *et al*., 2012; Patel, 2015); lower log scores were accepted only if at least five best matches were found to be *S. aureus*.

Subsequently, the standard method combined with the antibiotic susceptibility testing detected MRSA in 18 cases. At the same time, PBP2' latex agglutination assay performed from the sediments of the enrichment broths could detect 16 samples as MRSA positive (Table 8). In two cases, the amounts of sediments were not sufficient for the latex agglutination assay.

In some cases, centrifugation of the enrichment broth gave only a low amount of sediments and MALDI-TOF MS analysis was unsuccessful finding no peaks or yielding non-reliable identification. In all these cases, the standard MRSA screening method also gave negative results. At the same time, if MALDI-TOF MS performed directly from the sediment of the selective enrichment broth detected a non-*S. aureus* species, the standard MRSA screening also proved to be negative for MRSA. Distribution of these non-MRSA strains was the following, *Staphylococcus epidermidis*, *S. haemolyticus* and *S. hominis* were identified in 85, 19 and 1 samples, respectively; *Enterococcus faecalis* was found in 5 cases; *Klebsiella pneumoniae* and *Proteus mirabilis* were detected in 2-2 samples, while *Enterobacter aerogenes*, *Enterococcus faecium*, *Escherichia coli* and *Klebsiella oxytoca* were found in single samples, respectively (log scores: 1.282-2.3). Identification proved to be not reliable in case of 6 samples (log scores: 1.093-1.396) and no peaks were found in 82 cases.

Table 8 Results of MALDI-TOF MS identification combined with the PBP2' latex agglutination test directly from the selective enrichment broth and the standard culture-based MRSA screening in case of the 49 samples identified as *S. aureus*.

No.	Sample ID[a]	Sample type	MALDI-TOF MS combined with PBP 2' latex assay			Standard culture-based MRSA screening[b]
			Species identified by MALDI-TOF MS	Log-score	PBP 2' latex assay	
1	14675	nasal	*S. aureus*	1.238	+	+
2	8847	nasal	*S. aureus*	1.893	+	+
3	8993	nasal	*S. aureus*	1.567	+	+
4	14674	nasal	*S. aureus*	2.026	+	+
5	66653	nasal	*S. aureus*	2.003	+	+
6	83424	nasal	*S. aureus*	2.275	+	+
7	108686	nasal	*S. aureus*	2.068	+	+
8	112482	nasal	*S. aureus*	2.085	+	+
9	8996	throat	*S. aureus*	1.553	+	+
10	9291	throat	*S. aureus*	1.698	+	+
11	14502	throat	*S. aureus*	1.637	+	+
12	66648	throat	*S. aureus*	2.122	+	+
13	66654	throat	*S. aureus*	2.318	+	+
14	83423	throat	*S. aureus*	2.24	+	+
15	86741	throat	*S. aureus*	2.243	+	+
16	108682	throat	*S. aureus*	1.545	-	+
17	112277	throat	*S. aureus*	1.656	-	+
18	112484	throat	*S. aureus*	2.064	+	+
19	112247	throat	*S. aureus*	1.843	-	-
20	74967	throat	*S. aureus*	2.255	-	-
21	74963	nasal	*S. aureus*	2.272	-	-
22	85807	wound	*S. aureus*	1.941	-	-
23	83415	axilla	*S. aureus*	1.883	-	-
24	9220	nasal	*S. aureus*	1,972	-	-
25	66204	nasal	*S. aureus*	2.289	-	-
26	83417	nasal	*S. aureus*	1.975	-	-
27	84291	nasal	*S. aureus*	2.282	-	-
28	86910	nasal	*S. aureus*	2.237	-	-

29	107363	nasal	*S. aureus*	1.782	-	-
30	107487	nasal	*S. aureus*	1.882	-	-
31	84291	nasal	*S. aureus*	2.282	-	-
32	111816	nasal	*S. aureus*	1.682	-	-
33	112244	nasal	*S. aureus*	2.042	-	-
34	86551	wound	*S. aureus*	1.918	-	-
35	14498	throat	*S. aureus*	1.818	-	-
36	66203	throat	*S. aureus*	2.119	-	-
37	107364	throat	*S. aureus*	1.946	-	-
38	107488	throat	*S. aureus*	2.007	-	-
39	107550	throat	*S. aureus*	2.094	-	-
40	108553	throat	*S. aureus*	2.256		-
41	111804	throat	*S. aureus*	1.695	-	-
42	112243	throat	*S. aureus*	1.974	-	-
43	112389	throat	*S. aureus*	2.173	-	-
44	63024	throat	*S. aureus*	1.976	-	-
45	63028	throat	*S. aureus*	2.028	-	-
46	63025	nasal	*S. aureus*	1.359	-	-
47	74762	axilla	*S. aureus*	2.193	-	-
48	74964	throat	*S. aureus*	2.299	-	-
49	14885	nasal	*S. aureus*	1.348	-	-

[a]Sample ID used by the Institute of Clinical Microbiology Albert Szent-Györgyi Clinical Center of the University of Szeged (Szeged, Hungary).
[b]Standard culture-based MRSA screening means that MRSA was detected by MALDI TOF MS identification and antibiotic susceptibility testing of *Staphylococcus aureus* strains after enrichment and selective culturing.

In our study to evaluate of the method combining MALDI-TOF MS performed directly from the enrichment broth and PBP2' latex agglutination assay (n = 255), 99% of the tests gave the same results as the standard MRSA screening method. The numbers of the true positive, false positive, true negative and false negative samples were 16, 0, 237 and 2, respectively. Thus, sensitivity and specificity of the method were 89% and 100%, respectively, while the positive and negative predictive values proved to be 100% and 99%, respectively. If the sample was found to be MRSA positive by the MALDI-TOF MS identification combined with the PBP2' latex agglutination assay, the sample was MRSA positive by the conventional analysis too.

5. DISCUSSION

MALDI-TOF MS analysis has proven to be an efficient and reliable technique for the routine identification of bacteria (Lartigue *et al.,* 2009; Benagli *et al.,* 2011; Cherkaoui *et al.,* 2011). By now, this method has become an integrant and important tool of the clinical diagnostic laboratories and it is also well established in the routine practice of our institution (Virók *et al.,* 2014; Fenyvesi *et al.,* 2014; Nagy *et al.,* 2009, 2011, 2013, 2014, 2017). Besides its application for species-level identification, the technique can be used for strain typing to detect intraspecies groups, such as strains with special pathogenicity or antibiotic resistance characteristics, serogroups or sequence types (Bader, 2013; Kostrzewa *et al.,* 2013; Christner *et al.,* 2014; Tang *et al.,* 2019; Welker *et al.,* 2019). Strain typing is currently a rapidly developing area of the application of MALDI-TOF MS method. Another important field of the efforts to improve the technique is the development of methods to shorten the cultivation time prior to the MALDI-TOF MS or perform the analysis directly form the clinical specimens (Singhal *et al.,* 2015). Here, we describe two method optimizations to shorten the cultivation time for MALDI-TOF MS identification of *S. agalactiae* and MRSA bacteria. In the latter case, MALDI-TOF MS analysis was combined with the detection of methicillin resistance. Besides species-level GBS identification, MALDI-TOF MS was also used to identify two high risk sequence types of *S. agalactiae.*

5.1 Development of a MALDI-TOF MS method for rapid detection of *S. agalactiae* colonization of pregnant women

Determination of the GBS colonization status provides significant data to suggest an appropriate intrapartum antibiotic prophylaxis (Schrag and Verani, 2013). The current standard procedure for GBS colonization screening involves a selective enrichment in a broth medium for 18-24 h followed by a 24-h sub-culturing step (Centers for Disease Control and Prevention, 2010). Thus, identification requires two days. Moreover, further 24-h culturing can be needed if the primary culturing gives ambiguous results.

Identification of *S. agalactiae* isolates is routinely performed by MALDI-TOF MS analysis. This method is commonly accepted and efficiently and reliably identifies *S. agalactiae* (Lartigue *et al.,* 2009). However, previous MALDI-TOF MS identifications of *S. agalactiae* and β-haemolytic streptococci have used pure cultures obtained by the standard culturing

method and thus, the whole 2- or 3-days culturing procedure had been performed before each analysis (Benagli *et al*., 2011; Cherkaoui *et al*., 2011; Lartigue *et al.*, 2009).

Using the standard identification method, at least 48 h was spent with culturing between the sample collection and the identification. Considering the positive predictive value (100%) of our rapid MALDI-TOF MS method, if it indicates GBS positivity for a sample, GBS colonization can be reported already 18-24 hours after the sample collection (i.e. directly from the selective enrichment broth) (Ábrók *et al.,* 2015). Thus, the detection time of GBS colonization can be significantly shortened in most cases.

Several studies highlight the need of the shortening the sample preparation and incubation time for MALDI-TOF MS (Hernández et al. 2016) but until to date, only a few direct measurement methods have been developed or adapted (Byliński *et al.,* 2017; Tré-Hardy *et al.,* 2017). Direct identification from the selective enrichment has successfully been adapted to detect, among others, *Salmonella* sp. from stool (Sparbier *et al.,* 2012), *Listeria monocytogenes* from food samples (Jadhav *et al.,* 2014), or different bacteria from positive blood cultures (Kohlmann *et al.,* 2015; Verroken *et al.,* 2016; Curtoni *et al.,* 2017; Chew *et al.,* 2019). Furthermore, Tré-Hardy *et al.* (2017) described and validated a sample preparation procedure for MALDI-TOF MS identification directly from a non-selective enrichment broth used for cerebro-spinal fluid and sterile tissue samples.

Regarding the congruence of the MALDI-TOF MS identification directly from the selective enrichment broth and that from the standard culture-based procedure, our method gave the same results in the 93% of the tests as the standard GBS screening. Similar rate could previously be achieved for the *Salmonella* sp. direct detection (92.6%) (Sparbier *et al.,* 2012) and various other bacteria (97-98%) (Oviano *et al.,* 2018). In all described cases, the most important step of these studies was the optimization of the sample preparation, especially the assurance of sufficient amounts of bacteria, for example by centrifuging the liquid medium (Sparbier *et al.,* 2012; Ábrók *et al.,* 2015; Oviano *et al.,* 2018).

5.2 Comparative analysis of the results of GBS screening by MALDI-TOF MS among pregnant women at the Albert Szent-Györgyi Clinical Center (University of Szeged, Hungary) in the past years

GBS colonization level (i.e. the mean value) in the samples obtained from pregnant women was found to be 18.4% between the beginning of 2012 until the middle of 2018 (Ábrók *et al*., 2020). This value falls into the range of the rates observed earlier in other European

countries (i.e. from 6.5 to 36%) (Barcaite *et al.*, 2008; Shabayek and Spellerberg, 2018). However, it is somewhat higher than that reported recently in the neighboring country, Serbia (15%) (Gajic *et al.*, 2019). This relatively high colonization rate found in our samples underlines the importance of the regular and accurate GBS screening of pregnant women.

All *S. agalactiae* strains isolated in our tests proved to be susceptible to penicillin, cefuroxime and vancomycin. Susceptibility of GBS strains to β-lactams and vancomycin can be regarded as a common feature of *S. agalactiae* strains reported in many previous studies (Garland *et al.*, 2011; Nakamura *et al.*, 2011; De Francesco *et al.*, 2012; Morozumi *et al.*, 2014).

At the same time, relatively high level of resistance to erythromycin and clindamycin (34.9 and 34.6%, respectively) and, over the examined years, a clearly increasing tendency in the rate of the resistant strains have been observed. Other studies have also attracted attention to the spreading of macrolide and lincosamide resistance among *S. agalactiae* strains (Blaschke *et al.*, 2010; Capanna *et al.*, 2013; Capraro *et al.*, 2013) and highlighted the need of susceptibility testing of colonizing GBS strains for an effective intrapartum antibiotic prophylaxis (Capanna *et al.*, 2013; Di Renzo *et al*., 2015; Shabayek and Spellerberg, 2018, Gajic *et al.*, 2019). Majority of the erythromycin and clindamycin resistant strains proved to be co-resistant to these antibiotics and most of the detected clindamycin resistances proved to be constitutive.

5.3 Application of a MALDI-TOF MS-based method to identify the highly virulent ST-1 and ST-17 clones of *S. agalactiae*

The GBS sequence types, ST-1 and ST17 are known to be frequently associated to neonatal meningitis and invasive neonatal infections (Shabayek and Spellerberg, 2018). The method of Lartigue *et al*. (2011) was used for the MALDI-TOF MS-based identification of these sequence types. This method involves the analysis of the MS spectra to search for the presence of sequence type-specific protein peaks. In the experiments of Lartigue *et al.* (2011), sensitivity and specificity of this MALDI-TOF MS-based typing method were 100% and 95%, respectively, for ST-1 and 100% and 98%, respectively, for ST-17. The potential of this MS spectrum analysis-based detection for the routine ST-17 screening was acknowledged by the European consensus conference held in 2013 and dedicated to issues related to GBS screening and peripartum prophylaxis in European countries (di Renzo *et al.*, 2015).

In our study, MALDI-TOF MS data of 260 strains isolated from antenatal GBS screening in 2017 and 2018 were examined. Almost half (46.5%) of the analyzed *S. agalactiae*

MS spectra contained protein patterns being characteristic to either the ST-1 (27.3%) or the ST-17 (19.2%) clones. Compared them to those reported previously from other countries (Bisharat *et al*., 2005; Meehan *et al.,* 2014; Usein *et al.,* 2014; Moltó-García *et al.,* 2016), these rates indicate a high prevalence of these potentially virulent and invasive GBS strains in our region. In other studies, ST-1 has been found as one of the predominant GBS colonizers in pregnant women and it has proven to be one of the most frequent clones in vaginal samples (Manning *et al.,* 2008; Teatero *et al.,* 2014; Shabayek and Spellerberg, 2018). Our study also indicates a significant distribution of the ST-17 sequence type among the strains colonizing pregnant women. Similarly, high level of prevalence was previously detected for this sequence type in Ireland and Romania (21 and 24%, respectively) (Meehan *et al.,* 2014; Usein *et al.,* 2014) and recently, Gajic *et al.* (2019) found ST-17 as the most common sequence type in Serbian samples.

Besides the high prevalence of ST-1 in our samples, proportion of the erythromycin and clindamycin co-resistant strains was found to be extremely high within the ST-1 positive isolates (52.1%). The strong association of ST-1 clones with high level of erythromycin resistance was also observed by Bergseng *et al.* (2009) previously.

5.4 Development of a MALDI-TOF MS method for the rapid identification of methicillin-resistant *S. aureus* strains.

Fast and accurate detection of MRSA strains in clinical samples is highly important to choose the adequate therapy and to prevent the spread of the pathogen. Therefore, shortening the identification time or development direct MRSA screening methods is an extensively studied area (Ibrahim *et al.,* 2017; Quiao *et al.,* 2018). This goal is generally achieved by combining different phenotypic tests (Verroken *et al.,* 2016; Lüthje *et al.,* 2017; Rees and Barr, 2017; Ábrók *et al.,* 2018). However, such direct methods often involve sophisticated tools for MRSA identification. For example, the approach of Rees and Barr (2017) uses a phage amplification detection method in the presence and absence of cefoxitin treatment and combines it with MALDI-MS analysis of the trypsin digested phage proteins. Another method was developed by Quiao *et al.* (2018) who applied a novel aptamer-based fluorometric assay from clinical samples, which was coupled with immunomagnetic separation.

In our study, a simple sample preparation for MALDI-TOF MS directly from the selective *S. aureu*s enrichment broth was combined with the PBP2' latex agglutination assay to detect methicillin resistance (Ábrók *et al.,* 2018). Based on its positive predictive value (100%),

MALDI-TOF MS combined with the PBP2' latex agglutination assay is an efficient alternative of the molecular detection methods to shorten the duration and to improve the efficiency of the *mecA*-postive MRSA screening. Using this technique, MRSA colonization can already be reported 18-24 hours after the sample collection.

PCR-based assays are widely used rapid molecular methods for MRSA identification (Wolk *et al.*, 2009a; Luteijn *et al.*, 2011; Peacock and Paterson, 2015; Dupieux *et al.*, 2017). These methods have sensitivities and specificities ranging from 82 to 100% and 64 to 99%, respectively (Marlowe and Bankowski, 2011). Sensitivity and specificity of our method is comparable with these values being 89% and 100%, respectively.

A significant advantage of our approach is that it can be easily performed and involved in the routine practice combining it with the standard culture method.

6. CONCLUSIONS

The aim of this study was to improve and evaluate the MALDI-TOF MS-based species- and intraspecies-level identification of *Streptococcus agalactiae* and methicillin-resistant *Staphylococcus aureus* bacteria.

Main results and conclusions are the followings:

1. We developed a MALDI-TOF MS-based identification method for GBS screening of pregnant women. By the optimization of the sample preparation, we could shorten the identification process and MALDI-TOF MS can be carried out directly from the GBS selective enrichment broth. While the standard culture-based method followed by MALDI-TOF MS analysis requires two or three days to complete the identification, if our method detects GBS positivity, GBS colonization can be reported after 18-24 hours.

2. Comparative analysis of the results of the GBS screening among pregnant women performed at our institution between 2012 and 2018 revealed a 18.4% colonization rate (mean value). This colonization level, which only slightly fluctuated over the examined period, is within the range of rates detected previously in Europe but it can be regarded as a relatively high rate compared to those reported in other countries. The colonization rate was higher than 10% in each age group and the highest rates were observed in 26-30 and 31-35 age groups. These data underline the importance of the regular GBS screening among pregnant women.

3. As expected, all GBS strains isolated between 2012 and 2018 were sensitive to β-lactams and vancomycin. However, the average level of resistance to erythromycin and clindamycin during this period was found to be high (34.9 and 34.6%, respectively). Furthermore, during the examined years, frequency of the resistant strains clearly increased and reached around 40% for both antibiotics in the last two years. To our knowledge, this study is the first analysis of the GBS screening results and antibiotic susceptibility pattern of GBS strains colonizing pregnant women in our region. Increasing rates of the macrolide and lincosamide resistant GBS strains highlight the need of susceptibility testing of the detected GBS strains to suggest an effective intrapartum antibiotic prophylaxis.

4. We analyzed the MALDI-TOF MS spectra of 260 GBS strains detected during antenatal screening in 2017 and 2018 to search them for specific peaks indicating the invasive and highly virulent sequence types, ST-1 and ST-17. A high rate of these high-risk types was found among the tested strains as almost half of them proved to be belonging to either the ST-1 (27.3%) or the ST-17 (19.2%) sequence types. These rates correspond to the highest rates reported from other countries. To our knowledge, these results provide the first data about the frequency of the highly virulent ST-1 and ST-17 GBS clones in Hungary. This study also reinforces the value and applicability of MALDI-TOF MS identification in group B *Streptococcus* screening and demonstrates that examination of the MALDI-TOF MS spectra is a useful method for strain typing and to detect the high-risk ST-1 and ST-17 GBS strains. Almost a half part of the GBS-positive pregnant women can be colonized by these potentially highly virulent clones in our region, which highlights the importance of the accurate antenatal *S. agalactiae* screening.

5. We developed a MALDI-TOF MS method for rapid MRSA identification directly from the selective enrichment broth. This procedure involves an optimized sample preparation for the MALDI-TOF MS analysis and the application of the PBP2' latex agglutination assay to detect methicillin resistance. The advantage of this method is the simplicity and that it can be easily incorporate into the routine MRSA screening. Sensitivity and specificity of our method was comparable with those of other more complex techniques. Using this method, the identification time can be reduced from two-three days (the standard method) to 18-24 hours.

Conclusions of our study underline the importance and the value of the MALDI-TOF MS technique in the clinical diagnostic practice and raised that optimization of the standard sample preparation procedures for MALDI-TOF MS can significantly improve the identification process.

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