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1 **Impact of different amino acid substitutions in penicillin-binding protein 3 on**
2 **beta-lactam susceptibility in *Haemophilus influenzae***
3
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24

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25 **Abstract**

26 **Purpose:** Beta-lactam antibiotics in combination with a beta-lactamase inhibitor are the first-
27 line treatment option for *Haemophilus influenzae* infections. However, beta-lactamase-
28 independent resistance to beta-lactams is increasing. This resistance mechanism has been
29 linked to amino acid substitutions in the penicillin-binding protein 3 (PBP3), but how these
30 substitutions lead to decreased binding affinities to certain beta-lactam antimicrobials
31 remains unknown.

32 **Methods:** We investigated beta-lactam resistance and amino acid substitutions in PBP3
33 from fifty-three clinical isolates of *H. influenzae* collected in Switzerland from January to April
34 2016. Identification of key polymorphisms and classification of strains into PBP3 amino acid
35 substitution groups I, II, and M was done as previously described. Based on published PBP3
36 crystal structures, we investigated how the group-specific amino acid substitutions impact the
37 beta-lactam binding site.

38 **Results:** We found that both group I and group II substitutions disrupt the Asn526-Arg517-
39 Glu324 interaction, which might affect the configuration of the beta-lactam binding site.
40 Amino acid substitutions in group M strains are distant from the active site and have most
41 likely no impact on beta-lactam binding. In accordance with this observation, all group M
42 strains showed minimal inhibitory concentrations (MICs) within the susceptible range for all
43 tested antimicrobials and were not significantly different to the wild type (beta-lactamase
44 producers excluded), while group I and group II strains showed significantly higher MICs for
45 beta-lactam antimicrobials.

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46 **Conclusion:** Group M strains are phenotypically equal to the wild type, while amino acid
47 substitutions of group I and group II might affect the beta-lactam binding through a common
48 mechanism by disrupting the Asn526-Arg517-Glu324 interaction.

49

50 **Keywords:** *Haemophilus influenzae*, beta-lactam antimicrobials, beta-lactam resistance,
51 PBP3, genotype, phenotype

52

53 ## Introduction

54 Beta-lactam antibiotics are considered to be the first-line treatment option for
55 *Haemophilus influenzae* infections: aminopenicillins used in combination with a beta-
56 lactamase inhibitor for respiratory and other non-invasive infections; and third-generation
57 cephalosporins for invasive infections such as meningitis (1). Although the percentages of
58 beta-lactamase positive isolates in European countries are relatively low (Turkey 6.8%,
59 Germany 9.3%, France 11.9%, Poland 17.9%) (2-5), the percentages in some Asian
60 countries are much higher (China 31.0%, Vietnam 40.5%) (6, 7). There are two main beta-
61 lactamase types found in *H. influenzae*: TEM-1 and ROB-1 (8). According to several studies,
62 ROB-1 beta-lactamase is very rare and mostly found in North America, while TEM-1 type is
63 globally distributed (8-10). These beta-lactamases confer resistance to aminopenicillins and
64 are easily detected by using a simple nitrocefin-based beta-lactamase test.

65 However, the appearance and spreading of beta-lactamase-independent beta-lactam
66 resistance is more worrying (11, 12). The beta-lactam resistence in these isolates is mostly
67 due to amino acid substitutions in the *ftsI* gene which lead to alterations in penicillin-binding
68 protein 3 (PBP3) and consequently a decreased affinity for binding of certain beta-lactam

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69 antibiotics (13, 14). Based on the beta-lactamase production and presence of *ftsI* mutations,
70 the strains can be classified into four susceptibility groups (**Table 1a**) as previously described
71 by Ubukata et al., Dabernat et al. and García-Cobos et al. (15-17). The strains with low-level
72 PBP3-mediated resistance such as group I and II (low-rPBP3) show ampicillin minimal
73 inhibitory concentrations (MICs) between 0.5 and 2 mg/L, whereas strains with high-level
74 resistance (high-rPBP3) such as group III exhibit higher ampicillin MICs between 1 and 16
75 mg/L, often combined with reduced susceptibility to cephalosporins (11, 17-22). Although
76 group III isolates are mainly distributed in some Asian countries such as Japan and Korea
77 (23, 24), these more resistant strains, especially group III-like have been detected on rare
78 ocasions in some European countries (3, 14, 20, 25, 26).

79 PBPs are transpeptidases or carboxypeptidases involved in peptidoglycan
80 metabolism. They harbour three specific motifs: SXXK, (S/Y)XN and (K/H) (S/T)G, which
81 define the active site of the serine penicillin-recognizing enzyme family (15, 27). The serine in
82 the SXXK motif is crucial to the catalytic mechanism (27).

83 The aim of our study was to investigate the impact of PBP3 amino acid substitutions
84 in clinical isolates of *H. influenzae* on (i) antimicrobial susceptibility, (ii) the beta-lactam
85 binding site based on published PBP3 crystal structures, and (iii) to investigate, whether
86 there is an association between antimicrobial susceptibility and the location of the substituted
87 amino acids.

88

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89 ## Materials and methods

90 ### Clinical specimens and reference strains

91 The clinical isolates of *H. influenzae* were collected prospectively from January to April 2016
92 from in-patients of the University Hospital Basel, Switzerland. Three isolates originated from
93 patients of the Cantonal Hospital in Olten, Switzerland. One isolate originated from the UK
94 NEQAS external quality assessment services, Scotland. Only one sample per patient was
95 included in the study. Thirty-four samples were obtained from respiratory samples: sputum
96 (n=20), tracheal secretion (n=8), bronchial secretion (n=4), and bronchoalveolar lavage
97 (n=2). Nineteen samples were collected from other body sites: conjunctiva (n=8), ear canal
98 (n=3), cerebrospinal fluid (n=2) and others (n=6).
99

100 ### Isolation and storage of the isolates

101 The isolation of *H. influenzae* from clinical samples was carried out by standard protocols
102 including Haemophilus Chocolate 2 agar (HAE2) or chocolate PolyViteX (PVX) agar
103 (bioMérieux, Marcy-l'Étoile, France) incubated at 36 °C under the atmosphere containing 5%
104 CO_2. Colonies were identified with matrix-assisted laser desorption ionization time of flight
105 mass spectrometry (MALDI-TOF MS; Bruker, Bremen, Germany; MBT 6903 MSP Library
106 version). All *H. influenzae* isolates were frozen at -70 °C in cryogenic Microbank™ vials (Pro-
107 Lab Diagnostics, Birkenhead, UK). Prior to testing, the strains were cultured on PVX agar
108 with subculture after 24 hours.
109

110 ### Antimicrobial susceptibility testing

111 Minimal inhibitory concentrations (MICs) determination was performed with Etest®
112 (bioMérieux). The following antimicrobials were tested: ampicillin, amoxicillin, amoxicillin-

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113 clavulanic acid, piperacillin, piperacillin-tazobactam, cefuroxime, and meropenem. The MIC
114 breakpoints and screening results for beta-lactam resistance with 1 U benzylpenicillin disc
115 were interpreted as defined by the European Committee on Antimicrobial Susceptibility
116 Testing (EUCAST, version 7.1) (28), except for piperacillin-tazobactam which was interpreted
117 as defined by the Clinical and Laboratory Standards Institute (CLSI, 27th edition) (29) due to
118 no existing EUCAST interpretation criteria. Piperacillin was interpreted according to CLSI
119 guidelines for piperacillin-tazobactam, because there are no interpretation criteria in either
120 EUCAST or CLSI guidelines for this antimicrobial.
121
122 **Statistical analysis of MICs for comparison of strains with amino acid substitutions to**
123 **wild type strains**
124 Wilcoxon rank-sum test was used to compare MICs between strains as follows: For all tested
125 antimicrobials, we compared the MICs of group IIa, IIb, IId and group M strains to the
126 respective MICs of the wildtype. Group I strains were excluded from the analysis, because
127 we found only two strains in this group. Since we were interested in beta-lactamase
128 independent resistance, all beta-lactamase producing strains were excluded as well. To
129 correct the obtained p-values for multiple hypothesis testing, we used the Benjamini-
130 Hochberg-Yekutiely procedure that controls the false discovery rate (30). The analysis has
131 been done in R. All analysis scripts including raw data and figure-generating scripts are
132 available on gitlab: https://gitlab.com/csb.ethz/haemophilus-pbp3-manuscript (online
133 ressource **ESM1)**.
134

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135 **Phenotypic and genotypic determination of beta-lactamase production**

136 Beta-lactamase production was determined with BBL™ Cefinase™ Paper Discs (Beckton
137 Dickinson, Franklin Lakes, NJ, USA) according to manufacturer's instructions. Presence of
138 TEM-1 beta-lactamase encoding gene was determined by conventional PCR with primers
139 described by Dabernat et al. (16). The following reference strains were used as controls for
140 TEM-1 PCR: *H. influenzae* ATCC 49247 (BLNAR) and *H. influenzae* ATCC 35056 (BLPAR).

141

142 **Determination of *ftsI* mutations and identification of PBP3 amino acid substitution**
143 **groups**

144 Nucleic acid extraction was performed with Advanced XL EZ1 (Qiagen, Hilden, Germany).
145 The digestion step using proteinase K was performed for 10 minutes at 56 °C and 10
146 minutes at 95 °C followed by purification with EZ1 Tissue card according to the
147 manufacturer's protocol. DNA extracts were eluted in 100 µl elution buffer. Amplification of
148 *ftsI* gene was performed by using the conventional PCR assay described by Cerquetti et al.
149 (31). PCR products were purified with ExoSAP-IT® (USB Corporation, Cleveland, OH, USA)
150 purification kit. The sequencing of the PCR amplicon was performed by Microsynth AG
151 (Balgach, Switzerland) using Sanger sequencing (sequencing primer^{frw}: 5'-
152 GCGGATAAAGAACGAATTGC-3' (14), sequencing primer^{rev}: 5'-
153 CTGGATAATTCTGTCTCAGA-3' (31)). Sequences were aligned with Lasergene SeqMan
154 Pro (DNASTAR, Madison, WI, USA) and translated into amino acid sequences using the
155 ExPASy translate tool (http://web.expasy.org/translate/). To identify amino acid substitutions,
156 the translated amino acid sequences were aligned using Clustal Omega
157 (http://www.ebi.ac.uk/Tools/msa/clustalo/) to reference strain *H. influenzae* RD KW20 (Acc.

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158 No. Genbank NC_000907). Key amino acid changes were grouped as reported previously,
159 see **Table 1b** (15-17).
160

161 **Investigation of group-specific mutations in the PBP3 crystal structure**

162 At the time of this study, there were no crystal structures of PBP3 from *Haemophilus* spp. in
163 the Worldwide Protein Data Bank (wwPDB; www.wwpdb.org). Therefore, we selected
164 structures from other gram-negative rods *Escherichia coli* (PDB ID 4BJP, 2.5 Å resolution)
165 and *Pseudomonas aeruginosa* (PDB ID 3PBR, 1.95 Å resolution) for our analysis.
166 Fortunately, the amino acid sequences of the three conserved PBP3 domains of *E. coli* and
167 *P. aeruginosa* are identical to those of *H. influenzae*, except for one conservative amino acid
168 substitution in *P. aeruginosa*, where serine is replaced by threonine in the (K/H) (S/T)G motif.
169 In addition, the root mean square deviation (RMSD) of all matched atoms in the PBP3 crystal
170 structures from *E. coli* and *P. aeruginosa* is 1.166 Å, which stands for high structural
171 similarity (32). We used Clustal Omega to align the PBP3 amino acid sequences and to
172 determine the sequence identity between the different species, i.e. *H. influenzae* RD KW20
173 (UniProt Entry P45059), *Escherichia coli* (GI:635575685), and *Pseudomonas aeruginosa*
174 (UniProt Entry Q51504). Crystal structures of PBP3 from *E. coli* and *P. aeruginosa* were
175 aligned with PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger,
176 LLC). To investigate whether specific amino acid substitutions impact the meropenem
177 binding site, we constructed three-dimensonal models of amino acid substitutions in *H.*
178 *influenzae* based on the *P. aeruginosa* crystal structure (PDB ID code 3PBR).
179

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180 ## Results

181 ### Identification of PBP3 amino acid substitution groups

182 Based on the PBP3 amino acid sequence deduced from the *ftsI* gene sequence, we
183 assigned the investigated strains (n=53) to following groups as previously described (**Table**
184 **1b**): wild type (n=17), group I (n=2), group II (a, b and d; n=21) or group M (miscellaneous,
185 n=13) (**Table 2**). No isolates belonging to groups IIc, III or III-like were found. Eight out of 13
186 group M isolates with three different mutation patterns (Asp350Asn and Val547Ile; Val547Ile;
187 Asp350Asn and Val547Ile and Asn569Ser) were described previously by Cherkaoui et al.
188 (14, 33). The remaining substitution combinations in group M have been identified for the first
189 time in four strains of this study (**Table 2**).

190

191 ### MICs for beta-lactam antibiotics of different PBP3 amino acid substitution groups

192 All determined MICs are summarized in **Table 3** and shown in **Figure 1**. All strains with
193 amino acid substitutions in PBP3 belonging to groups I, II and M (beta-lactamase producers
194 excluded) showed very low MICs for piperacillin and piperacillin-tazobactam (range 0.016-
195 0.25 and <0.016-0.25 mg/l, respectively), which was not observed for any other penicillin with
196 or without beta-lactamase inhibitor (**Table 3 and Figure S1**). For all tested antimicrobials, we
197 compared the MICs of group IIa, IIb, IId and group M isolates (beta-lactamse producers
198 excluded) to the respective MICs of the wildtype. Group I isolates have been excluded due to
199 low sample size (n=2). We found no significant difference between group M and the wild type
200 isolates. In contrast, all group II isolates showed significantly higher MICs (p-values ranging
201 from 0.007 to 0.016, see **Figure 1**) except for piperacillin and piperacillin-tazobactam.

202

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203 In the benzylpenicillin disc screen, all wild type and group M isolates (except beta-lactamase
204 producing strains) were categorized as beta-lactam susceptible (**Figure S2**). All group I and
205 group II isolates were categorized as resistant in the beta-lactamase screen (zone diameter
206 <=11mm), except for two strains with zone diameters of 12 mm (on the breakpoint) which
207 were categorized as susceptible.

208

209 **Impact of group-specific amino acid substitutions on the PBP3 crystal structure**

210 We investigated the position of the identified group-specific amino acid substitutions and their
211 impact on the PBP3 binding site (**Figure 2**). Since there was no PBP3 crystal structure of *H.*
212 *influenzae* available at the time of this study, we selected PBP3 crystal structures from *E. coli*
213 and *P. aeruginosa* for our analysis, which amino acid sequences of the three conserved
214 PBP3 domains were identical to those of *H. influenzae*, except for one conservative amino
215 acid substitution in *P. aeruginosa*, where serine is replaced by threonine in the (K/H) (S/T)G
216 motif. The overall PBP3 amino acid sequence identity between *H. influenzae* and *E. coli* is
217 54%, between *H. influenzae* and *P. aeruginosa* 39%, and between *E. coli* and *P. aeruginosa*
218 47%. These identities are sufficient for our analysis, because we are interested in the
219 position of mutations in the three-dimensional structure and protein structure is more
220 conserved than protein sequence (32, 34). Crystal structure alignment of PBP3 of *E. coli* and
221 *P. aeruginosa* confirmed this statetement: the RMSD (Root Mean Square Deviation) of the
222 alignment of all matched atoms is 1.166 Å (see **Figure 2a**), which stands for a good
223 accordance (32). Because the *P. aeruginosa* crystal structure (PDB ID code 3PBR) contains
224 a representation of the binding of beta-lactam meropenem and has a high resolution (1.95
225 Å), we used this structure to model the amino acid substitutions in *H. influenzae*.

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226 Interestingly, all relevant amino acids were identical between the two strains, i.e. the amino
227 acids that we found to be directly or indirectly affected by the group-specific mutations
228 Arg517His, Asn526Lys, and Val547Ile (**Figure S3**).
229 **Figure 2b** shows the location of the conserved catalytic domains of the beta-lactam binding
230 site. None of the found mutations were directly located at the binding site but all substitutions
231 of group M were distant from the beta-lactam binding site, whereas group I and II
232 substitutions were closer to the binding site (**Figure 2c**).
233 The amino acid substitution Val547Ile were found in almost all group M strains and some
234 group II strains (see **Table 2**). This conservative mutation has most likely no impact on
235 protein structure: Val547 is located in a hydrophobic pocket where it is surrounded by
236 aliphatic amino acids except for Thr532 and Thr281 (**Figure 2d**) the substitution of the
237 aliphatic amino acid valine by the aliphatic amino acid isoleucine has most likely no influence
238 on the protein structure.
239 The amino acid substitution Arg517His occured in group I strains, and the substitution
240 Asn526Lys occurred in group II strains. These substitutions are adjacent and lie in
241 neighboring beta turns of the beta sheet that harbours the KTG motif. We speculate that
242 Arg517 and Asn526 are part of an important Arg517-Asn526-Glu324 interaction (**Figure 2e**,
243 left panel) for the following reasons: (a) Arg517 and Asn526 are able to build a hydrogen
244 bond and to connect the two beta turns, thus stabilizing this important region; (b) Arg517 is
245 able to build a hydrogen bond to Glu324, thus connecting the alpha helix that harbours the
246 STVK motif to the beta sheet that harbors the KTG motif. In addition, the positive charge of
247 arginine and the negative charge of glutamate attract each other. These interactions between

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248 Arg517, Asn526 and Glu324 could stabilize the configuration of the two functional motifs
249 KTG and STVK.
250 As shown in the middle panel of **Figure 2e**, the group I mutation Arg517His results in the
251 loss of the electrostatic attraction to Glu324; His517 is able to replace Arg517 as a proton
252 acceptor in the hydrogen bond to Asn526, and can serve as a proton donor in the hydrogen
253 bond to Glu324. Yet, this substition might impact the protein structure, because (a) hydrogen
254 bonds are weaker than electrostatic interactions, and (b) histidine is less flexible than
255 arginine, which means that the Arg517-His517-Glu324 interaction has less degrees of
256 freedom than the Arg517-Asn526-Glu324 bridge.
257 As shown in the right panel of **Figure 2e**, the group II mutation Asn526Lys results in the loss
258 of the hydrogen bond to Arg517. In addition, lysine is negatively charged such as arginine,
259 which leads to an electrostatic repulsion. As a result, the two beta turns might now be
260 repelled from each other instead of attracted.
261
262 **Genotypic and phenotypic detection of beta-lactamase production**
263 In 25% of the isolates tested (13/53), beta-lactamase was detected by Cefinase^{TM} test. The
264 same 13 strains were also found positive with TEM-1 PCR, and the remaining 40 strains
265 were TEM-1 negative (**Table 2**).
266
267 **Susceptibility according to four aminopenicillin susceptibility groups**
268 Based on the MIC distributions, results of benzylpenicillin disc screen and conclusions drawn
269 from the crystal structure binding sites, group M isolates were considered to belong either to
270 gBLNAS or gBLPAR, while group I and II isolates were considered to belong to gBLNAR or

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271 gBLPACR. The classification of the investigated strains according to beta-lactamase
272 production and amino acid substitution groups is as follows: 36% (19/53) gBLNAS, 39%
273 (21/53) gBLNAR, 21% (11/53) gBLPAR and 4% (2/53) gBLPACR. The results of MIC testing
274 according to four aminopenicillin susceptibility groups is shown in **Table 4:** 81% (17/21) of
275 gBLNAR strains were categorized as amoxicillin and amoxicillin-clavulanic acid susceptible
276 and 9.5% (2/21) as ampicillin susceptible. With EUCAST breakpoints for *H. influenzae*
277 infections other than meningitis, all gBLNAR strains were meropenem susceptible, while
278 when breakpoints for meningitis were applied, 15% (3/20) were categorized as intermediate
279 (**Table 4**). The number of gBLPACR strains in the study was only two, and therefore no
280 reliable classification according to susceptibility phenotype could be made for this group.
281

282 Discussion

283 In recent years, alterations in PBP3 leading to decreased binding affinity of certain beta-
284 lactam antibiotics have emerged as a prevalent resistance mechanism in *H. influenzae* (35).
285 In this study, we investigated the mutations in the *ftsI* gene encoding for PBP3 within a
286 collection of clinical isolates from two Swiss hospitals. To our knowledge, our study
287 investigated for the first time the location and potential impact of the polymorphisms in the
288 PBP3 crystal structure of clinical isolates of *H. influenzae* with a detailed description of amino
289 acid interactions. The most frequently found group of amino acid substitutions belonged to
290 group II (19/53; 35.8%), which was also observed in studies from other European countries
291 (3, 14, 20, 21, 36). We could not detect strains of the group III, which harbours high-level
292 PBP3 alterations (11, 35).

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293 All wild type strains showed MICs within the susceptible range for all tested antimicrobials
294 and were categorized as beta-lactam susceptible with benzylpenicillin disc screen, except, as
295 anticipated, beta-lactamase producing isolates. Surprisingly, the same results were obtained
296 for strains with amino acid substitutions of the group M (see **Figure 1**). Based on our crystal
297 structure analysis, we suggest that the group I and group II substitutions Arg517His and
298 Asn526Lys influence the binding of beta-lactam antimicrobials trough a common mechanism
299 by disrupting the Asn526-Arg517-Glu324 interaction (see **Figure 2e**). Since these three
300 amino acids are able connect the beta sheet and the alpha helix that harbour the KTG and
301 STVK motifs, their interaction might be important for a proper binding site formation. Further
302 molecular investigations including site-directed mutagenesis of these amino acids are clearly
303 needed to test this hypothesis. Interestingly, a just recently published study on the crystal
304 structure of *H. influenzae* comes to a similar conclusion that mutations Arg517His and
305 Asn526Lys might lead to resistance through long-range structural rearrangements (37). Our
306 study complements this finding and proposes a mechanistic explanation, i.e. the disruption of
307 the Asn526-Arg517-Glu324 bridge.

308 Group M substitutions are distant to the binding site and shown in **Figure 4** and have
309 probably no influence on the configuration of the binding site. We propose, that strains
310 belonging to group M are phenotypically equal to the wild type, since an impact on the beta-
311 lactam binding site is unlikely. This was confirmed by low MICs in antimicrobial susceptibility
312 testing of these isolates (**Figure 1**) and categorization as beta-lactam susceptible in
313 benzylpenicillin disc screen (**Figure S2**).

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314 Investigation of group III alterations (even though not found in this study) based on PBP3
315 structure of *P. aeruginosa* was not possible due to differences in amino acid sequences
316 between *P. aeruginosa* and *H. influenzae* in this region.
317 Although Ellington *et al.* concluded that prediction of antimicrobial resistance based on whole
318 genome sequencing data is currently not feasible and requires collection of extensive data
319 sets (38), the mechanistic understanding on how single point mutations impact binding
320 affinities of antibiotic drugs may help to predict antimicrobial resistance in the future.
321 All isolates in this study showed very low MICs to piperacillin-tazobactam (range <0.016-0.25
322 mg/l), which was also documented in european and asian studies (14, 39). Piperacillin MICs
323 were also very low in non-beta-lactamase-producing strains (range <0.016-0.25 mg/l), which
324 implicates that there is no intrinsic activity of tazobactam against *Haemophilus* strains.
325 Nevertheless, no EUCAST nor CLSI breakpoints for *H. influenzae* have been issued to date
326 for piperacillin, and only CLSI breakpoints for piperacillin-tazobactam have been published
327 (29).
328
329 Reliable detection of beta-lactam resistance in *H. influenzae* is essential, since beta-lactam
330 antimicrobials represent a first line treatment for infections caused by this bacterium.
331 Detection of *H. influenzae* strains producing beta-lactamase with nitrocefin-based tests is
332 rapid and reliable. In 34 out of 36 isolates with PBP3 alterations, EUCAST screening for
333 beta-lactam susceptibility with benzylpenicillin disc was able to separate between PBP3
334 alterations with no impact on the beta-lactam binding (group M) and the alterations leading to
335 decreased affinity to beta-lactams (group I and II). All isolates containing amino acid
336 substitutions of group M were categorized as beta-lactam susceptible (showing zone

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337 diameters of 15 to 19 mm), together with wild type isolates (exception: beta-lactamase
338 producing strains) as shown in **Figure S2**. Two isolates belonging to groups I and II were
339 falsely categorized in screening as beta-lactam susceptible, but it needs to be mentioned that
340 they both exhibited zone diameter on the breakpoint (12 mm), and that technical factors
341 could be responsible for this borderline zone diameter. Further studies on larger collection of
342 clinical isolates are needed to investigate this observation. Furthermore, our study showed
343 that some gBLNAR strains (belonging to group I and II) are categorized as susceptible for
344 amoxicillin, ampicillin and amoxicillin-clavulanic acid when applying EUCAST interpretation
345 criteria (**Table 4**). Although clinical impact of these in vitro findings needs some further
346 investigations, two pragmatic approaches were recommended by Skaare et al. (35): (a) *H.*
347 *influenzae* isolates with altered PBP3 positive by screening for beta-lactam resistance with
348 benzylpenicillin 1U disc should be categorized as cefuroxime resistant and always be
349 reported as ampicillin resistant in cases of meningitis; (b) a comment should be added
350 recommending high-dose aminopenicillin therapy or the use of other agents in severe
351 infections caused by screening-positive isolates categorized as susceptible to
352 aminopenicillins by disc or gradient diffusion.
353
354 Emergence of strains with resistance to carbapenems is another worrying development. The
355 study of Cherkaoui and colleagues provides evidence that development of imipenem
356 heteroresistance depends on a combination of altered PBP3, slowed drug influx and its
357 enhanced efflux due to the loss of regulation of the efflux pump (33). In the present study, we
358 found that 15% of BLNAR strains were categorized as meropenem intermediate when
359 interpreted with EUCAST meningitis breakpoints (**Table 4**). In contrast, meropenem resistant

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360 isolates were reported in the 7.4% of the gBLNAR isolates from a Portugese study (21). The
361 same study showed that the MIC_{50} and MIC_{90} values of meropenem were both four times
362 higher for gBLNAR isolates compared to gBLNAS. The same pattern was also observed for
363 the strains of this study, but no strains with resistant phenotype were observed (**Table 4**).
364
365 Our study has the following limitations: (a) the number of isolates examined in this study is
366 limited, but the results are in accordance with the findings of other Swiss and European
367 studies (3, 5, 14, 17); (b) no group III or group III-like isolates were included in the analysis,
368 since these isolates are still rarely found in Europe (3, 14, 17); (c) besides the heterogenous
369 impact of various alterations in PBP3 with multiple changes in amino acids also other
370 possible resistance mechanisms like efflux pumps might further act on the MICs of
371 antimicrobials (17, 33, 40).
372
373 In conclusion, based on our molecular and phenotypic findings, we assume that strains
374 belonging to M group are phenotypically equal to wild type, while amino acid substitutions of
375 group I and group II affect the beta-lactam binding through a common mechanism by
376 disrupting the Asn526-Arg517-Glu324 interaction. Not all PBP3 alterations have an influence
377 on the resistant phenotype in *H. influenzae*. When EUCAST interpretation criteria were
378 applied, some gBLNAR strains (all harbouring group I and II mutations) were categorized as
379 susceptible to ampicillin, amoxicillin or amoxicillin-clavulanic acid.
380

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383

384 **Compliance with Ethical Standards**

385 **Conflict of Interest:** The authors declare that they have no conflict of interest.

386 **Ethical approval:** This article does not contain any studies with human participants or
387 animals performed by any of the authors.

388 **Informed consent:** Not applicable (this article does not contain any studies with human
389 participants by any of the authors).

390

391 **Online ressources and electronic supplementary material (ESM)**

392 **ESM1:** Repository with raw data including all analysis scripts and figure-generating scripts in
393 R: https://gitlab.com/csb.ethz/haemophilus-pbp3-manuscript

394 **ESM2:** Supplementary figures can be found at the end of this document.

395 **Fig. S1** Determined MIC values for all isolated strains (*bla*-positive and *bla*-negative).

396 **Fig. S2** Distribution of investigated *H. influenzae* strains (n=53) according to benzylpenicillin
397 1 U disc zone diameter (screening for beta-lactam resistance according to EUCAST).

398 **Fig. S3** Location of amino acid substitutions in the protein sequence of the reference strain
399 *H. influenzae* RD KW20 (UniProt Entry P45059).

400 **ESM3:** Annotated sequence aligment between *H. influenzae* RD KW20 (UniProt entry
401 P45059) and *P. aeruginosa* (UniProt entry Q51504). The file can be downloaded from the
402 gitlab repository: https://gitlab.com/csb.ethz/haemophilus-pbp3-manuscript

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539 **Table 1. (a)** Categorization of *H. influenzae* strains into four aminopenicillin susceptibility
540 groups based on the beta-lactamase production and presence of *ftsI* mutations. **(b)** PBP3
541 mutation groups and corresponding key amino acid substitutions.
542

(a)

Abbreviation	Genotype
gBLNAS	Beta-lactamase negative and ampicillin susceptible without resistance mechanism
gBLNAR	Beta-lactamase negative and ampicillin resistant with PBP3 mutations
gBLPAR	Beta-lactamase positive and ampicillin resistant without PBP3 mutations
gBLPACR	Beta-lactamase positive and amoxicillin-clavulanic acid resistant with PBP3 mutations

(b)

PBP3 mutation group	Key amino acid substitution
I[1]	Arg517His
II[1]	Asn526Lys
IIa[2]	Asn526Lys (the only substitution)
IIb[2]	Asn526Lys, Ala502Val
IIc[2]	Asn526Lys, Ala502Thr
IId[2]	Asn526Lys, Ile449Val
III[1]	Met377Ile,Ser385Thr, Leu389Phe, Asn526Lys
III-like[3]	Group III mutations without Asn526Lys or with additional combinations at the KTG or STVK motif
M[3]	Miscellaneous

543
544 [1]According to Ubukata et al. (15)
545 [2]According to Dabernat et al. (16)
546 [3]According to Garcia-Cobos et al. (17)
547
548

549 **Table 2.** Amino acid substitutions in the transpeptidase domain of the *ftsI* gene found in the strains of this study (n=53).
550

Group	ID[e]	No. of strains		Amino acid substitutions												
						Near STVK motif[a]	Near SSN motif[a]			Near KTG motif[a]						
		bla -[b]	*bla* +[b]	Ala-294[c]	Lys-310	Asp-350	Met-377	Ile-449	Gly-490	Ala-502	Arg-517	Asn-526	Ala-530	Val-547	Val-562	Asn-569
Wild type	A	11	6													
I	B	1								Val	His					
	C	1									His					
IIa	D	4	1			Asn			Glu			Lys	Ser			
	E	1				Asn						Lys			Leu	
	F	1										Lys	Ser	Ile		
IIb	G	2				Asn	Ile		Glu	Val		Lys		Ile		Ser
	H	1	1			Asn	Ile			Val		Lys		Ile		Ser
	I	2								Val		Lys				
IId	J	6						Val				Lys		Ile		Ser
	K	2					Ile	Val				Lys		Ile		Ser
M	L		2			Asn								Ile		
	M		2[d]		Glu									Ile		

	N	5												Ile		
	O	1 [d]				Asn								Ile		Ser
	P	1 [d]												Ile		Ser
	Q	1 [d]	1 [d]	Ser												

[a] The conserved catalytic motifs are encoded in the transpeptidase amino acid sequence as follows: STVK 327-330 amino acid, SSN 379-381 amino acid, KTG 512-514 amino acid.
[b] bla-: beta-lactamase negative (TEM-1 negative); bla+: beta-lactamase positive (TEM-1 positive).
[c] Amino acid substitution described for the first time in this study.
[d] Amino acid substitution combinations described for the first time in strains of this study.
[e] ID according to the amino acid substitution

579
580 **Table 3.** Results of minimal inhibitory concentration (MIC) testing according to amino acid substitution groups.
581

Group	ID[g]	No. of isolates *bla* -[b]	No. of isolates *bla* +[b]	Ampicillin	Amoxicillin	Amoxicillin-clavulanic acid	Piperacillin	Piperacillin-tazobactam	Cefuroxime (iv[c])	Meropenem (infections other than meningitis)	Meropenem (meningitis)
Wild type	A		6	4->256	3->256	0.38-1.5	2-24	0.016- 0.064	0.38-1	0.032-0.064	0.032-0.064
	A	11		0.25-0.5	0.25-0.75	0.19-0.75	<0.016-0.032	<0.016-0.064	0.19-1	0.016-0.064	0.016-0.064
I	B	1		1.5	1.5	1.5	0.032	0.023	2	0.064	0.064
	C	1		0.75	1	0.75	0.064	0.047	1.5	0.064	0.064
IIa	D		1	>256	>256	1	8	0.064	2	0.19	0.19
	D	4[e]		1- 2	0.75-1.5	0.75-1.5	0.032-0.094	0.016-0.064	1.5-2	0.125-0.38	0.125 -0.38
	E	1		3	4	4	0.064	0.032	12	0.38	0.38
	F	1		1.5	1	1	n.d.[d]	0.047	1.5	n.d.[d]	n.d.[d]
IIb	G	2		1-2	1- 3	1- 3	0.016-0.094	0.016-0.064	4-16	0.094-0.25	0.094-0.25
	H		1	>256	>256	3	>256	0.25	16	0.094	0.094
	H	1		3	2	2	0.125	0.19	12	0.094	0.094
	I	2		1.5-2	1.5- 3	1.5- 3	0.032-0.047	0.032-0.064	2- 4	0.094-0.25	0.094-0.25
IId	J	6[f]		1.5-2	1.5-2	1.5-2	0.032-0.25	<0.016-0.125	2- 6	0.094-0.19	0.094-0.19
	K	2		2-3	2- 4	2- 3	0.064-0.094	0.064	6	0.19-0.38	0.19 -0.38

M	L		2	>256	>256	1.-1.5	2-8	<0.016-0.016	1	0.094-0.125	0.094-0.125
	M		2	>256	>256	1	8-64	0.016-0.023	0.75	0.064	0.064
	N	5		0.38-0.5	0.25-0.75	0.38-0.75	0.016-0.047	<0.016-0.032	0.75	0.032-0.094	0.047-0.094
	O	1		0.75	0.75	0.75	0.023	0.016	0.75	0.064	0.064
	P	1		0.5	0.75	0.75	0.023	0.016	0.75	0.064	0.064
	Q		1	>256	>256	1	32	0.023	0.75	0.047	0.047
	Q	1		0.38	0.38	0.5	0.016	<0.016	0.75	0.047	0.047

[a] green: susceptible; yellow: intermediate; red: resistant.
[b] bla-: beta-lactamase negative; bla+: beta-lactamase positive.
[c] iv: refers to intravenous administration of cefuroxime;
[d] n.d.: not done.
[e] ampicillin: one isolate susceptible, three isolates resistant; meropenem: one isolate intermediate, three isolates susceptible
[f] cefuroxime (i/v): four isolates intermediate, two isolates resistant
[g] ID according to the amino acid substitution

597 **Table 4.** Results of MIC testing according to four aminopenicillin susceptibility groups (gBLNAS n=19, gBLNAR n=21, gBLPAR n=11, gBLPACR
598 n=2).

	MIC (mg/l)				Susceptibity category (%)		
Antimicrobial	Genotypic category	MIC_{50}	MIC_{90}	MIC range	S	I	R
Ampicillin	gBLNAS	0.38	0.5	0.19-0.75	100	0	0
	gBLNAR	1.5	3	0.75-3	9.5	0	90.5
	gBLPAR	>256	>256	4->256	0	0	100
	gBLPACR	n.a.[a]	n.a.[a]	>256	0	0	100
Amoxicillin	gBLNAS	0.5	0.75	0.25-0.75	100	0	0
	gBLNAR	1.5	3	0.75-4	81	0	19
	gBLPAR	>256	256	3->256	0	0	100
	gBLPACR	n.a.[a]	n.a.[a]	>256	0	0	100
Amoxicillin-clavulanic acid	gBLNAS	0.5	0.75	0.19-0.75	100	0	0
	gBLNAR	1.5	3	0.75-4	81	0	19
	gBLPAR	1	1	0.38-1.5	100	0	0
	gBLPACR	n.a.[a]	n.a.[a]	1-3	50	0	50
Piperacillin[d]	gBLNAS	0.016	0.023	<0.016-0.047	100	0	0
	gBLNAR	0.047	0.094	0.016-0.25	100	0	0
	gBLPAR	8	32	2-64	0	0	100
	gBLPACR	n.a.[a]	n.a.[a]	8->256	0	0	100
Piperacillin-tazobactam	gBLNAS	<0.016	0.032	<0.016-0.064	100	0	0
	gBLNAR	0.047	0.125	<0.016-0.19	100	0	0

	gBLPAR	0.023	0.047	<0.016-0.064	100	0	0
	gBLPACR	n.a.[a]	n.a.[a]	0.064-0.25	100	0	0
Cefuroxime (iv[c])	gBLNAS	0.75	0.75	0.25-1	100	0	0
	gBLNAR	2	12	1.5-16	0	57	43
	gBLPAR	0.75	1	0.38-1	100	0	0
	gBLPACR	n.a.[a]	n.a.[a]	2-16	0	0	0
Meropenem (meningitis/ infections other than meningitis)	gBLNAS	0.047	0.064	0.016-0.094	100 / 100	0 / 0	0 / 0
	gBLNAR	0.125	0.38	0.016-0.19	85 / 100	15 / 0	0 / 0
	gBLPAR	0.064	0.094	0.032-0.125	100 / 100	0 / 0	0 / 0
	gBLPACR	n.a.[a]	n.a.[a]	0.064-0.25	100 / 100	0 / 0	0 / 0

599
600 [a] n.a.: not applicable because only two strains tested.
601 [b] One BLNAR strain was not tested for piperacillin and meropenem.
602 [c] iv: refers to intravenous administration of cefuroxime.
603
604

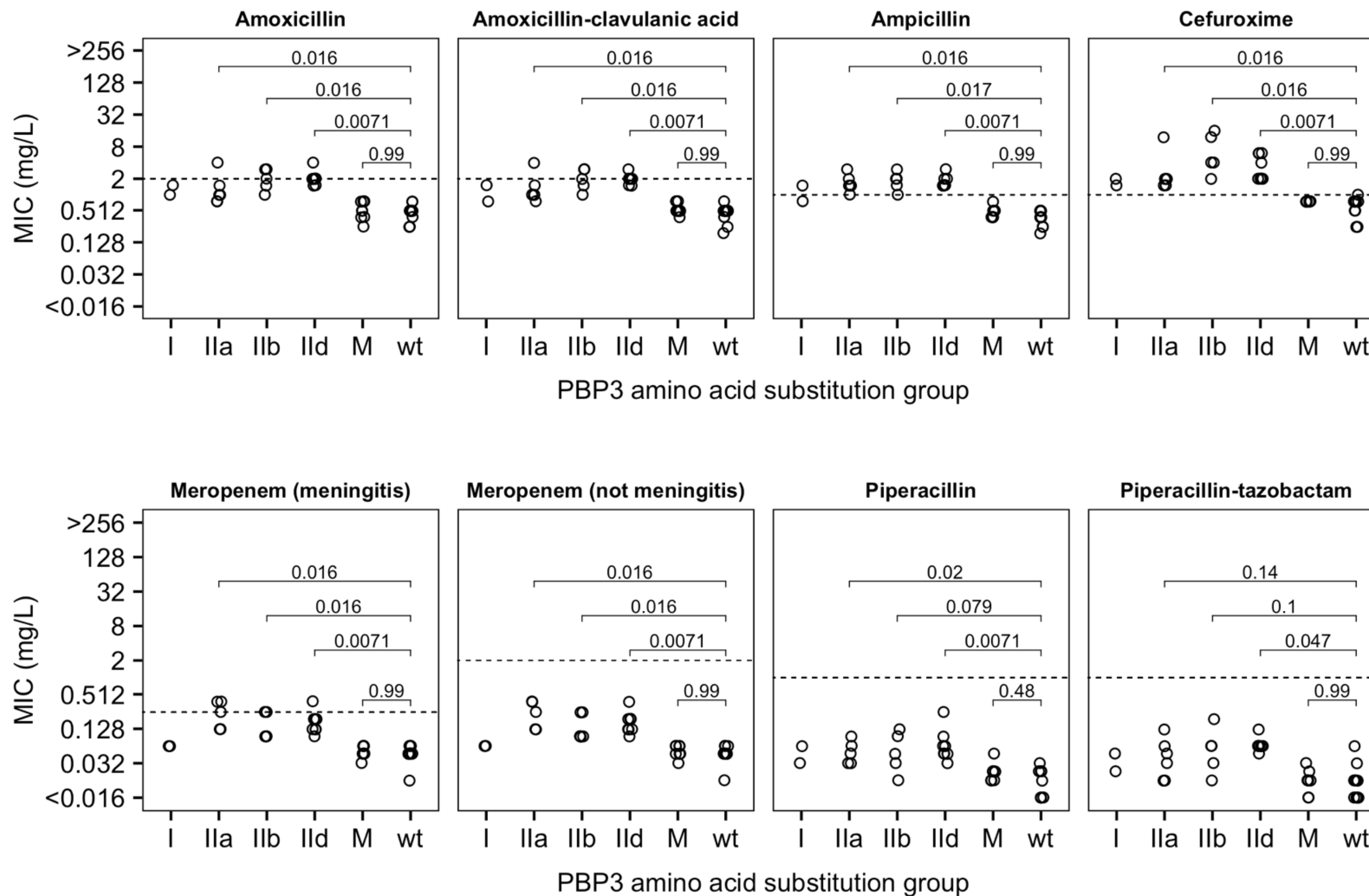

Fig. 1 MIC values of beta-lactamase negative strains. Wilcoxon rank-sum test was used to compare MIC values of strains with amino acid substitutions to the wildtype. The numbers show the adjusted p-values (Benjamini-Hochberg-Yekutiely correction). The dotted line indicates the MIC breakpoint or, if applicable, the lower limit of the intermediary region. All antibiotic breakpoints are as defined by EUCAST with the exception of piperacillin and piperacillin-tazobactam which are according to CLSI.

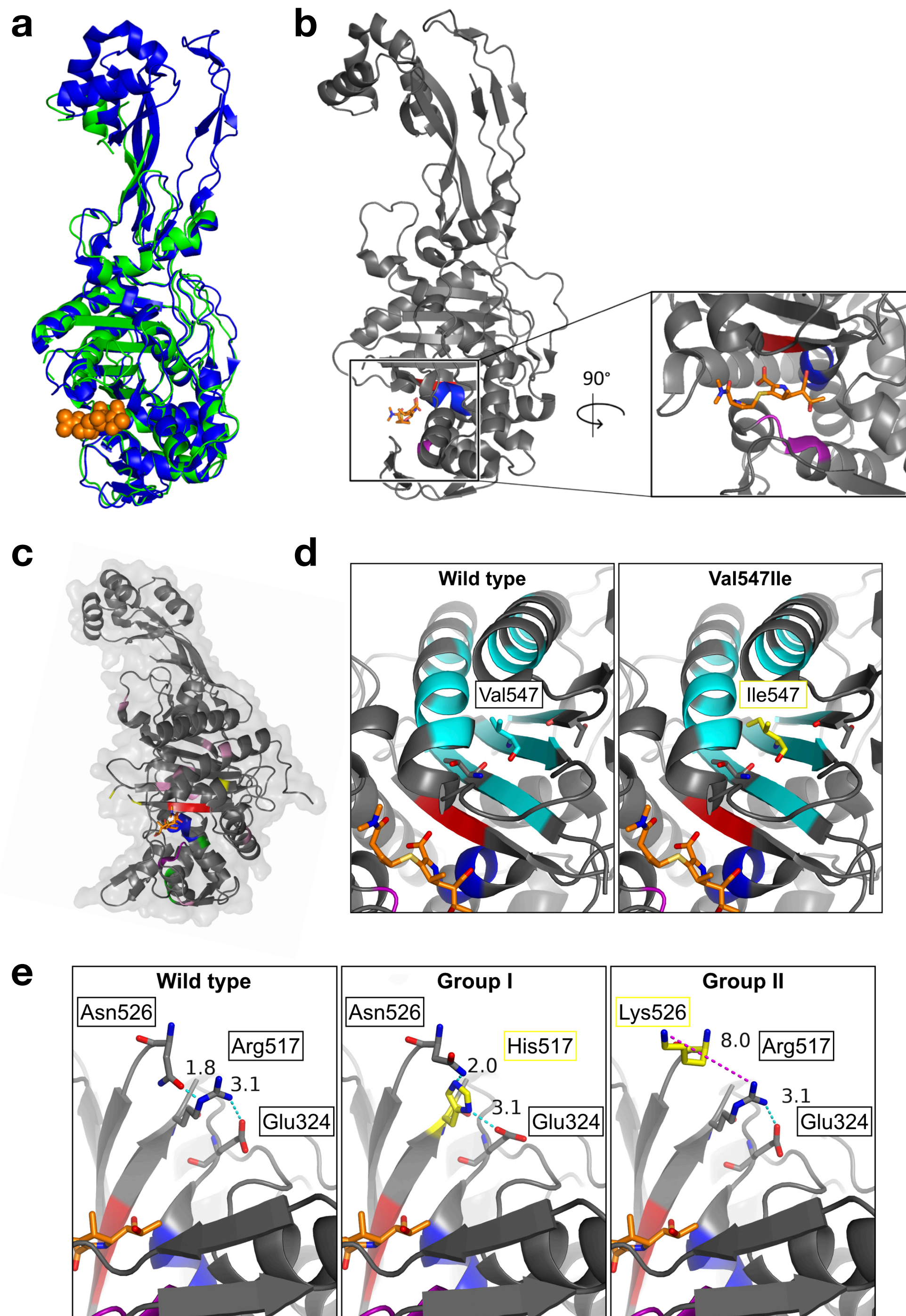

Fig. 2 PBP3 crystal structure analysis**. (a)** Crystal structure alignment of PBP3 of *Escherichia coli* (green, PDB ID 4BJP, solved at 2.50 Å) and of *Pseudomonas aeruginosa* (blue, PDB ID 3PBR, solved at 1.97 Å). The meropenem molecule is shown in orange. The root mean square deviation of all matched atoms (2185 atoms) is 1.166 Å. **(b)** PBP3 crystal structure of *P. aeruginosa* with a molecule of meropenem (orange) bound at the active site. The transpeptidase domain belongs to the active C-terminus. The three catalytic domains are highlighted in blue (SXXK), purple ((S/Y)XN) and red ((K/H)(S/T)G motif). **(c)** Localization of amino acid substitutions of PBP3 groups in the PBP3 crystal structure of *P. aeruginosa* (PDB ID 3PBR): group I and II are highlighted in yellow, group III in green and group M in pink. The three catalytic domains are highlighted in blue (SXXK), purple ((S/Y)XN) and red ((K/H)(S/T)G motif). The meropenem molecule is shown in orange. **(d)** Graphic representation of the most common group M mutation Val547Ile (shown in yellow) in the PBP3 crystal structure of *P. aeruginosa* (PDB ID 3PBR). Val547 lies in a hydrophobic pocket where it is surrounded by aliphatic amino acids (shown in cyan), except for Thr532 and Thr281, which are polar. The substitution of an aliphatic amino acid by another aliphatic amino acid (valine to isoleucine) has most likely no impact on the protein structure. The three catalytic domains are highlighted in blue (SXXK), purple ((S/Y)XN) and red ((K/H)(S/T)G motif). The meropenem molecule is shown in orange. **(e)** Investigation of the group I mutation Arg517His and group II mutation Asn526Lys (both shown in yellow) based on the PBP3 structure of *P. aeruginosa* (PDB ID 3PBR). Attracting interactions are shown in cyan (i.e. hydrogen bonds and electrostatic attraction between Arg-Glu), repelling interactions in pink (electrostatic repulsion between Lys-Arg). Numbers indicate the distance between atoms in Ångström. Both mutations are able to disrupt the Arg517-His517-Glu324 interaction. The three catalytic domains are highlighted in blue (SXXK), purple ((S/Y)XN) and red ((K/H)(S/T)G motif). The meropenem molecule is shown in orange.

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Supplementary Material

of the manuscript

Impact of different amino acid substitutions in penicillin-binding protein 3 on beta-lactam susceptibility in *Haemophilus influenzae*

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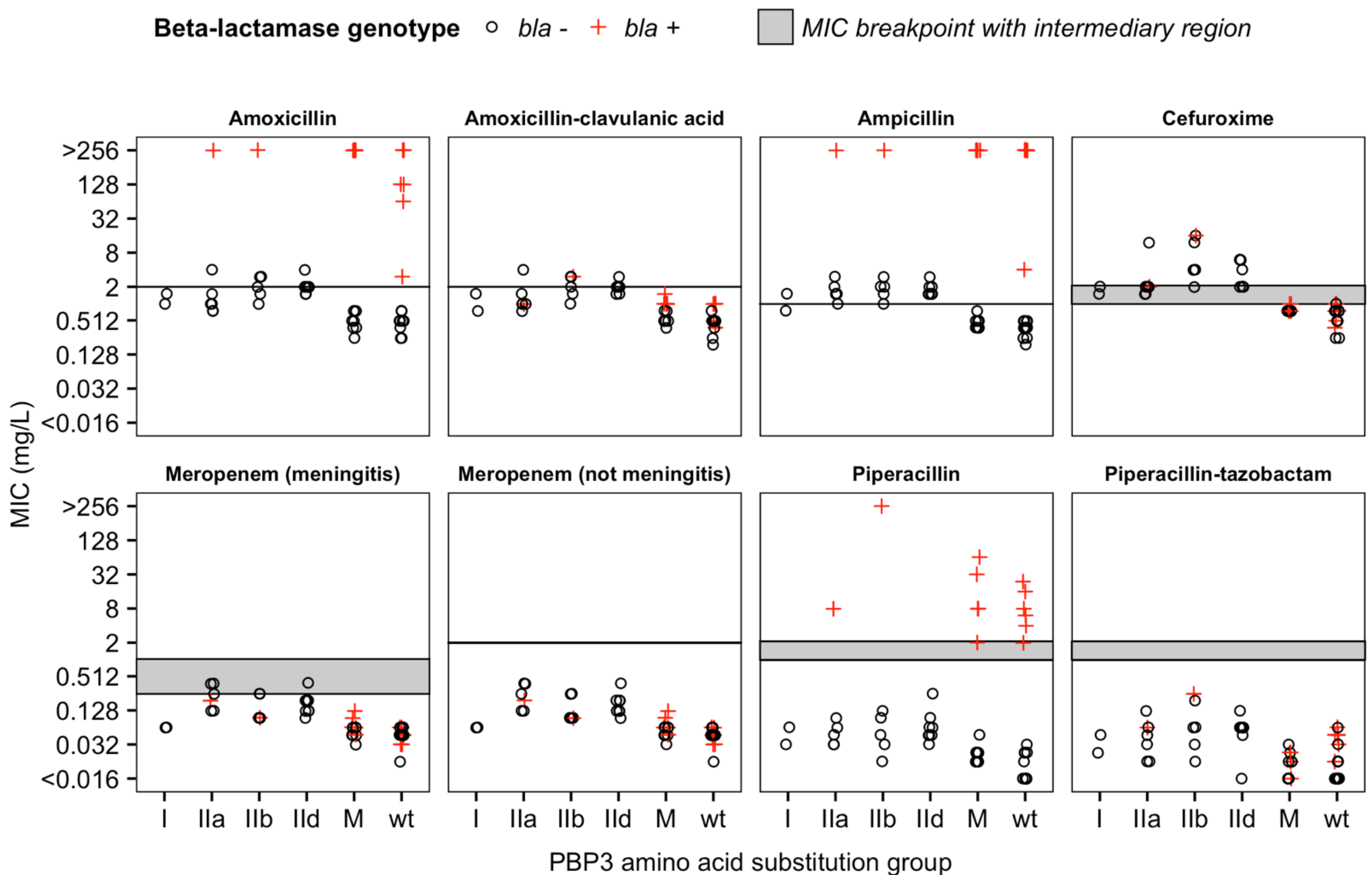

Fig. S1 Minimal inhibitory concentrations (MIC) of all isolated strains for tested antibiotics (amoxicillin, amoxicillin-clavulanic acid, ampicillin, cefuroxime, meropenem (meningitis), meropenem (not meningitis), piperacillin, piperacillin-tazobactam). The horizontal line indicates the MIC breakpoint and, if applicable, the grey region indicates the intermediary region. All antibiotic breakpoints are according to EUCAST with the exception of piperacillin and piperacillin-tazobactam which are according to CLSI.

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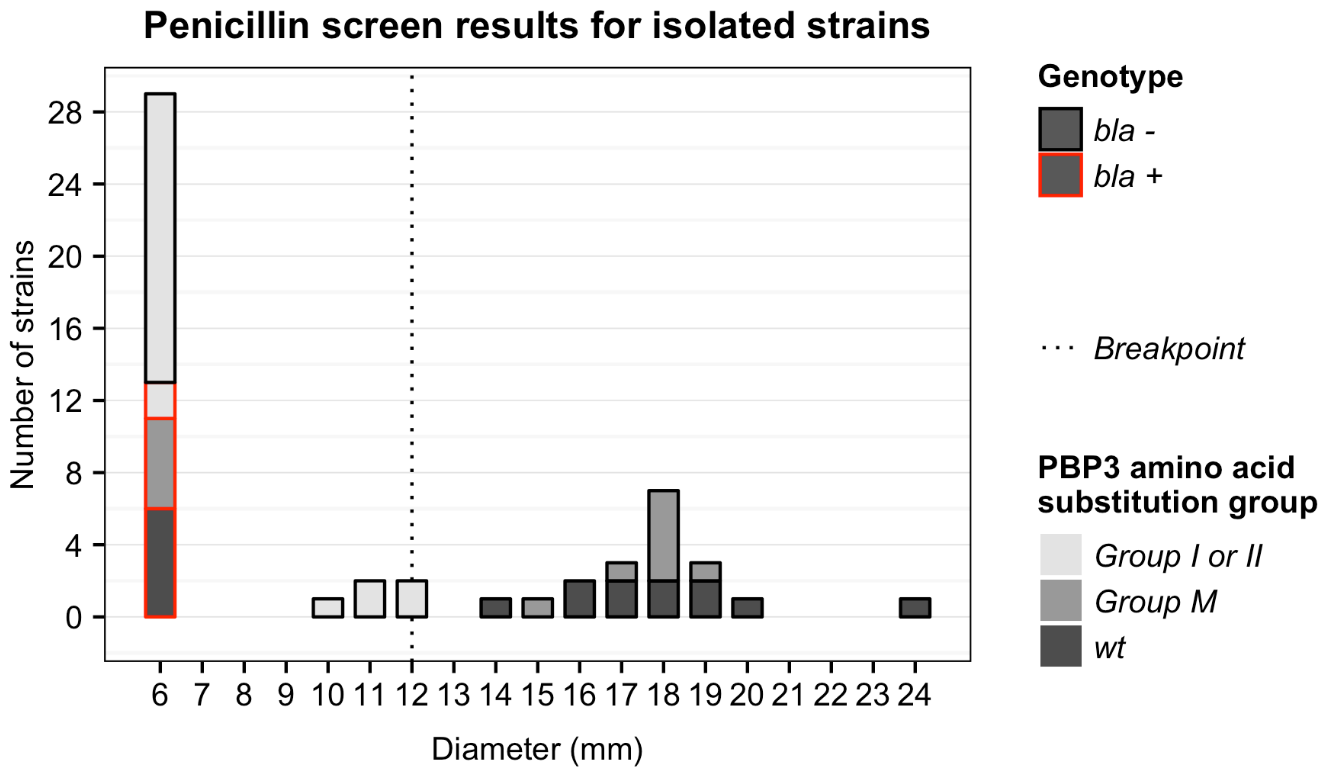

Fig. S2 Distribution of investigated *H. influenzae* strains (n=53) according to benzylpenicillin 1 U disc zone diameter (screening for beta-lactam resistance according to EUCAST). Wildtype: n=17; group M: n=13; group I and II: n=23; beta-lactamase positive isolates (red border): n=13. The dotted line indicates the diameter breakpoint (resistant ≤11 mm, susceptible ≥12 mm).

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        10         20         30         40         50
MVKFNSSRKS GKSKKTIRKL TAPETVKQNK PQKVFEKCFM RGRYMLSTVL
        60         70         80         90        100
ILLGLCALVA RAAYVQSINA DTLSNEADKR SLRKDEVLSV RGSILDRNGQ
       110        120        130        140        150
LLSVSVPMSA IVADPKTMLK ENSLADKERI AALAEELGMT ENDLVKKIEK
       160        170        180        190        200
NSKSGYLYLA RQVELSKANY IRRLKIKGII LETEHRRFYP RVEEAAHVVG
       210        220        230        240        250
YTDIDGNGIE GIEKSFNSLL VGKDGSRTVR KDKRGNIVAH ISDEKKYDAQ
       260        270        280        290        300
DVTLSIDEKL QSMVYREIKK AVSENNAESG TAVLVDVRTG EVLAMATAPS
       310        320        330        340        350
YNPNNRVGVK SELMRNRAIT DTFEPGSTVK PFVVLTALQR GVVKRDEIID
       360        370        380        390        400
TTSFKLSGKE IVDVAPRAQQ TLDEILMNSS NRGVSRLALR MPPSALMETY
       410        420        430        440        450
QNAGLSKPTD LGLIGEQVGI LNANRKRWAD IERATVAYGY GITATPLQIA
       460        470        480        490        500
RAYATLGSFG VYRPLSITKV DPPVIGKRVF SEKITKDIVG ILEKVAIKNK
       510        520        530        540        550
RAMVEGYRVG VKTGTARKIE NGHYVNKYVA FTAGIAPISD PRYALVVLIN
       560        570        580        590        600
DPKAGEYYGG AVSAPVFSNI MGYALRANAI PQDAEAAENT TTKSAKRIVY
       610
IGEHKNQKVN
```

Fig. S3 Protein sequence of the reference strain *H. influenzae* RD KW20 (UniProt Entry P45059). The group-specific amino acid substitutions Arg517His, Asn526Lys and Val547Ile are shown in green and their suggested interaction partners Arg517 and Glu324 are highlighted in yellow. The three motifs that define that active site of the serine penicillin-recognizing enzyme family are shown in blue, purple and red. The amino acid substitution Arg517His were found in all group I strains and might lead to a loss of an electrostatic interaction to Glu324. Substitution Asn526Lys were found in all group II strains and might lead to a loss of a hydrogen bond to Arg517. Substitution Val547Ile were found in all group M and in some group II strains and has most likely no impact on the protein structure. For more information on the identified mutations, see **Table 1**. For a representation of the mutations in the three-dimensional protein structure, see **Figures 4-6**. Amino acids in grey belong to a beta-loop that is not represented in the crystal structure that was used for structure analysis (PDB ID 3PBR).