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 Thesis

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1. INTRODUCTION

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) is a powerful technique to identify bacteria and it has become an important part of the routine diagnostic arsenal of the clinical laboratories. 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.

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

1.2.1 Species-level identification

To generate species specific protein mass fingerprints of bacteria, constantly expressed, highly abundant proteins in a size range between 2 and 20 kDa are used [1]. As the recorded spectrum is never completely identical with that in the reference database, the software analyzing the MS data gives a score value (log score) to the spectrum based on its similarity to the reference spectrum. Practically, these score values are considering 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 at the genus level [2].

The sample is generally a small portion of the pure culture of an isolated bacterium. The colony can be examined directly after it was spotted onto the target plate ("direct transfer method") [3]. In some cases, direct transfer should be extended with a rapid on-target extraction using 70% formic acid to increase the efficiency of the identification ("direct transfer-formic acid preparation") [3]. Alternatively, an on-target ethanol-formic acid extraction can also be used [3]. Certain bacteria cannot be lysed directly on the target plate efficiently [5]. Instead, protein extraction is carried out before the sample is subjected onto the target plate [5]. MALDI TOF MS identification generally takes 1 h or less. However, as accurate result can be obtained from pure cultures, culturing steps cannot be omitted. Thus, we should count with at least 18-48 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 [4].

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 [6-8]. 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 [9].

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 one of the most frequent agents of invasive diseases in neonates [10] and is among the leading causes of neonatal sepsis and meningitis [11-12]. GBS can be present as a commensal of the gastrointestinal and genitourinary tracts and about 10-30% of pregnant women are colonized by GBS without any symptoms [10,13]. However, these women are considered to be at increased risk of premature delivery and stillbirth [12].

1.3.2 Intraspecific groups of Streptococcus 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 [14,15]. Regarding the pathogenicity, serotype III is considered as the predominant invasive type in neonates [16,17].

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 [18]. Majority of the STs from human origin can be grouped into 6 clonal complexes [19]. Among them, ST-17 is well known as a clone, which is strongly associated with neonatal early-onset meningitis and GBS late-onset disease [10] (Furfaro *et al.*, 2018). It belongs to serotype III and is referred as “highly virulent”. Several studies have highlighted the need of the rapid identification of the ST-17 lineage if the GBS screening is positive [6]. As the causative agent of neonatal invasive diseases, another important clone is ST-1, which mostly belongs to the serotype V [20].

1.3.3 GBS screening of pregnant women

GBS screening of pregnant women has been implemented in many countries [10]. If the screening detects vaginal or rectal GBS colonization, an intrapartum antibiotic prophylaxis (IAP) is administered to prevent the infection of the neonates [10]. The standard method to detect the colonization is a culturing method, which involves firstly a selective enrichment in a broth medium (e.g. 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 (e.g. on Columbia agar plates containing 5% sheep blood) for another 24-48 h [21]. 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.

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 [18]. However, as this method uses pure cultures, the above-mentioned culturing steps should be performed prior to the analysis [18,22]. MALDI-TOF MS also proved to be applicable to detect certain MLSTs of *S. agalactiae*. Lartigue *et al.* [6] 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.

Since *S. agalactiae* is susceptible to β-lactams, penicillins are the first line antibiotics of choice for IAP and to treat GBS infections [23]. In penicillin-allergic women, application of clindamycin is recommended and in case of the detection of clindamycin resistance, vancomycin treatment is used [23]. However, an increasing resistance to antibiotics used as alternatives of β-lactams has been detected [23,24]. Therefore, susceptibility testing of strains isolated from antenatal GBS screening is important and applied in the routine diagnostics.

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 commensal and human pathogenic. Methicillin-resistant *S. aureus* (MRSA) is notorious about causing nosocomial and community acquired infections worldwide [25].

Generally, methicillin resistance of MRSA 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 [26]. 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 [27]. Methicillin resistance also means resistance practically to all β-lactam antibiotics [26]. 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 [28]. Thus, MRSA infections are frequently difficult to treat; however, a delay in the appropriate antibiotic therapy may highly and negatively affect the clinical outcome [29]. Therefore, the rapid and accurate detection is an important prerequisite for an efficient infection control.

1.4.2 MRSA identification

Standard MRSA surveillance uses a culture-based screening strategy consisting of a selective enrichment followed by a sub-culturing on an MRSA selective medium [25,30]. 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.

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 commonly used [25,29].

MALDI-TOF MS is an efficient tool for *S. aureus* identification [31,32]. However, as this method uses pure cultures, the enrichment and sub-culturing steps should be performed prior to the analysis.

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.* [6] 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

To optimize the MALDI-TOF MS sample preparation methods for GBS screening, 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. 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

All GBS samples were screened by the standard culturing-based method according to the recommendations of CDC 2010 guideline [21]. For MRSA detection, all samples were screened by the standard culturing-based method [30,33].

3.3 Antibiotic susceptibility testing

Antimicrobial susceptibility tests were performed by the disk diffusion method according to the EUCAST recommendations (http://www.eucast.org/) [34].

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.

3.5 MALDI-TOF MS analysis

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 [3]. MALDI-TOF MS identification was performed according to Wieser *et al.* [31]. using a Microflex MALDI Biotyper mass spectrometer (Bruker Daltonics, Germany).

3.6 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 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) [35]

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

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. agalatiae* 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. Based on these tests, a procedure for sample preparation directly from the Todd-Hewitt enrichment broth was proposed 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.

The above described method was tested on 100 vaginal samples and compared to the results of the standard GBS screening. The standard culture-based method proved GBS 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). 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 *S. aureus* in one case (log score: 1.636). 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. This means that 93% of the rapid MALDI-TOF MS tests gave the same results as the standard GBS screening. When the two methods led to different results, bacteria other than GBS were also present in the samples. 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) [36]

Results of the GBS screening among pregnant women and antibiotic susceptibility testing performed between January 2012 and July 2018 were evaluated. 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.

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.

Antibiotic susceptibility of the strains isolated from the GBS positive samples (n = 3554) 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. 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%.

A clear increasing tendency could 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%.

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.* [6], 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. 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. 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. 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) [37]

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 [38], 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. 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. 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.

To test the method, 255 samples were collected from MRSA screening performed from 2015 to 2017 at our institution. 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. 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. 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.

In 99% of cases, MALDI-TOF MS directly from the enrichment broth combined PBP2' latex agglutination assay gave the same results as the standard MRSA screening. Numbers of the true positive, false positive, true negative and false negative samples were 16, 0, 237 and 2,

respectively. 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 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 [22]. It is also well established in the routine practice of our institution [39,40].

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 [41]. Identification of *S. agalactiae* by MALDI-TOF MS is a commonly accepted method, which efficiently identifies this bacterium [18]. However, MALDI-TOF MS identification uses pure cultures obtained by the standard culturing method and thus, the whole 2- or 3-days culturing procedure should be performed before the analysis [18,22]. 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). 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 but until to date, only a few direct measurement methods have been developed [42,43]. Direct identification from the selective enrichment has successfully been adapted to detect, among others, *Salmonella* sp. from stool [44], *Listeria monocytogenes* from food samples [45], or different bacteria from positive blood cultures [46-49]. 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%) [44] and various other bacteria (97-98%) [50]. 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 [44,50].

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 [36] This value falls into the range of the rates observed earlier in other European countries (i.e. from 6.5 to 36%) [51]. However, it is somewhat higher than that reported recently in the neighboring country, Serbia (15%) [52].

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 previous studies [53,54].

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 [24,55] and highlighted the need of susceptibility testing of colonizing GBS strains for an effective intrapartum antibiotic prophylaxis [23,51,52]. 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 [51]. The method of Lartigue *et al*. [6] 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.* [6], 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 [23].

In our study, 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. 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 [51]. Our study also indicates a significant distribution of the ST-17 sequence type among the strains colonizing pregnant women. Recently, Gajic *et al.* [52] 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.* [56] previously.

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

Shortening the identification time or development direct MRSA screening methods is an extensively studied area [57]. This goal is generally achieved by combining different phenotypic tests [58]. However, such direct methods often involve sophisticated tools. For example, the approach of Rees and Barr [58] 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 Qiao *et al.* [57] 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 [37]. 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 of the *mecA*-postive MRSA screening. Using this technique, MRSA colonisation can already be reported 18-24 hours after the sample collection. 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. A MALDI-TOF MS-based identification method for GBS screening of pregnant women was developed. We could shorten the identification process and MALDI-TOF MS could 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. This colonization level is within the range of rates detected previously in Europe. 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.

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 was found to be high (34.9 and 34.6%, respectively). Furthermore, 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 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 belonged to either the ST-1 (27.3%) or the ST-17 (19.2%) 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 to detect the high-risk ST-1 and ST-17 strains. Almost a half part of the GBS-positive pregnant women can be colonized by

these potentially highly virulent clones in our region, which emphases the importance of the accurate antenatal *S. agalactiae* screening.

5. A MALDI-TOF MS method was developed for rapid MRSA identification directly from the selective enrichment broth. The procedure involves an optimized sample preparation for the MALDI-TOF MS 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.

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LIST OF PUBLICATIONS

Publications in referred journals related to the subject of the thesis

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**

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