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Marie-Frédérique Lartigue, Geneviève Héry-Arnaud, Eve Haguenoer, Anne-Sophie Domelier, Pierre-Olivier Schmit, Nathalie van Der Mee-Marquet, Philippe Lanotte, Laurent Mereghetti, Markus Kostrzewa, Roland Quentin

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1 **JCM00175-09 - Revised Manuscript.**

2 **Identification of *Streptococcus agalactiae* isolates from various phylogenetic**
3 **lineages by Matrix-Assisted Laser Desorption/Ionization -Time of Flight Mass**
4 **Spectrometry**

5 Marie-Frédérique Lartigue^{1,2}, Geneviève Héry-Arnaud¹, Eve Haguenaer¹, Anne-
6 Sophie Domelier^{1,2}, Pierre-Olivier Schmit³, Nathalie van der Mee-Marquet^{1,2}, Philippe
7 Lanotte¹, Laurent Mereghetti¹, Markus Kostrzewa⁴, and Roland Quentin^{1,2}.

8

9 ¹ Université François-Rabelais de Tours, UFR de Médecine, EA 3854 « Bactéries et
10 risque materno-foetal », Institut Fédératif de Recherche 136 « Agents Transmissibles et
11 Infectiologie », 37032 Tours Cedex, France

12 ² Service de Bactériologie et Hygiène Hospitalière, Hôpital Trousseau, CHRU de Tours,
13 37044 Tours, France.

14 ³ Bruker Daltonique, 34 rue de l'Industrie, 67166 Wissembourg Cedex, France

15 ⁴ Bruker Daltonik GmbH, Permoser Str. 15, D-04318 Leipzig, Germany

16

17 **Corresponding author. Mailing address :**

18 Marie-Frédérique Lartigue

19 Service de Bactériologie-Hygiène

20 CHRU Trousseau

21 37044 Tours cedex 9

22 France.

23 Phone : 33 2 47 47 81 13

24 Fax : 33 2 47 47 85 30

25 E-mail : lartigue@med.univ-tours.fr

26 Abstract

27 Variations in proteins related to bacterial diversity may affect species
28 identification performed using matrix-assisted laser desorption/ionization-time of flight
29 mass spectrometry (MALDI-TOF MS). Using this method, we identified 110
30 *Streptococcus agalactiae* isolates characterized by serotyping and MLST. Serotype III
31 and ST23 strains expressed the widest variations in molecular weights of putative
32 “species-identifying” biomarker ions. Recognition of the diversity of MALDI patterns
33 observed in strains that represent all major intraspecies lineages assists in the
34 constitution of an optimal reference database.

35 *Streptococcus agalactiae* is the main cause of neonatal infections and has
36 emerged as an increasingly frequent pathogen in non-pregnant humans (15). Several
37 studies have identified diversity in *S. agalactiae* species, first by serotyping, and then
38 extensively by multilocus enzyme electrophoresis (MLEE) (12, 13). A large number of
39 different Sequence Types (STs) distributed over several major phylogenetic lineages or
40 clonal complexes (CC) have recently been identified by multilocus sequence typing
41 (MLST) (<http://pubmlst.org/sagalactiae/>). STs 17 and 19 account for the majority of
42 cases of *S. agalactiae* meningitis in infants (7). CCs 1, 12, 17, 19 and 23 are mostly
43 associated with infections in adults (7).

44 Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
45 (MALDI-TOF MS) has emerged as a new technique for species identification. By
46 measuring the exact molecular masses of many peptides and small proteins, it is
47 possible to determine the species within a few minutes, whether the analysis is started
48 with whole cells, cell lysates, or crude bacterial extracts (4, 6, 9, 11). Nevertheless, wide
49 variations in protein expression have been reported, specifically according to the
50 distribution of strains in various phylogenetic lineages that make up many species. For
51 example, we have shown such variations in the expression of metabolic enzymes (12,
52 13), in catabolic functions (3), in the expression of surface-exposed bacterial proteins
53 involved in the adhesion and/or invasion of host cells (14). In addition, the diversity of
54 the rRNA gene region found in *S. agalactiae* species (2) may induce variations in the
55 expression of the ribosomal proteins detected by MALDI-TOF MS, a method based
56 mainly on the detection of ribosomal protein fractions of bacteria (1, 4, 11).

57 The aim of this study was therefore to determine whether variations in protein
58 expression related to the phylogenetic position of strains affect the results obtained by
59 MALDI-TOF MS when used to identify *S. agalactiae* isolates. One hundred and ten

60 strains were selected from an epidemiologically unrelated national collection (10, 13)
61 isolated from the vagina, the anatomical site at which the genetic diversity of strains is
62 the highest. As usually performed in medical laboratories, strains were plated on 5%
63 sheep's blood agar (laboratoire bioMérieux, Marcy l'Etoile, France). Serotyping was
64 performed with a Pastorex rapid latex agglutination test (Bio-Rad, Hercules, Calif.) and
65 by a previously reported PCR serotype identification method (8). Forty-five isolates
66 were from serotype III (40.9% of isolates), 17 from serotype II (15.5%), 16 from
67 serotype V (14.5%), 15 from serotype Ia (13.6%), 7 from serotype Ib (6.4%), and 7
68 from serotype IV (6.4%). Three isolates were not typeable (2.7%). MLST analysis,
69 carried out with the standard MLST scheme (7), identified 38 STs for the 110 strains
70 (Fig. 1). The relationships between STs were defined by UPGMA analysis and
71 represented as a tree generated from allelic profile data using Phylodendron
72 (<http://pubmlst.org/sagalactiae/>) (Fig. 1). By adding together data from serotyping and
73 MLST, 52 patterns were obtained that represent the wide diversity of the *S. agalactiae*
74 population studied and the major STs and CCs (Fig. 1).

75 For MALDI-TOF-MS analysis, cell extracts were prepared using ten colonies for
76 each preparation. Samples were prepared according to the microorganism profiling
77 « ethanol/formic acid extraction » procedure as recommended by the manufacturer, with
78 minor modifications as recently described (1). After drying and addition of a chemical
79 matrix, the samples were analyzed by MALDI-TOF MS on a Bruker Ultraflex
80 TOF/TOF III in positive linear mode (Bruker Daltonique, Wissembourg, France). The
81 spectra were recorded as recently described (1). For each spectrum, 500 laser shots were
82 collected and analyzed. For automated data analysis, raw spectra were processed using
83 the MALDI Biotyper 1.1 software (Bruker Daltonique, France) with default settings. To
84 identify bacteria, the peak lists generated were used for matches against the initial

85 MALDI Biotyper reference library (*S. agalactiae* ATCC 27956 THL and *S. agalactiae*
86 DSM 2134) directly using the integrated pattern-matching algorithm of the software (1).
87 In a typical analysis of *S. agalactiae* strains by MALDI-TOF MS, 70 prominent ion
88 peaks were noted in the spectra in the region between 2,000 and 20,000 Da, the highest-
89 intensity peaks being consistently in the range of 3,000 to 10,000 Da. The log(score) of
90 the MALDI Biotyper pattern matching algorithm is calculated according to the log of
91 the product of three factors: the matches of the unknown spectrum against the reference
92 spectrum in the database (main spectrum), the reverse matches of the main spectrum
93 with the unknown spectrum, and the correlation of relative intensities of the unknown
94 spectrum and the main spectrum. The product has a maximum value of 1000, leading to
95 a maximum log(score) of 3. Differences in the distribution of the strains in log (score)
96 groups were tested by the Chi² test (STAT-ITCF software, Paris). A p value < 0.05 was
97 considered to indicate statistical significance.

98 On this basis, the log (score) values obtained by MALDI-TOF MS correctly
99 identified all 110 *S. agalactiae* isolates at the species level [log (score), ≥ 2.0]; 86 of the
100 110 (78%) being identified with excellent scores [log (score), ≥ 2.3] (Fig. 1, Table 1).
101 Nevertheless, significant variations in the log (score) were observed according to
102 serotypes and major STs. Indeed, excellent scores [log (score) ≥ 2.3] were obtained
103 significantly less frequently (64%) for serotype III strains than for the other strains (73
104 to 100%) (Table 1) ($p = 0.0027$). Similarly, excellent scores [log (score), ≥ 2.3] were
105 obtained less frequently for ST23 strains (43%) than for the other major STs (77 to
106 88%) (Table 1) (Chi² test, $p = 0.025$).

107 To test the reproducibility of the method, five strains representative of the five
108 main STs (ST1, ST10, ST17, ST19, and ST23) were grown in quintuplicate and
109 analyzed. The mass spectrum was measured five times for each replicate of each of the

110 five representative strains of main STs (125 measurements). The log(score) calculated
111 using the mass spectra data allowed determination of the coefficient of variation,
112 $CV=\delta/\mu$ in which δ is the standard deviation and μ is the average. The CV for intra runs
113 (5 measurements for each replicate) varied from 0.006 to 0.025 and the highest CV for
114 inter runs (5 different replicas) was 0.027. Moreover, reproducibility tests were also
115 performed by cultivation of bacteria on different culture media (5% sheep blood agar,
116 CPS ID 2®, chocolate PVX agar (bioMérieux), Granada® (Biolys, Taluyers, France),
117 and Mueller Hinton agar). A strain of GBS ST17 was grown in triplicate on each
118 medium. All the log(score) were above 2.3. The low values of the intra runs and inter
119 runs CV and the low variations in the scores obtained using various culture media
120 indicate that the reproducibility of the method was high. Thus the variations found
121 between populations of various serotypes or STs could not be related to the procedure
122 used for preparing the samples.

123 This study confirmed variations in expression of proteins according to the
124 distribution of strains in serotypes and in various phylogenetic lineages that make up *S.*
125 *agalactiae* species. Strains of serotype III and of the phylogenetic lineage ST23
126 expressed greater variations in the molecular weights of putative “species-identifying”
127 biomarker ions than strains of other serotypes and STs. The ability to produce an intense
128 peak in the protein pattern correlates with the ionization efficiency, combined with the
129 protein quantity used. The variations observed for serotype III strains may be explained
130 by the greater genetic diversity of this serotype population compared to the other
131 serotype populations, as shown in Figure 1 and as previously described (7, 12, 13). As
132 shown previously, CC23 (that mainly contains ST23 strains) is genetically a highly
133 divergent clone in the species (Fig. 1) (5, 7, 16). This characteristic may explain why
134 ST23 strains express differences in the nature or quantities of proteins when compared

135 to other major ST populations that are genetically more closely related. These variations
136 did not affect the ability of MALDI-TOF MS combined with the MALDI Biotyper
137 software method to identify *S. agalactiae* at a species level; log (score) values were ≥ 2.0
138 for all strains. Nevertheless, to optimize the ability of MALDI-TOF MS to identify *S.*
139 *agalactiae* strains whatever their phylogenetic origin, we selected five strains to
140 constitute a new reference database on the basis of the variations observed for each
141 lineage. These strains were retained because they generated the best representative
142 spectra for each major intraspecies lineage (ST1, ST10, ST17, ST19, and ST23). The
143 log (score) values obtained by using the new reference database identified 109 of the
144 110 *S. agalactiae* isolates (>99%) with a higher log (score) (≥ 2.3) (Fig. 1, Table 1).

145 In conclusion, genetic diversity between bacterial species affects the diversity of
146 MALDI patterns observed when using MALDI-TOF MS. Consequently, the most
147 effective performance for identification of bacterial strains at the species level was
148 obtained by using a reference database designed, not by selecting one or two strains
149 randomly, but by choosing them from the major phylogenetic lineages that represent the
150 species studied.

151

152 Conceived and designed the experiments: MFL, RQ. Performed the experiments: MFL,
153 GH, EH, ASD, NVDM, PL, LM (serotyping and MLST), MFL, POS (MALDI-TOF
154 MS). Analyzed the data: MFL, MK, RQ. Wrote the paper: MFL, RQ.

155

156

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224 margin, and genital body sites. J. Clin. Microbiol. **46**:2906-2911

225 **Figure Legend**

226

227 Figure 1. MLST UPGMA tree of 110 *S. agalactiae* strains used to test MALDI-TOF
228 MS for identification of *S. agalactiae* strains and log (score) obtained for each strain
229 analyzed.

230 ^a The MALDI-TOF MS analysis calculates the log (score) that allows classification of
231 the results of identification in four categories: i) a log (score) ≥ 2.3 indicates a highly
232 probable level of species identification, ii) a log (score) ≤ 2.299 and ≥ 2.0 indicates a
233 highly probable level of genus identification and probable species identification, iii) a
234 log (score) ≤ 1.999 and ≥ 1.7 indicates a probable genus identification and iv) a log
235 (score) ≤ 1.699 indicates non-reliable identification. Whatever the phylogenetic origin of
236 the strain tested, no log (score) ≤ 1.999 was found.

237 ^b Initial MALDI Biotyper reference library consisted of main spectra of *S. agalactiae*
238 ATCC 27956 THL and *S. agalactiae* DSM 2134.

239 ^c New reference library consisted of the 5 newly created mass spectra for *S. agalactiae*
240 strains representative of each major intraspecies lineage (ST1, ST10, ST17, ST19, and
241 ST23).

242 Table 1. Identification scores produced by the pattern-matching algorithm against the
 243 initial MALDI Biotyper reference library (main spectra of *S. agalactiae* ATCC 27956
 244 THL and *S. agalactiae* DSM 2134) and against a new reference library (mass spectra of
 245 five *S. agalactiae* strains selected to represent each major intraspecies lineage ST1,
 246 ST10, ST17, ST19, and ST2) for 110 *S. agalactiae* strains, according to serotypes and
 247 major intraspecies STs.

	Log(score)			
	Initial reference library		New reference library	
<i>S. agalactiae</i>	≥ 2.3	$< 2.3 - \geq 2.0$	≥ 2.3	$< 2.3 - \geq 2.0$
strains (N)	N (%)	N (%)	N (%)	N (%)
All (110)	86 (78)	24 (22)	109 (99)	1 (1)
Serotype Ia (15)	11 (73)	4 (27)	15 (100)	0
Serotype Ib (7)	7 (100)	0	7 (100)	0
Serotype II (17)	15 (88)	2 (12)	17 (100)	0
Serotype III (45)	29 (64)	16 (36)	44 (98)	1 (2)
Serotype IV (7)	7 (100)	0	7 (100)	0
Serotype V (16)	15 (94)	1 (6)	16 (100)	0
ST1 (16)	13 (81)	3 (19)	16 (100)	0
ST10 (8)	7 (88)	1 (12)	8 (100)	0
ST17 (15)	12 (80)	3 (20)	15 (100)	0
ST19 (13)	10 (77)	3 (23)	13 (100)	0
ST23 (7)	3 (43)	4 (57)	7 (100)	0

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