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Polysaccharide capsule composition of pneumococcal serotype 19A subtypes: Unaltered among subtypes and independent of the nutritional environment

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Running title: 19A capsule subtypes structures

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26 **ABSTRACT**

27 Serotype 19A strains have emerged as a cause of invasive pneumococcal disease after the
28 introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) and serotype 19A
29 has now been included in the recent thirteen-valent vaccine (PCV13). Genetic analysis has
30 revealed at least three different capsular serotype 19A subtypes and nutritional environment
31 dependent variation of the 19A capsule structure has been reported. Pneumococcal vaccine
32 effectiveness and serotyping accuracy might be impaired by structural differences in
33 serotype 19A capsules. We therefore analyzed the distribution of 19A subtypes collected
34 within a Swiss national surveillance program and determined capsule composition in
35 different nutritional conditions with high-performance liquid chromatography (HPLC), gas
36 chromatography – mass spectrometry (GC-MS) and nuclear magnetic resonance
37 spectroscopy (NMR). After the introduction of PCV7 a significant relative increase of subtype
38 19A-II and decrease of 19A-I occurred. Chemical analyses showed no difference in the
39 composition as well as the linkage of 19A subtype capsular saccharides grown in defined
40 and undefined growth media being consistent with a trisaccharide repeat unit composed of
41 rhamnose, N-acetyl-mannosamine and glucose. In summary, our study suggests that no
42 structural variance dependent of the nutritional environment or the subtype exists. The
43 serotype 19A subtype shift observed after the introduction of the PCV7 can therefore not be
44 explained by selection of a capsule variant. However, capsule composition analysis of
45 emerging 19A clones is recommended in cases where there is no other explanation for a
46 selective advantage such as antibiotic resistance or loss or acquisition of other virulence
47 factors

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INTRODUCTION

The polysaccharide capsule is a major virulence factor of the human pathogen *Streptococcus pneumoniae* (pneumococcus) and more than 90 different capsular types are known today, which differ in the chemical structure of their capsular polysaccharides (1). These differences are reflected in the type-specific reaction with anticapsular antibodies, by which a serotype is determined and cross-reactive serotypes are pooled into serogroups. The serogroup 19 contains, among others, serotypes 19F and 19A, which belong to the clinically most relevant serotypes (2). Based on both genetic background and chemical analyses the serotype 19A and 19F oligosaccharide repeating unit structures have been determined to be trisaccharides of glucose (Glc), rhamnose (Rha) and N-acetyl-mannosamine (ManNAc), differing only in the glycosidic linkage between glucose and rhamnose:

19F: →2)-α-L-Rha-(1→PO_4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→

19A: →3)-α-L-Rha-(1→PO_4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ (1, 3-5).

Based on chemical analyses two types of 19A oligosaccharide structures have been described (6, 7). In addition to the genetically proposed structure above, an alternative with a serotype 19F backbone and two side chains of β-D-GlcNAc-(1→3)-β-D-Gal- (1→P→2) and α-L-Fuc-(1→P→3) has been reported based on chemical analysis (7). The polysaccharide structures appear to vary with different *in vitro* growth conditions (6). Influence of the nutritional environment on the pneumococcal polysaccharide capsule could have biological consequences, as this would potentially impair any intervention or test targeting the pneumococcal capsule. For example, the fungus *Cryptococcus neoformans* is known to be able to change the capsule structure in vitro and also during infection (8-11), and those changes have been shown to lead to altered antigenicity (8, 11).

Because polysaccharides for pneumococcal vaccine production are derived from *in vitro* cultures, a nutrient-dependent variation could lead to antigenic preparations which differ from the *in vivo* antigen.

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76 Capsule variation could also impair diagnostic procedures such as classical serotyping,
77 which is based on polysaccharide specific antigen-antibody reaction.

78 Serotype 19A strains have emerged after the introduction of the seven-valent pneumococcal
79 vaccine, and were subsequently included in the thirteen-valent vaccine now recommended
80 in most countries (12, 13). The emergence of serotype 19A after the introduction of the
81 seven-valent vaccine was surprising, as a cross-protection was expected due to close
82 chemical similarity to the serotype 19F capsule as it was observed for serotypes 6A and 6B
83 which also differ only by one glycosidic linkage (14, 15). Furthermore, 19F-19A cross-
84 protection had been observed to a certain degree in an animal model (16). Recent work
85 suggests a conformational difference in polysaccharide structure, which might explain the
86 reduced cross-protection (17, 18).
87 At least three different 19A capsule subtypes are known based on the genetic arrangement
88 of the capsule gene locus compared to a reference strain (19). In addition to various SNPs
89 along the capsule operon, most characteristically, subtypes I and II have an inverted rmlD
90 gene, which is the last gene in the rhamnose synthesis pathway (3). To our knowledge, no
91 variations in the capsule structure have been described for different subtypes.

92 Given the recent discovery of 19A capsular subtypes and previous reports of structural
93 variants as well as the introduction of serotype 19A in the 13- valent pneumococcal
94 conjugate vaccine (PCV13) we aimed to determine the epidemiology and capsule
95 composition of different 19A subtypes in different nutritional environments.

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96 **MATERIAL AND METHODS**

97 **Bacterial strains and serotype 19A subtype analysis.** Serotype 19A subtype strains were
98 selected from a Swiss national pneumococcal surveillance program (20). In order to detect
99 the different described 19A subtypes (19) we analyzed 158 pneumococcal serotype 19A
100 isolates derived from the upper respiratory tract of infant and adult outpatients with signs of
101 upper respiratory tract infection (20). Antimicrobial susceptibility for penicillin,
102 sulfamethoxazole / trimethoprim (SXT) and erythromycin was determined as previously
103 described (20). In brief, minimum inhibitory concentration (MIC) for penicillin non-
104 susceptibility was ≥ 0.06 mg/L while for erythromycin and SXT the disk diffusion method was
105 performed (intermediate and resistant were considered as non-susceptible). A two-step PCR
106 protocol was used to determine the 19A subtype of each strain using the following
107 conditions. The first PCR was done with two primer pairs: rmlb_1_f -GAT GGT GAG AAG
108 AAC AAT AAG; rmlb_2_f - GAC GGT GAG AAG AAC AAC AAG; rmld_1_r- CTT CAT TAC
109 GTT CAT CCA ATA and rmld_2_r CAG CTG AAG ACA CCA CTT GGT. PCR conditions
110 were initial heat activation 6 min 95°C, 30 cycles of 30s 95°C, 20s of 60°C and 90s of 72°C
111 with a final extension of 5 min 72°C. The reaction mixture contained 2.5 µL of FastStart *Taq*
112 reaction buffer without $MgCl_2$, 2.7 µL of 25 mM $MgCl_2$ stock solution, 4 µL of 1.25 mM
113 deoxynucleoside triphosphates (0.2 mM final), 0.2 µL (1 U) of FastStart *Taq* polymerase (all
114 from Roche Molecular Biochemicals, Rotkreuz, Switzerland), and 0.25 µL of each primer
115 (100 µM stock resulting in 1 µM final concentration, Microsynth AG, Balgach, Switzerland) in
116 a total volume of 25 µL. PCR products were visualized on 1% agarose gels. A resulting PCR
117 product of 560 bp indicated subtypes 19A-I and 19A-II whereas a band of 425 bp indicated
118 subtype 19A-III. For isolates within the first group (19A-I/19A-II), a second PCR reaction was
119 performed to discriminate between the two groups using the following primers: wzg_2_f -
120 AGT TGA TTC GTC CAT CCA CAC T; wzg_3_f -GGA ATT GAC ACA TAT GGT CCT and
121 wzh_r -GCC AAG AGA GCC TTG CTT TCC. The resulting PCR products were 654 bp and
122 833 bp for types 19A-I and 19A-II, respectively.

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123 Strains were further characterized with *plyNCR*-RFLP as previously described (21, 22). We
124 selected the following strains for further analysis of the capsule composition (Table 1):
125 109.44 and 501.14 (subtype 19A-I), 501.24 (subtype 19A-II), and 412.49 (subtype 19A-III).
126 For all selected subtype test strains, multi locus sequence typing (MLST) was determined as
127 previously described (22). In addition, we included the internationally spread Hungary-19A-6
128 strain (23), which was classified as a 19A III. Serotype 19F strains 505.32 and B201.73 were
129 selected for comparison as the polysaccharide repeat unit contains the same backbone
130 monosaccharides but different glycosidic linkages between the repeat units. Both strains are
131 clinical isolates derived from Swiss national surveillance programs (20, 24). In addition a
132 capsule knockout mutant of strains B201.73 was generated as previously described (25, 26)
133 to assess the amount of background signal in capsule extracts. Commercially available
134 capsule polysaccharide of serotype 19A from the American Type Culture Collection (ATCC,
135 Molsheim Cedex, France) was used as a reference standard.

136 Chi-Square and Fisher exact tests were used to calculate p values for epidemiological
137 analyses. A value of $p \leq 0.05$ was considered significant. In addition, we used a multivariate
138 logistic regression model to ascertain the strength of the association between PCV7 era and
139 19A subtypes, and adjusted for potential confounders like age (0-1 (base), 2-4, 5-15 and >15
140 years), sex (male gender as base), penicillin resistance (susceptible chosen as base),
141 erythromycin resistance (susceptible chosen as base), SXT resistance (susceptible chosen
142 as base) and geopraphical origin (east Switzerland as base). Adjusted odds ratios (aOR)
143 with 95% confidence intervals (95%CI) were received (table S1). Trends over time in the
144 prevalence of different 19A subtypes before the introduction of PCV7 were analyzed using
145 linear regression.

146 **Growth conditions, polysaccharide purification, hydrolysis and high performance**
147 **liquid chromatography (HPLC).** Strains were handled and grown as described previously
148 (25). The undefined growth medium pneumococcal inoculation medium (PIM) which has
149 been reported to alter 19A capsule composition (6), as well as a chemically defined medium

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150 (CDM) by van den Rijn and Kessler (27) were used. CDM was supplemented with 5 mg/L
151 choline chloride (28), but made without monosaccharides to allow modification of the type
152 and concentration of the carbon source for each experiment. Monosaccharides were added
153 to the CDM and the mixture was sterile filtrated using a 0.22 µm filter unit (TPP,
154 Trasadingen, Switzerland). Two forms of CDM were created, one supplemented with 55 mM
155 Glucose and the other one mimicking the composition of the salivary mucin MG1, i.e.: N-
156 aceteylneuraminic acid (NeuNAc):fucose (Fuc):galactose (Gal):N-acetylglucosamine
157 (GlcNAc):N-acetylgalactosamine (GalNAc) = 1:5:4:3:1 (29)). For one experiment pooled
158 human saliva from ten healthy volunteers was collected using Salivette (Sarstedt,
159 Nümbrecht, Germany). After centrifugation with 1500 x g for 2 minutes samples were pooled
160 and sterile filtrated with 0.22µm centrifugal filter units (Millipore, Billerica, MA) at 5000 x G for
161 20 minutes as previously described (30).
162 Capsules of strains grown in different growth media were released by overnight incubation in
163 1% phenol, separated from the bacteria by centrifugation and filtration, and then purified with
164 sodium acetate/ethanol precipitation, followed by protease and nuclease digestion of
165 remaining contaminants, and finally cut-off filtrated as described (25). The extracted
166 polysaccharides were then completely hydrolyzed by trifluoroacetic acid (TFA) (31). The
167 monosaccharide composition of capsular polysaccharides of strains grown in CDM was
168 determined by high performance liquid chromatography (HPLC) analysis of fluorescently
169 labelled monosaccharides as previously described (25, 32-35), whereas the PIM extracts
170 were analyzed on a system consisting of an ASI-100 autosampler and P680 HPLC pump
171 (DIONEX, Sunnyvale, CA, USA) with an injection volume of 20 µL per sample. Separation of
172 the monosaccharide was done with a flow rate of 0.85 mL/min as follows: 6% solvent B
173 isocratic for 35 minutes followed by a linear gradient from 6 to 12% solvent B over 20
174 minutes. Then, the column was washed with 100% solvent B for 10 minutes and 100%
175 solvent A for 15 minutes followed before re-equilibrating the system with 6% solvent B for 10
176 minutes. Total run time was 90 minutes and data was collected for 55 minutes using an L-
177 7480 Fluorescence Detector (Merck Hitachi, Darmstadt, Germany). A Luna 5 mm, C18

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178 column (Phenomenex, Torrance, CA) was used for separation and column temperature was
179 maintained at 24 °C using a TCC-100 column oven (Thermo Scientific Dionex, Reinach,
180 Switzerland). Peaks were identified by comparing retention time with monosaccharide
181 standards analyzed in the same run (Figure S1A). As N-acetylated aminosugars are
182 deacetylated during hydrolysis with TFA (36), deacetylated aminosugars were used as
183 standards for their acetylated counterpart (e.g., mannosamine for N-acetyl-mannosamine).
184 Negative controls included in each experiment were medium negative control for growth,
185 extraction, hydrolysis and HPLC. HPLC raw data was exported into GraphPad Prism
186 (Version 5, GraphPad Software, Inc.) for creating figures.

187 **Gas chromatography – mass spectrometry (GC-MS).** GC-MS analysis of alditol acetates
188 of the polysaccharide hydrolysates from strains 109.44 grown in PIM and 501.24 grown in
189 PIM, pooled saliva, and CDM glucose was performed as a control, as previously described
190 (37, 38).

191 **Nuclear magnetic resonance spectroscopy (NMR).** NMR data were collected on a Bruker
192 Avance II (500 MHz; ^{1}H) spectrometer equipped with a 1.7 mm triple-resonance (^{1}H, ^{13}C,
193 ^{31}P) microprobe head. The samples were extracted as described above and prepared as
194 follows: The full amount of each capsule extract (~4 – 5 mg) was dissolved in 50 µl of D_2O
195 and 40 µl of the resulting mixtures were transferred into 1.7 mm NMR tubes. The water
196 resonance was suppressed using a classical presaturation scheme. HSQC spectra were
197 collected on a Bruker Avance III HD (600 MHz) spectrometer equipped with an inverse 5mm
198 TCI helium cryoprobe. All spectra were acquired at a regulated temperature of 298 K and
199 calibrated to the residual water peak (4.766 ppm). For the interpretation of the received
200 carbon and hydrogen shifts, results from previous studies were used as guidance for the
201 capsule structure determination of the 19A capsule extracts (4, 39, 40).
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204 **RESULTS**

205 **Epidemiology of non-invasive 19A subtypes.** We first aimed at analyzing the distribution
206 of the different pneumococcal serotype 19A subtypes within the Swiss Sentinel Network
207 collection of upper respiratory tract isolates. PCV7 had been recommended in Switzerland
208 since late 2005 to all children under the age of 24 months in a three dose schedule given at
209 2, 4 and 12 months and since August 2006 the vaccine has been fully reimbursed by the
210 mandatory Swiss health insurance. Serotype 19A strains isolated from the upper respiratory
211 tract between 1998 and 2011 were analyzed. In total, 158 19A isolates were screened for
212 the three described subtypes using a two-step PCR protocol described above. Between
213 1998 and 2005, we identified 30 (57.7%), 5 (9.6%) and 17 (32.7%) isolates belonging to
214 subgroups 19A-I, 19A-II, and 19A-III, respectively. After 2006 until 2011, a significant relative
215 difference was noted overall (p=0.02) but also individually for the subgroups 19A-I (p=0.02)
216 and 19A-II (p=0.02) as 40 (37.7%), 28 (26.4%) and 38 (35.8%) isolates were detected with
217 the 19A-I, 19A-II, and 19A-III subtypes, respectively (Figure 1A). There was still a significant
218 shift even after data from the PCV7 introduction year (2006) were excluded (p=0.03). We
219 found no evidence for a difference of 19A subtypes distribution according to age (Pre versus
220 post introduction of PC7 eras over age groups of ≤1, 2-4, 5-15 and >15 years; Fisher's exact
221 test; p= 0.3). In addition, there was no indication of a time trend for the frequency of 19A
222 subtypes before the PCV7 was present (Data not shown). As for molecular types derived by
223 *plyNCR*-RFLP typing, *plyNCR*-RLPF types 1 (55.7%) and 16 (18.4%) were the most
224 frequent within the 158 strains but we did not detect a significant shift of molecular types
225 between pre and post PCV7 introduction era (Figure 1B). In addition we revealed that
226 antibiotic resistances within the 19A strains were generally high and that 19A II is
227 significantly more susceptible towards penicillin, erythromycin, and SXT as compared to the
228 other subtypes (Figure 1C). However, calculating a multivariate logistic regression model we
229 confirmed that there was strong evidence that, compared to 19A I, the odds of observing
230 subtype 19A II after introduction of PCV7 was 6 times higher than before PCV7, and that this

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231 association was independent of antimicrobial resistance, geographical region, age or sex
232 (p = 0.005; **Table S1**).

233 We then selected at least one strain of each subtype for further laboratory analysis to
234 determine capsular composition of the different subtypes (Table 1). Selected subtype strains
235 represented different genetic backgrounds as indicated by MLST and *plyNCR*-RFLP
236 analysis (Table 1).

237 **Method validation.** To assess the degree of contamination of capsule extracts with cell wall
238 and other components, we first compared the monosaccharide composition of hydrolyzed
239 capsule extracts of a serotype 19F clinical isolate B201.73 with extracts of its isogenic
240 capsule knockout mutant (B201.73Δcps) by HPLC with fluorescence detection (Figure S1B).
241 No rhamnose peaks were detected for the strain without a capsule, but small amounts of
242 glucose and higher amounts of amino sugars including mannosamine could be detected,
243 most likely deriving from cell wall polysaccharide (CWPS) and from murein layer.
244 Furthermore, hydrolysis with TFA is known to produce varying amounts of hydrolysis-derived
245 (di)saccharides (Figure S1B). This contamination was usually present in capsule extracts
246 from tested strains and was higher in amount as compared to the commercially available
247 purified pneumococcal polysaccharide (Figure 2). Furthermore, after complete hydrolysis a
248 linkage analysis is not possible and only monosaccharide determination can be done (e.g.,
249 same chromatogram for serotype 19A and 19F). Therefore, we used the HPLC method only
250 as a screening tool for additional neutral monosaccharides under different growth conditions
251 (i.e. galactose and fucose).

252 **Capsule composition analysis of 19A subtypes by HPLC and GC-MS.** We then analyzed
253 the capsule composition of a strain for each subgroup (109.44 and 501.14 (subtype 19A-I),
254 501.24 (subtype 19A-II), and Hungary-19A-6 and 412.49 (subtype 19A-III) grown in CDM
255 supplemented with 55 mM glucose. We did not detect additional neutral monosaccharides
256 and comparison of the used isolates revealed no differences among the strains indicating

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257 the same monosaccharide backbone built of glucose, rhamnose and (N-acetyl)-
258 mannosamine. As expected, HPLC chromatograms were not able to differentiate 19A from
259 19F as both serotypes contain the same monosaccharide compositions (Figure 2A). We
260 then analyzed the capsular extracts from the ATCC standard (Figure 2B) and from 19A
261 subtypes grown in the undefined medium PIM (Figure 2C). Again, no additional neutral
262 monosaccharides were detected and thus reported additional monosaccharides (galactose
263 and fucose would have been expected on the neutral side based on the literature (6)) were
264 not seen in our preparations. As HPLC allows only the identification of peaks based on their
265 retention time in comparison to standards, the presence of the neutral monosaccharides in
266 capsule extracts was confirmed for 109.44 (19A-I) grown in CDM with 55 mM Glucose
267 (Figure S3A) and PIM (Figure S3B) and 501.24 (19A-II) grown in PIM (Figure S3C) with GC-
268 MS (which revealed glucose and rhamnose in all preparations). GC-MS also confirmed a
269 lower degree of contamination with cell wall and other compounds in the ATCC standard
270 compared to subtype capsule extracts (Figure S3D).
271 To mimic saccharide nutrients present in the natural human environment of *S. pneumoniae*,
272 109.44 (19A-I) was grown in CDM with monosaccharides contained in human mucin (5.5
273 mM total concentration of the mucin building monosaccharides in ratios as determined for
274 the salivary mucin MG1) and 501.24 (19A-II) was also grown in pooled human saliva
275 collected from 10 healthy volunteers after sterile filtration. No additional neutral
276 monosaccharides were identified to be present in the polysaccharide capsule extracted from
277 the strains grown under these conditions compared to capsule extracted from strains grown
278 in CDM supplemented with glucose or PIM but the signal to noise ratio was much lower in
279 saliva grown capsule (Figure S2 and S3E).

280 **NMR analyses reveal no structural difference between subtypes.**

281 In order to confirm the proposed structures of the oligosaccharides we compared the
282 anomeric region (4.8-6.0 ppm) in the ^{1}H-NMR of all strains grown in CDM supplemented with
283 55mM glucose (Figure 3A). We were able to clearly differentiate the capsule of serotype 19A

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284 strains from 19F capsule. Comparison of carbon and hydrogen shifts with previously
285 reported NMR spectra confirmed the following structures for serotype 19F: →2)-α-L-Rha-
286 (1→PO4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ and for serotype 19A: →3)-α-L-Rha-
287 (1→PO4→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→ . In addition, no differences among the
288 serotypes 19A subtypes were detected. We then performed an identical ^{1}H-NMR analysis of
289 all strains grown in PIM which has been reported to alter the composition of 19A capsule (6).
290 The spectra were again consistent within 19A subtypes but different from serotype 19F
291 capsular polysaccharide (Figure 3A). Based on those results, it can be stated that the
292 capsule composition is independent of tested growth medium as the patterns were identical
293 to each other and to the ATCC reference 19A polysaccharide. Only a slight shift was
294 observed for the capsule analysis of the Hungary 19A-6 strain. However, the overlay of the
295 2D ^{1}H-^{13}C HSQC-NMR spectra of this isolate grown in PIM and CDM supplemented with
296 glucose revealed identical patterns for the H-atoms of the anomeric region as illustrated for
297 rhamnose, N-acetyl mannosamine and glucose (Figure 3B). The full 2D Spectrum is shown
298 in Figure S4.
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300 **DISCUSSION**

301 In this study we demonstrated that the distribution of pneumococcal serotype 19A subtypes
302 changed after the implementation of PCV7 in Switzerland. However, all analyzed capsular
303 extracts from serotype 19A subtypes grown in defined medium CDM and the undefined
304 medium PIM showed a capsule composition which was consistent with the one proposed
305 based on genetic analysis. Furthermore, we did not find any evidence for additional side
306 chains altered repeat units or linkage changes under different nutritional conditions. .

307 During the time of widespread use of PCV7 in Switzerland the serotype 19A subtype
308 distribution changed substantially compared to the pre-vaccine period. Similar shifts with
309 decreasing 19A-I and increasing 19A-II subtypes after the introduction of PCV7 have also
310 been reported in the Netherlands (19). Furthermore, although classified as group III by PCR,
311 based on its genome sequence the Hungary 19A-6 strain contains an additional insertion
312 element downstream of the last cps gene (*rmlD*) and differs from the subgroup III strains as
313 defined by Elberse et al. (19) which could suggest an additional serotype 19A subgroup.

314 Given the identical capsule structure for all tested 19A subtypes as revealed for the first time
315 in our study using NMR, HPLC and GC-MS, no alteration of vaccine effectiveness due to
316 capsular differences between individual subtypes is suggested. Furthermore, the lack of anti-
317 19F antibody cross-protection against serotype 19A can therefore not be explained by
318 selection of 19A subtypes with altered capsule structures which supports recent discoveries
319 suggesting a general low protection due to conformational difference in polysaccharide
320 structures of 19F and 19A (17, 18).
321

322 However, other factors could explain the observed subtype shift after the introduction of
323 PCV7 in Switzerland. For example, changes in 19A subtypes could be due to changes in
324 clonal distribution although we did not detect a significant shift of molecular types as
325 determined by *plyNCR*-RFLP. However, additional typing methods or whole genome

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326 sequencing might be more appropriate to investigate this hypothesis. As for antibiotic
327 resistance being a potential driver for the subtype shifts, we revealed that 19A II is
328 significantly more susceptible to antibiotics as compared to the other subtypes indeed, but
329 as 19A II increases in the PCV7 era, increasing antibiotic resistance can therefore not
330 explain the changes in the distribution, though antibiotic resistance within serotype 19A
331 strains is generally high as previously shown (41). A subtype redistribution under increasing
332 vaccine selection pressure after the introduction of PCV13 would be unexpected, although it
333 has been speculated that there might be a difference in capsule thickness for different
334 subtypes which might lead to differences in opsonophagocytotic susceptibility (19).

335 Previously reported experiments showing additional side chains in 19A isolates grown in PIM
336 could not be replicated (6). An explanation for this finding might be a strain-specific genetic
337 alteration in previous studies. Indeed, it has been recently reported that serotype 11D has
338 two different capsular polysaccharide repeating units in a ratio of 1:3 (25% and 75%,
339 respectively) due to a bispecific transferase WcrL (42). Although precursors of capsular
340 galactose and N-acetyl-glucosamine might be available due to their synthesis in other
341 pathways (43), the (environment-dependent) addition of 2 side chains comprised of three
342 additional monosaccharides is expected to be reflected with at least additional
343 glycosyltransferase enzymes within the capsule locus involved in their linkage, and a switch
344 of the glycosidic linkage between rhamnose and glucose would suggest mutations resulting
345 in bispecificity or two copies of the wzy polymerase (3). To our knowledge, neither of these
346 two possibilities have been detected in 19A strains (3). However, variation of the repeat unit
347 structure would be suspected to have an influence on the antigenicity thus resulting in
348 suspect serotyping results (i.e., less reactive with anti-19A antiserum) which were not
349 observed in this study. However, given the increasing vaccine selection pressure after the
350 introduction of PCV13, we recommend capsule structure determination of emerging clones
351 and/or serotypes given recent discoveries of novel serotypes and capsule variants (42, 44-
352 47). The importance of chemical capsule structure analysis is also highlighted by a recent

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353 analysis of serotype “6E” capsule, demonstrating that this potential new serotype determined
354 at the genetic level produces capsular polysaccharide identical to 6B capsular
355 polysaccharide (48).

356 A particular strength of this study is the use of multiple techniques for capsule structure
357 determination, which enhances the reliability of reported findings. We first used an HPLC
358 based approach to determine if additional neutral sugars were incorporated into the capsule
359 and validated the methodology with GC-MS. As this technique requires complete hydrolysis
360 a linkage analysis was not possible in this first step. Furthermore, although this method has
361 been demonstrated to have high normalized recoveries of neutral monosaccharides
362 rhamnose and glucose of serotype 19F monosaccharides (32), TFA hydrolysis has also
363 been reported to be less satisfactory for polysaccharides with aminosugar moieties (32) and
364 it has been reported that disaccharides can be formed during hydrolysis based on different
365 stability of intramolecular bonds (32) which also occurred in our experiments.
366 Results showed traces of contamination of cell-wall components in our preparations, which
367 is a common co-extracted compound in pneumococcal capsule preparations probably
368 because the capsule is covalently linked to the cell wall (32, 49, 50). To determine linkage
369 analysis and further characterization of the capsule oligosaccharide repeat units, we
370 performed additional 1D and 2D NMR analysis of polysaccharides from unhydrolyzed
371 capsule extracts. The major limitation of this study is that the number of different 19A
372 subtype strains analyzed was rather small. However, we chose at least one sample of each
373 of the currently known subtypes and it can therefore be expected that our study is
374 representative. Although we did not find evidence for nutrition-dependent variations of
375 capsule structures, this cannot be considered as final proof for an absence of such
376 variations. Furthermore, we did not have the same strains or capsule extracts for which a
377 nutrient-dependent variation has been reported as a reference for the assays used (6).

378 In summary, the polysaccharide capsule composition for tested serotype 19A subtypes was
379 consistently composed of the same trisaccharide repeat unit. Although we therefore do not

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380 expect a structural advantage for certain subtypes it remains to be determined how the 19A
381 subtype distribution will be affected by PCV13. We did not detect any nutritional
382 environment-dependent alterations of the capsule composition. However, given the genetic
383 plasticity of *S. pneumoniae* and current vaccine selection pressure, we propose to test the
384 capsule composition of emerging serotype 19A clones, especially in cases where there is no
385 other explanation for a selective advantage such as antibiotic resistance or loss or
386 acquisition of other virulence factors.

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558 **TABLE 1. *Streptococcus pneumoniae* strains used in this study**

ID	Serotype	Subtype*	*plyNCR*-RFLP**	MLST
109.44	19A	19A-I	16	ST 276
501.14	19A	19A-I	1	ST 416
501.24	19A	19A-II	1	ST 199
412.49	19A	19A-III	14	ST 1151
Hungary 19A-6 (ATCC 700673)	19A	19A-III	20	ST 268***
505.32	19F	19F	4	ST 179
B201.73	19F	19F	40	ST 43

559

560 **ND: Not done; MLST: Multi Locus Sequencing Typing; ST: Sequence Type; RFLP:**
561 **Restriction Fragment Length Polymorphism**

562 *** Defined as previous described (19)**

563 **** Done as previously described (21).**

564 ***** According to: http://spneumoniae.mlst.net/sql/fulldetails.asp?id=689**

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569 **FIGURE LEGENDS**

570 **FIGURE 1. A. Epidemiology of 19A subtypes (19A-I, 19A-II, and 19A-III) 1998-2011.** A
571 total of 158 serotype 19A strains isolated within the Swiss Sentinel Network (outpatients with
572 upper respiratory tract infections) were analyzed. 1998-2005 was considered as pre-
573 conjugate vaccine (PCV7) era. The % of tested isolates and subgroup assignment for both
574 periods is shown. There was an overall significance as derived by Fisher exact test
575 (P=0.02). Chi-Square test revealed significant changes in relative frequency between the
576 two periods for subtypes 19A-I (p=0.02) and 19A-II (p=0.02). 95% confidence intervals (CI)
577 are indicated. **B. Distribution of molecular types as determined by *plyNCR*-RFLP 1998-**
578 **2011.** Shown are the most frequent *plyNCR*-RFLP types (Types 1 and 16). The remaining
579 types were pooled within 'others'. There was no overall significance as derived by Fisher
580 exact test for the two different eras. 95% confidence intervals (CI) are indicated. **C.**
581 **Antibiotic resistance of serotype 19A isolates against penicillin, erythromycin, and**
582 **sulfamethoxazole / trimethoprim (SXT), 1998-2011.** Minimum inhibitory concentration
583 (MIC) for penicillin non-susceptibility was ≥ 0.06 mg/L while for erythromycin and SXT the
584 disk diffusion method was performed (intermediate and resistant were considered as non-
585 susceptible. 19A II is significantly more susceptible towards penicillin (Fisher exact test; p<
586 0.001), erythromycin (p= 0.014), and SXT (p< 0.001) as compared to the other subtypes.
587 95% confidence intervals (CI) are indicated.

588 **FIGURE 2. HPLC chromatograms of serotype 19A subtypes and 19F strain B201.73**
589 **grown in CDM and PIM.** HPLC composition analysis of hydrolyzed polysaccharide capsule
590 of clinical isolates of serotype 19A subtypes grown in chemically defined medium with 55
591 mM glucose (A) compared to ATCC purified pneumococcal serotype 19A polysaccharide
592 (B), and the pneumococcal inoculation medium PIM (C). Y-axis shows fluorescence (FU)
593 and chromatograms were stacked to facilitate comparison. Peaks of mannosamine (Man-N),
594 rhamnose (Rha) and glucose (Glc) are labeled.

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595 **FIGURE 3. A. 1D NMR spectra of serotype 19A subtypes.** Shown are 1H NMR spectra of
596 capsular polysaccharide purified from each subtype grown in CDM 55 mM glucose (black)
597 and PIM (red) compared to serotype 19F and ATCC 19A purified pneumococcal
598 polysaccharide. **B. 2D NMR.** Shown is a superimposition of ^{1}H-^{13}C HSQC-NMR spectra of
599 the anomeric region from PS from Hungary 19A-6 capsule grown in CDM and PIM.

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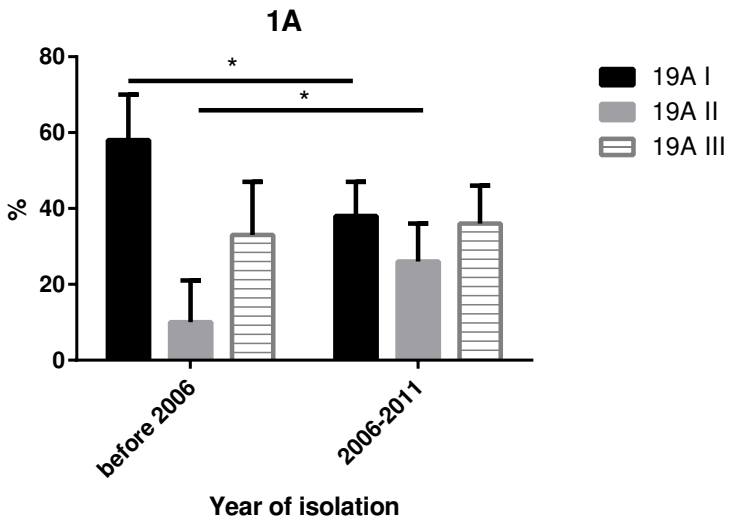

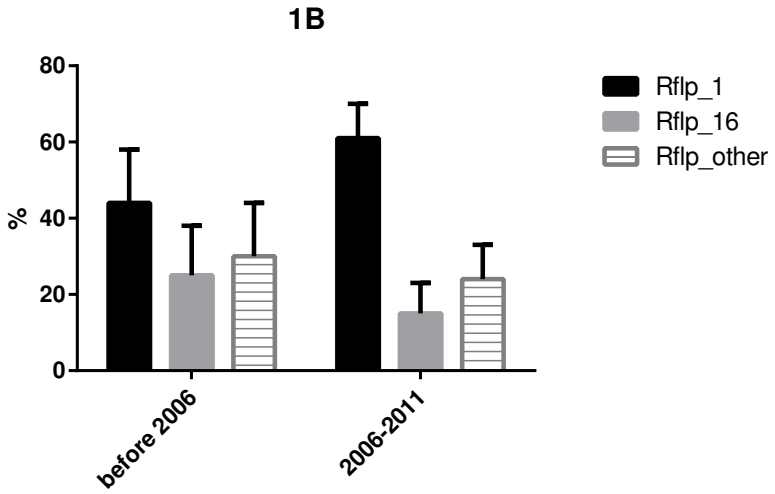

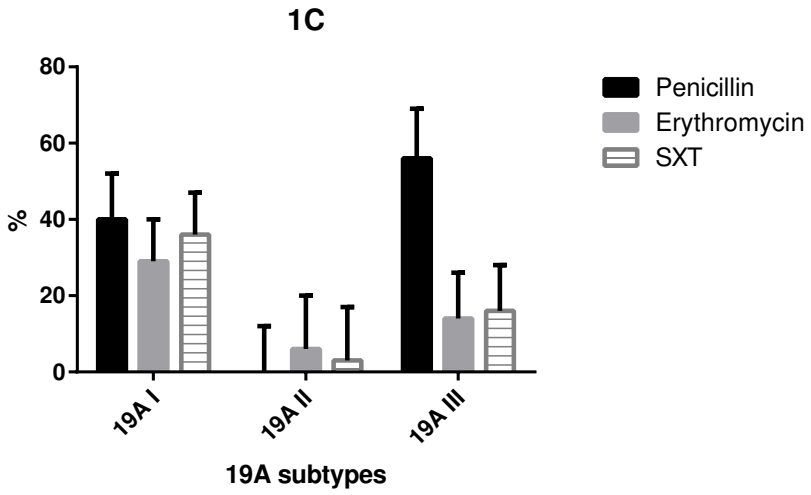

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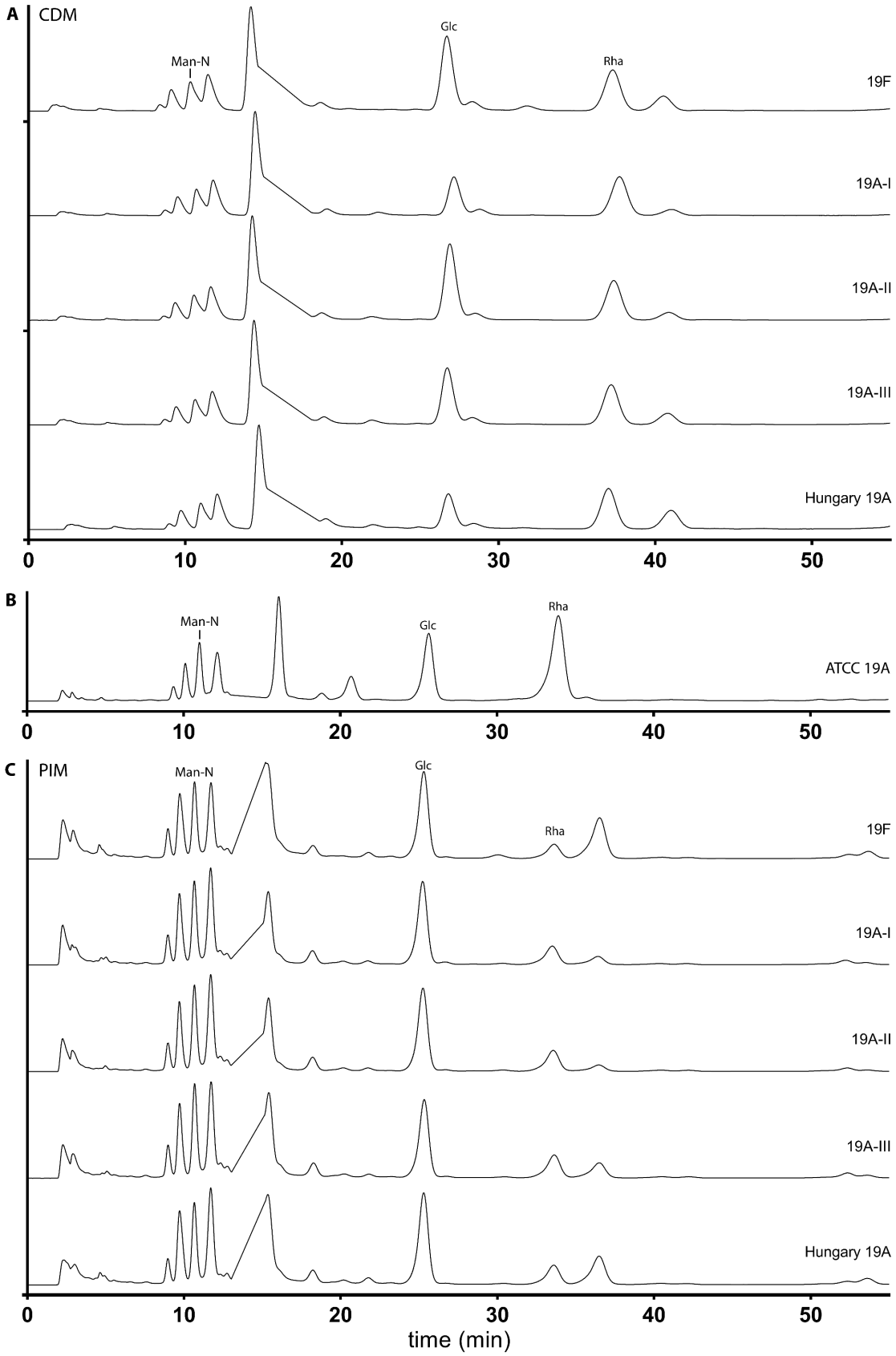

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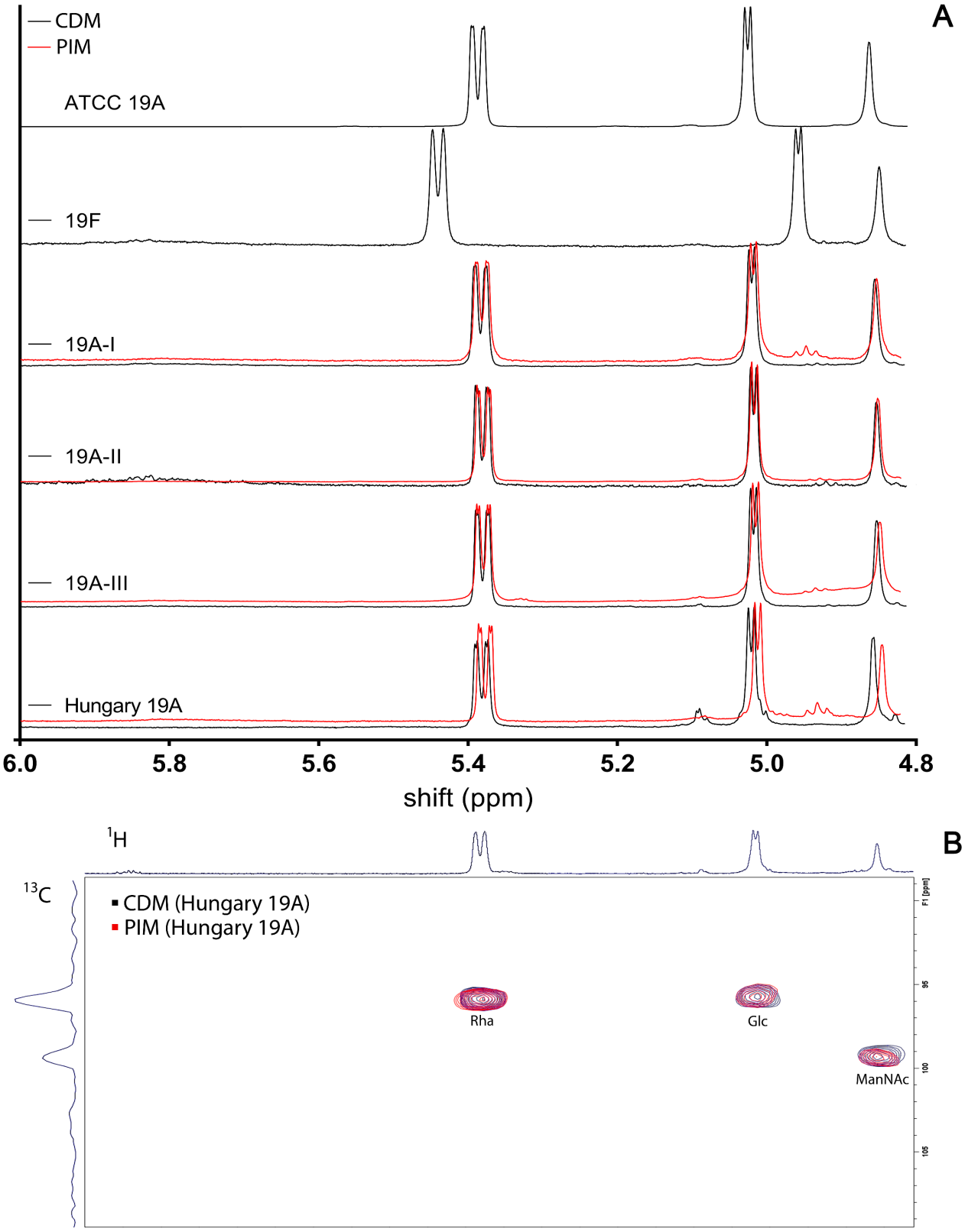

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