

1 **Chromosomally and extrachromosomally mediated high-level gentamicin**  
2 **resistance in *Streptococcus agalactiae***

3  
4  
5 Parham Sendi,<sup>a</sup> Martina Furitsch,<sup>b</sup> Stefanie Mauerer,<sup>b</sup> Carlos Florindo,<sup>c</sup> Barbara C. Kahl,<sup>d</sup>  
6 Sarah Shabayek,<sup>b,e</sup> Reinhard Berner<sup>f</sup> and Barbara Spellerberg<sup>b#</sup>

7  
8 Department of Infectious Diseases, University Hospital of Bern, and Institute for Infectious  
9 Diseases, University of Bern, Bern, Switzerland<sup>a</sup>; Institute of Medical Microbiology and  
10 Hygiene, University of Ulm, Ulm, Germany<sup>b</sup>; National Institute of Health Department of  
11 Infectious Diseases, Lisboa, Portugal<sup>c</sup>; Institute of Medical Microbiology, University Hospital  
12 of Münster, Münster, Germany<sup>d</sup>; Microbiology and Immunology Department, Faculty of  
13 Pharmacy, Suez Canal University, Egypt<sup>e</sup>; Clinic and Polyclinic of Pediatrics and Adolescent  
14 Medicine, Technische Universität Dresden (Carl Gustav Carus University Hospital), Dresden,  
15 Germany<sup>f</sup>

16  
17 Running Head: High-Level Gentamicin Resistance in *S. agalactiae*

18  
19  
20 #Address correspondence to Barbara Spellerberg, [barbara.spellerberg@uniklinik-ulm.de](mailto:barbara.spellerberg@uniklinik-ulm.de)

21 Institute of Medical Microbiology and Hygiene,  
22 University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany  
23 Phone: +49 (0) 731-50065333, Fax: +49 (0) 731-50065302

24  
25 Keywords: *Streptococcus agalactiae*, high-level gentamicin resistance, gentamicin,  
26 aminoglycosides.

27 **Abstract:**

28 *Streptococcus agalactiae* (group B *Streptococcus*, GBS) is a leading cause of sepsis in  
29 neonates. The rate of invasive GBS disease in non-pregnant adults also continues to climb.  
30 Aminoglycosides alone have little or no effect on GBS, but synergistic killing with penicillin  
31 has been shown *in vitro*. High-level gentamicin resistance (HLGR) in GBS isolates, however,  
32 leads to loss of a synergistic effect. We therefore performed a multicentre study to determine  
33 the frequency of HLGR GBS isolates and to elucidate the molecular mechanisms leading to  
34 gentamicin resistance. From eight centres in four countries, 1128 invasive and colonizing  
35 GBS isolates were pooled and investigated for the presence of HLGR. We identified two  
36 strains that displayed HLGR (BSU1203 and BSU452), both of which carried the *aacA-aphD*  
37 gene, typically conferring HLGR. Though, only one strain (BSU1203) also carried the  
38 previously described chromosomal gentamicin resistance transposon, designated Tn3706. In  
39 the other strain (BSU452), plasmid purification and subsequent DNA sequencing resulted in  
40 the detection of plasmid pIP501 carrying a remnant of a Tn3 family transposon. Its ability to  
41 confer HLGR was proven by transfer into an *Enterococcus faecalis* isolate. Conversely, loss  
42 of HLGR was documented after curing both GBS BSU452 and the transformed *E. faecalis*  
43 strain from the plasmid. This is the first report showing a plasmid mediated HLGR in GBS.  
44 Thus, in our clinical GBS isolates HLGR is mediated both chromosomally and  
45 extrachromosomally.

46

47

48

49

50

51

52

53 **Introduction**

54 *Streptococcus agalactiae*, alternatively designated group B *Streptococcus* (GBS), is a leading  
55 cause of morbidity and mortality in neonates and pregnant women. Recommendations for  
56 diagnosing maternal GBS colonization and administering intrapartum antimicrobial  
57 prophylaxis have led to a significant decrease in these infections (1). The rate of invasive  
58 GBS disease in non-pregnant adults, however, continues to climb (2). Elderly persons and  
59 those with underlying diseases – two expanding segments of the population – are at increased  
60 risk (3). Treatment concepts for invasive GBS infections in non-pregnant adults have not been  
61 established. Clinical isolates of GBS are susceptible to penicillin, the antimicrobial agent of  
62 choice for treating invasive diseases. Several publications advocate the addition of an  
63 aminoglycoside to penicillin or ampicillin for infective endocarditis (4) and periprosthetic  
64 joint infections (5), although aminoglycosides have ototoxic and nephrotoxic side effects, in  
65 particular in the elderly. Aminoglycosides alone have little or no effect on GBS, but  
66 synergistic killing with penicillin has been shown *in vitro* (6). In case of the presence of high-  
67 level gentamicin resistance (HLGR) in a bacterial isolate, there is a lack of a synergistic  
68 effect.

69 While HLGR in *Enterococcus* spp. is frequently found (7), to the best of our knowledge, only  
70 two HLGR GBS strains have been previously reported (8, 9). Most diagnostic laboratories do  
71 not test routinely for HLGR in GBS. Thus, little is known about the frequency of HLGR  
72 GBS, the mechanisms of acquiring HLGR and the potential to spread genetic elements  
73 associated with HLGR.

74 The aim of this study was to estimate the frequency of HLGR GBS isolates (i) in  
75 systematically and continuously collected GBS isolates from colonized pregnant and non-  
76 pregnant women and (ii) in GBS isolates pooled in a collection that stems from various  
77 selected patient populations. Upon detection of HLGR isolates, we elaborated the molecular  
78 mechanism conferring this resistance.

79 **Materials and Methods**

80 ***GBS isolates***

81 The study consisted of 1128 GBS isolates. Of these, 464 (41%) were pooled from various  
82 GBS collections (Table 1). These isolates stem from various centres and were previously  
83 investigated in another context (10-15). The other 664 (59%) GBS isolates were prospectively  
84 collected and screened for the presence of HLGR. The origin of GBS isolation, the  
85 association with diseases or colonization, and the sampling period are presented in Table 1.

86 ***Definition and identification of HLGR in GBS***

87 No definition of HLGR in GBS has been published. According to the recommended screening  
88 tests for the detection of HLGR in *Enterococcus* spp., the resistant isolates have an MIC  $\geq$ 500  
89 mg/L (16). In addition, HLGR isolates with an MIC  $>$ 500 mg/L have been reported (17, 18).  
90 Therefore, HLGR in GBS was defined when the gentamicin MIC determined by Etest was  
91  $\geq$ 512 mg/L. All MIC determinations were confirmed with  $\geq$ 3 measurements.

92 Two different methods for the identification of HLGR in GBS were applied. Five hundred  
93 sixty-one isolates (49.7%) were plated on Mueller Hinton agar supplemented with 256 mg/L  
94 of gentamicin. Subsequently, the MIC of growing GBS colonies was determined by Etest. For  
95 567 (50.3%) isolates, the MIC was primarily determined by Etest without a prior HLGR  
96 screening test.

97 ***Bacterial strains***

98 The strains used in this study are presented in Table 2. The plasmid-free recipient used in the  
99 mating experiments was *E. faecalis* (BSU386), a clinical blood culture isolate without HLGR.

100 ***Genetic basis of HLGR***

101 Standard recombinant DNA techniques were used for nucleic acid preparation and analysis.  
102 Plasmid DNA was isolated and purified using the QIAprep Spin Miniprep Kit (QIAGEN,  
103 Hilden, Germany), according to the manufacturer's instructions. PCR was performed with  
104 *Taq* polymerase according to the manufacturer's protocol (Roche Diagnostics, Mannheim,

105 Germany), with 35 cycles of amplification steps consisting of 1 min at 94°C, 1 min at 55°C  
106 and 1 min at 72°C. PCR products were sequenced on an ABI PRISM 310 Genetic Analyzer,  
107 using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems,  
108 Weiterstadt, Germany). To identify HLGR resistance gene, we performed multiplex PCR as  
109 described by Vakulenko *et al.*(19). For the detection of Tn3706 specific nucleotide sequences,  
110 we used PCR with the primers O1, O2 and O3, as described by Horaud *et al* (20). The primers  
111 used for this study are presented in Table 3. Detection of open reading frames (ORFs) was  
112 carried out by using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Sequence  
113 comparison was performed by using the BLAST system  
114 (<http://www.ncbi.nlm.nih.gov/BLAST/>). Nucleotide sequences were submitted to GenBank  
115 under the accession number (KP698941).

#### 116 ***Transfer and mobilization of the genetic element conferring HLGR***

117 To investigate the potential transfer of resistance, we transformed *E. faecalis* (BSU386) with  
118 plasmid DNA, as described previously (21). Curing the transformed *E. faecalis* strain and  
119 GBS BSU452 with HLGR was achieved as follows. The strains were exposed in an overnight  
120 culture to serial twofold dilutions of ciprofloxacin in Todd Hewitt broth plus 0.5% yeast  
121 extract and Luria-Bertani medium. Bacterial cultures containing the highest subinhibitory  
122 concentration of ciprofloxacin (i.e. 0.625 mg/L for GBS BSU452 and 10 mg/L for *E. faecalis*  
123 BSU580) were plated on antibiotic-free tryptic soy agar blood plates and grown overnight at  
124 37°C. Single colonies were then tested for loss of resistance to gentamicin by subculturing  
125 onto Mueller Hinton agar plates containing 256 mg/L gentamicin. The MIC for gentamicin  
126 was then determined by Etest.

127

128

129

130

131 **Results**

132 ***GBS isolates with HLGR***

133 Among 1128 GBS isolates, two (0.17%) strains with HLGR were identified. One strain  
134 (BSU1203, MIC >1024 mg/L) was obtained from a 35-year-old Swiss woman during prenatal  
135 screening. The second HLGR GBS strain (BSU452, MIC 512 mg/L) was isolated in a  
136 respiratory specimen from a 26-year old man with cystic fibrosis.

137 ***PCR detection of genes conferring HLGR in GBS***

138 To investigate the resistance determinants in HLGR GBS strains, we performed PCRs that  
139 were specific for the *aac(6')-Ie-aph(2'')-Ia* gene and the flanking IS256 element (19). The  
140 *aac(6')-Ie-aph(2'')-Ia* gene was readily detected in both strains, yielding the expected product  
141 of 348 bp (19).

142 ***Detection of an old transposon and a novel element***

143 A PCR with a primer set annealing on the structural gentamicin resistance gene and the IS256  
144 sequence, located downstream of this gene, showed the expected 369-bp product for strain  
145 GBS BSU1203 (19), indicating the presence of the previously described chromosomal  
146 transposon Tn3706. For further characterization of the resistance determinant, PCRs that were  
147 specific for insertion sequence elements of Tn3706 were performed, as published previously  
148 (20). These PCRs were positive in GBS strain BSU1203 and matched those previously  
149 described in HLGR GBS strain B128 (20), confirming the presence of Tn3706. In GBS strain  
150 BSU452 the *aac(6')-Ie-aph(2'')-Ia* gene was found, but none of the PCR reactions specific for  
151 the transposon structures of Tn5281, Tn4001 or Tn3706 (20), yielded a product, suggesting  
152 the presence of a novel HLGR resistance determinant in this strain.

153 ***Characterization of a novel mobile genetic element conferring HLGR in GBS***

154 To identify the genetic structure of GBS strain BSU452 carrying the *aac(6')-Ie-aph(2'')-Ia*  
155 gene, we performed an inverse PCR on a plasmid preparation of BSU452. Primers annealing  
156 to the gentamicin resistance gene (Table 3) and directed towards DNA regions upstream and

157 downstream of *aac(6')-Ie-aph(2'')-Ia* and yielded a PCR product about 2 kb in length, which  
158 was completely sequenced. Nucleotide comparison with the GenBank database revealed a  
159 100% identity of nucleotides 1-253 and 721-2029 with plasmid pTEF1 of *E. faecalis* strain  
160 V583 (AE016833.1). Several ORFs were identified on the 2 kb PCR product and comparison  
161 of the deduced amino acids with the GenBank database revealed a DNA resolvase fragment  
162 and one copy of an insertion sequence element with high homology to *IS1216*. This structure  
163 displayed high similarities to a Tn3 family transposon.

#### 164 ***Transfer and mobilization of the resistance determinant in association with HLGR***

165 The HLGR resistance genes and the flanking DNA sequences in GBS BSU1203 matched  
166 those previously identified in GBS B128, and because thorough molecular analyses on the  
167 acquisition of HLGR have been published for that strain (8, 22, 23), further investigations  
168 focussed on GBS BSU452. Tn3 family transposons are typically located on plasmids. To  
169 investigate if this is the case in strain GBS BSU452 and to characterize the potential of  
170 spreading HLGR to other isolates, we transformed the gentamicin susceptible *E. faecalis*  
171 strain BSU386 with the plasmid preparation obtained from GBS BSU452. Positive clones (i.e.  
172 designated *E. faecalis* BSU580, which carries the mobile element of BSU452) were obtained  
173 upon plating the transformed strain onto the HLGR screening agar, as described above. A  
174 subsequent gentamicin evaluation revealed an increase in MIC from 12 mg/L to  $\geq 1024$  mg/L  
175 (Table 2). To ensure that the increased MIC was due to the uptake of the plasmid DNA,  
176 plasmid preparations were subjected to gel electrophoresis (Figure 1), which showed the  
177 presence of large plasmids in the HLGR strains BSU452 and BSU580. Further confirmation  
178 of the successful transfer was achieved by PCR showing the presence of the *aac(6')-Ie-*  
179 *aph(2'')-Ia* gene and a lack of any flanking *IS256* sequences in *E. faecalis* strain BSU580. To  
180 confirm that the newly detected mobile genetic element is indeed located on a plasmid, we  
181 attempted to cure GBS BSU452 and the transformed *E. faecalis* BSU580 from the plasmid.  
182 This was successfully achieved by growing the strains in subinhibitory conditions of

183 ciprofloxacin, as described by Eliopoulos *et al.* (24). Under these conditions, clones of both  
184 GBS BSU452 and the transformed *E. faecalis* strain BSU580 lost their elevated resistance to  
185 gentamicin. The MICs decreased in GBS strain BSU729 (i.e. strain BSU452 after plasmid  
186 curing) from 512 mg/L to 24 mg/L and in *E. faecalis* BSU720 (i.e. strain BSU580 after  
187 plasmid curing) from  $\geq 1024$  mg/L to 12 mg/L (Table 2). In addition, the lack of a plasmid in  
188 the cured strains could be demonstrated by gel electrophoresis (Figure 1). Plasmid loss in the  
189 presence of subinhibitory ciprofloxacin occurred at a frequency of about 0.02% (1 in 4500  
190 colonies in GBS and 1 in 6000 colonies of *E. faecalis*). One of the most commonly found  
191 plasmids in GBS and enterococci is pIP501. To determine, if the detected plasmid is pIP501,  
192 the plasmid preparation obtained from GBS BSU 452 was subjected to PCR as detailed  
193 elsewhere (25). PCR products were sequenced and the presence of pIP501 in GBS BSU 452  
194 confirmed.

195

## 196 **Discussion**

197 In contrast to screens for *Enterococcus* spp., GBS surveillance schemes usually do not include  
198 gentamicin susceptibility testing, and screening for HLGR is often omitted in clinical  
199 laboratories. The prevalence of HLGR in GBS is, therefore, unknown. Previously, two HLGR  
200 GBS strains were reported. One of them (B128) was isolated from an infected leg wound in  
201 1987 (8), the other from a 49-year-old woman with a urinary tract infection, published in  
202 2002 (9). We identified two further colonizing strains in a collection of over 1000 isolates  
203 (0.17%). This proportion is in contrast to a previously published Argentinian study (18).  
204 Among 141 strains, the authors found 13.5% HLGR GBS. Although no firm epidemiological  
205 conclusion about the frequency of HLGR isolates can be made on the basis of these two  
206 studies, it should be noted that up to 20% – 35% of women are colonized with GBS (26), and  
207 that the absolute number of HLGR GBS isolates may be much higher than previously  
208 estimated (27). Thus, for a patient receiving penicillin-gentamicin combination therapy for



209 invasive GBS infection, and considering the potential side effects of aminoglycosides, the  
210 presence of HLGR is of significant clinical relevance.

211 Investigations on the genetic basis for HLGR in *E. faecalis* led to the identification of the  
212 *aacA-aphD* gene (28). It is typically found on the composite transposon Tn5281. The  
213 transposon resembles Tn4001 in *Staphylococcus aureus* and is characterized by the presence  
214 of two IS256 copies flanking the transposon structure. The *aacA-aphD* gene, later designated  
215 *aac(6')-aph(2'')*, encodes a bifunctional enzyme with an acetyltransferase and a  
216 phosphotransferase function. The enzyme catalyzes inactivation of the vast majority of  
217 aminoglycosides with the exception of streptomycin. In most *Enterococcus* spp. with HLGR,  
218 the transposon harbouring the *aac(6')-aph(2'')* gene is found on a plasmid (29). Truncated  
219 forms of Tn4001 are typically located on plasmid DNA (30). Intact Tn4001 transposons can  
220 also be located on chromosomal DNA. In the previously described HLGR GBS strain B128,  
221 the *aacA-aphD* gene was found on a Tn4001 derivative (designated Tn3706), located on  
222 chromosomal DNA (8). In one of our strains (BSU1203), the finding of transposon Tn3706  
223 conferring HLGR is in agreement with the previously published findings about HLGR GBS  
224 strain B128 (8, 22, 23)). Horaud *et al.* (23) described that its transposition from *E. faecalis*  
225 occurred on GBS plasmid pIP501. However, after conjugative transfer between GBS strains,  
226 the hybrid replicons pIP501::Tn3706 were found to be structurally unstable. This observation  
227 indicated that streptococcal pIP501-like plasmids do not constitute appropriate delivery  
228 vectors for the dissemination of Tn3706 among GBS, and therefore, HLGR is found relatively  
229 rarely among GBS (23). Although these arguments speak against a high potential for spread,  
230 the persistence of HLGR in GBS128 and BSU1203 indicates that Tn3706 can be stably  
231 integrated into the chromosome.

232 In GBS BSU452, we identified a different mobile genetic element. The genes surrounding the  
233 *aac(6')-aph(2'')* gene did not display the structures of transposon Tn4001, or any of the  
234 closely related derivatives or its truncated forms. We detected plasmid pIP501, which is a

235 conjugative plasmid that often carries multiresistance genes. It has previously been described  
236 in *S. agalactiae* in association with HLGR and belongs to the Inc18 group of plasmids (25).  
237 Tn3 family transposons are commonly associated with Inc18 plasmids and often confer  
238 antibiotic resistance in *Enterococcus* spp. (31). They are, however, typically associated with  
239 glycopeptide and macrolide resistance (32) and not HLGR. Investigators have previously  
240 reported the presence of an *IS1216* transposase on Tn3-like remnants,(33) as we found in our  
241 GBS BSU452 strain; however, *IS1216* is typically associated with tetracycline resistance in  
242 streptococcal species (34). To the best of our knowledge, the detection of the *aacA-aphD* gene  
243 on a Tn3-like transposon and the presence of *IS216* in association with HLGR is a novel  
244 finding. It has been reported neither for enterococci nor for GBS.

245 The resistance determinant in GBS BSU452 shows close homologies to parts of the  
246 enterococcal resistance plasmid pTEF1 of the *E. faecalis* strain V583 (35), suggesting that it  
247 may have been transferred through horizontal gene transfer. This is, however, speculative for  
248 GBS strain BSU452, since the presence of a HLGR Tn3-like transposon in GBS has not been  
249 previously described. Nevertheless, horizontal gene transfer of resistance genes from  
250 *Enterococcus* spp. to other gram-positive bacteria by mobile genetic elements is a well-  
251 described mechanism in the spread of antibiotic resistance (31, 32). Horizontal gene transfer  
252 has recently been suggested for the acquisition of vancomycin resistance genes in GBS (36).  
253 GBS strain BSU452 was isolated from the sputum of a cystic fibrosis patient, but there was  
254 no evidence of enterococcal colonization. Considering that patients with cystic fibrosis are  
255 often treated with antibiotics (including aminoglycosides), and their microbiome in the  
256 respiratory tract is different from that of untreated healthy patients, it is possible that  
257 horizontal gene transfer to GBS originated from the selected flora. However this hypothesis  
258 cannot be proven in our case and remains speculation. Though, a plasmid-borne HLGR has  
259 high potential for further spread in a GBS population, the concern of this phenomenon cannot  
260 be predicted yet. In this study, we demonstrated that pIP501, including the HLGR resistance

261 determinant of GBS452, could easily be transferred to *E. faecalis*. Thus, it is conceivable that  
262 transfer to other GBS isolates is also possible, especially in view of the fact that pIP501 is a  
263 broad host range plasmid, well established in GBS and enterococci.

264 In conclusion, the overall frequency of HLGR GBS in our large collection of isolates was  
265 low. Molecular investigations revealed a transposon located on the chromosome, as  
266 previously described in a single isolate, (8, 22, 23) and a Tn3 family transposon conferring  
267 HLGR in association with pIP501. These findings point towards a new dimension of potential  
268 spread of HLGR within GBS.

269

#### 270 **Acknowledgement**

271 We are indebted to Professor Alessandra Carattoli for valuable comments and critical review  
272 of the manuscript.

#### 273 **Funding and Transparency Declarations**

274 This work was supported in part by the Velux Foundation, Zurich, Switzerland (Proj. No. 724  
275 to P.S.).

276 Conflicts of interest: none.

277

278

279

280

281

282

283

284

285

#### 286 **References**

- 287 1. **Schrag SJ, Zell ER, Lynfield R, Roome A, Arnold KE, Craig AS, Harrison LH,**  
288 **Reingold A, Stefonek K, Smith G, Gamble M, Schuchat A.** 2002. A population-  
289 based comparison of strategies to prevent early-onset group B streptococcal disease in  
290 neonates. *N Engl J Med* **347**:233-239.
- 291 2. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S,**  
292 **Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA,**  
293 **Zell ER, Schuchat A, Schrag SJ.** 2008. Epidemiology of invasive group B  
294 streptococcal disease in the United States, 1999-2005. *Jama* **299**:2056-2065.
- 295 3. **Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison**  
296 **LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER,**  
297 **Jackson D, Thompson T, Schrag SJ.** 2009. Increasing burden of invasive group B  
298 streptococcal disease in nonpregnant adults, 1990-2007. *Clin Infect Dis* **49**:85-92.
- 299 4. **Westling K, Aufwerber E, Ekdahl C, Friman G, Gardlund B, Julander I, Olaison**  
300 **L, Olesund C, Rundstrom H, Snygg-Martin U, Thalme A, Werner M, Hogevisk H.**  
301 2007. Swedish guidelines for diagnosis and treatment of infective endocarditis. *Scand*  
302 *J Infect Dis* **39**:929-946.
- 303 5. **Zimmerli W, Trampuz A, Ochsner PE.** 2004. Prosthetic-joint infections. *N Engl J*  
304 *Med* **351**:1645-1654.
- 305 6. **Baker CN, Thornsberry C, Facklam RR.** 1981. Synergism, killing kinetics, and  
306 antimicrobial susceptibility of group A and B streptococci. *Antimicrob Agents*  
307 *Chemother* **19**:716-725.
- 308 7. **Schouten MA, Voss A, Hoogkamp-Korstanje JA.** 1999. Antimicrobial  
309 susceptibility patterns of enterococci causing infections in Europe. The European VRE  
310 Study Group. *Antimicrob Agents Chemother* **43**:2542-2546.
- 311 8. **Buu-Hoi A, Le Bouguenec C, Horaud T.** 1990. High-level chromosomal gentamicin  
312 resistance in *Streptococcus agalactiae* (group B). *Antimicrob Agents Chemother*  
313 **34**:985-988.
- 314 9. **Liddy H, Holliman R.** 2002. Group B *Streptococcus* highly resistant to gentamicin. *J*  
315 *Antimicrob Chemother* **50**:142-143.
- 316 10. **Brimil N, Barthell E, Heindrichs U, Kuhn M, Luticken R, Spellerberg B.** 2006.  
317 Epidemiology of *Streptococcus agalactiae* colonization in Germany. *Int J Med*  
318 *Microbiol* **296**:39-44.
- 319 11. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-**  
320 **Pereira F, Castro R, Borrego MJ, Santos-Sanches I.** 2014. Epidemiological  
321 surveillance of colonising group B *Streptococcus* epidemiology in the Lisbon and  
322 Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type  
323 IV/clonal complex 17 clone. *Euro Surveill* **19**.
- 324 12. **Fluegge K, Siedler A, Heinrich B, Schulte-Moenting J, Moennig MJ, Bartels DB,**  
325 **Dammann O, von Kries R, Berner R, German Pediatric Surveillance Unit Study**  
326 **G.** 2006. Incidence and clinical presentation of invasive neonatal group B  
327 streptococcal infections in Germany. *Pediatrics* **117**:e1139-1145.
- 328 13. **Fluegge K, Wons J, Spellerberg B, Swoboda S, Siedler A, Hufnagel M, Berner R.**  
329 2011. Genetic differences between invasive and noninvasive neonatal group B  
330 streptococcal isolates. *Pediatr Infect Dis J* **30**:1027-1031.
- 331 14. **Eickel V, Kahl B, Reinisch B, Dubbers A, Kuster P, Brandt C, Spellerberg B.**  
332 2009. Emergence of respiratory *Streptococcus agalactiae* isolates in cystic fibrosis  
333 patients. *PLoS One* **4**:e4650.
- 334 15. **Shabayek S, Abdalla S, Abouzeid AM.** 2014. Serotype and surface protein gene  
335 distribution of colonizing group B streptococcus in women in Egypt. *Epidemiol Infect*  
336 **142**:208-210.

- 337 16. **Clinical and Laboratory Standards Institute.** 2014. Performance Standards for  
338 Antimicrobial Susceptibility Testing: Twenty-Fourth Information Supplement M100-  
339 S24, Screening Test for Detection of High-Level Aminoglycoside Resistance (HLAR)  
340 in *Enterococcus* species. CLSI, Wayne, PA, USA.
- 341 17. **Weinbren MJ, Johnson AP, Woodford N.** 2000. Defining high-level gentamicin  
342 resistance in enterococci. *J Antimicrob Chemother* **45**:404-405.
- 343 18. **Villar HE, Jugo MB.** 2013. [Emergence of high-level resistance to gentamicin and  
344 streptomycin in *Streptococcus agalactiae* in Buenos Aires, Argentina]. *Rev Esp*  
345 *Quimioter* **26**:112-115.
- 346 19. **Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA,**  
347 **Chow JW.** 2003. Multiplex PCR for detection of aminoglycoside resistance genes in  
348 enterococci. *Antimicrob Agents Chemother* **47**:1423-1426.
- 349 20. **Horaud T, de Cespedes G, Trieu-Cuot P.** 1996. Chromosomal gentamicin resistance  
350 transposon Tn3706 in *Streptococcus agalactiae* B128. *Antimicrob Agents Chemother*  
351 **40**:1085-1090.
- 352 21. **Friesenegger A, Fiedler S, Devriese LA, Wirth R.** 1991. Genetic transformation of  
353 various species of *Enterococcus* by electroporation. *FEMS Microbiol Lett* **63**:323-327.
- 354 22. **Kaufhold A, Podbielski A, Horaud T, Ferrieri P.** 1992. Identical genes confer high-  
355 level resistance to gentamicin upon *Enterococcus faecalis*, *Enterococcus faecium*, and  
356 *Streptococcus agalactiae*. *Antimicrob Agents Chemother* **36**:1215-1218.
- 357 23. **Horaud T, de Cespedes G, Trieu-Cuot P.** 1996. Chromosomal gentamicin resistance  
358 transposon Tn3706 in *Streptococcus agalactiae* B128. *Antimicrob Agents Chemother*  
359 **40**:1085-1090.
- 360 24. **Eliopoulos GM, Wennersten C, Zigelboim-Daum S, Reiszner E, Goldmann D,**  
361 **Moellering RC, Jr.** 1988. High-level resistance to gentamicin in clinical isolates of  
362 *Streptococcus (Enterococcus) faecium*. *Antimicrob Agents Chemother* **32**:1528-1532.
- 363 25. **Brantl S, Nuez B, Behnke D.** 1992. In vitro and in vivo analysis of transcription  
364 within the replication region of plasmid pIP501. *Mol Gen Genet* **234**:105-112.
- 365 26. **Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L,**  
366 **Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in  
367 European countries. *Acta Obstet Gynecol Scand* **87**:260-271.
- 368 27. **Murdoch DR, Reller LB.** 2001. Antimicrobial susceptibilities of group B  
369 streptococci isolated from patients with invasive disease: 10-year perspective.  
370 *Antimicrob Agents Chemother* **45**:3623-3624.
- 371 28. **Ferretti JJ, Gilmore KS, Courvalin P.** 1986. Nucleotide sequence analysis of the  
372 gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-  
373 aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and  
374 identification and cloning of gene regions specifying the two activities. *J Bacteriol*  
375 **167**:631-638.
- 376 29. **Horodniceanu T, Bougueleret L, El-Solh N, Bieth G, Delbos F.** 1979. High-level,  
377 plasmid-borne resistance to gentamicin in *Streptococcus faecalis* subsp. zymogenes.  
378 *Antimicrob Agents Chemother* **16**:686-689.
- 379 30. **Casetta A, Hoi AB, de Cespedes G, Horaud T.** 1998. Diversity of structures  
380 carrying the high-level gentamicin resistance gene (*aac6-aph2*) in *Enterococcus*  
381 *faecalis* strains isolated in France. *Antimicrob Agents Chemother* **42**:2889-2892.
- 382 31. **Palmer KL, Kos VN, Gilmore MS.** 2010. Horizontal gene transfer and the genomics  
383 of enterococcal antibiotic resistance. *Curr Opin Microbiol* **13**:632-639.
- 384 32. **Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A.** 2010. Mobile  
385 genetic elements and their contribution to the emergence of antimicrobial resistant  
386 *Enterococcus faecalis* and *Enterococcus faecium*. *Clin Microbiol Infect* **16**:541-554.

- 387 33. **Tanous C, Chambellon E, Sepulchre AM, Yvon M.** 2005. The gene encoding the  
388 glutamate dehydrogenase in *Lactococcus lactis* is part of a remnant Tn3 transposon  
389 carried by a large plasmid. *J Bacteriol* **187**:5019-5022.
- 390 34. **Novais C, Freitas AR, Silveira E, Baquero F, Peixe L, Roberts AP, Coque TM.**  
391 2012. Different genetic supports for the tet(S) gene in Enterococci. *Antimicrob Agents*  
392 *Chemother* **56**:6014-6018.
- 393 35. **Paulsen IT, Banerjee L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE,**  
394 **Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L,**  
395 **Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W,**  
396 **Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T,**  
397 **Radune D, Ketchum KA, Dougherty BA, Fraser CM.** 2003. Role of mobile DNA  
398 in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299**:2071-  
399 2074.
- 400 36. **Park C, Nichols M, Schrag SJ.** 2014. Two cases of invasive vancomycin-resistant  
401 group B streptococcus infection. *N Engl J Med* **370**:885-886.
- 402 37. **Frohlicher S, Reichen-Fahrni G, Muller M, Surbek D, Droz S, Spellerberg B,**  
403 **Sendi P.** 2014. Serotype distribution and antimicrobial susceptibility of group B  
404 streptococci in pregnant women: results from a Swiss tertiary centre. *Swiss Med Wkly*  
405 **144**:w13935.
- 406  
407

408 **Table 1.** The pooled collection of isolates investigated for the presence of high-level gentamicin resistance

Ref <sup>a</sup>	n <sup>a</sup>	Disease/Case definition	Origin of GBS isolation	Study type	Collection periods	Geographic origin	HLGR
(10)	75	No disease/colonization	Vaginal and rectal swabs from pregnant and non-pregnant women	Cross-sectional study	2001 – 2003	Aachen and Munich, Germany	0
(12)	60	EOD with invasive neonatal GBS infections	Isolation of GBS from blood or CSF and other sterile body fluids	Part of the prospective active surveillance study	2001 – 2003	Freiburg, Germany	0
(13)	50 <sup>b</sup>	Suspicion of EOD without proven invasive GBS disease	GBS isolates from non-sterile sites	Part of the prospective active surveillance study	2001 – 2003	Freiburg, Germany	0
(14)	30	Patients with cystic fibrosis	Respiratory samples	Collection of isolates <sup>c</sup>	2002 – 2008	Münster, Germany	1
(11)	150	No disease/colonization	Rectovaginal specimens from pregnant and non-pregnant women	Part of the national surveillance study	2005 – 2009	Lisbon, Portugal	0
-	97	No disease/colonization	Vaginal swabs from pregnant and non-pregnant women	- <sup>d</sup>	2009	Ulm, Germany	0
(15)	99	No disease/colonization	Vaginal swabs from pregnant and non-pregnant women	Cross-sectional study	2010	Ismailia, Egypt	0
(37)	364	No disease/colonization	Vaginal swabs from pregnant women	Cross-sectional study	2009 – 2010	Bern, Switzerland	1
-	203	Invasive group B <i>Streptococcus</i> infections	Isolation of GBS from blood, CSF and other sterile body fluids	- <sup>e</sup>	1998 – 2013	Bern, Switzerland	0
Total	1128						2

409 GBS, Group B *Streptococcus*; HLGR, high-level gentamicin resistance; Ref, reference; n, number of GBS isolates; EOD, early-onset disease.410 <sup>a</sup> GBS isolates investigated and published in a context other than the presence of HLGR. The number of GBS isolates investigated for HLGR may

411 vary from the number in the source publication for technical reasons.

412 <sup>b</sup> For this study, only GBS isolates with serotype III were available.

413 <sup>c</sup> GBS isolation occurred during a routine visit or during a visit due to an exacerbation of clinical symptoms.

414 <sup>d</sup> Prospective collection during routine diagnostic microbiology laboratory analysis. GBS isolates were investigated for this study.

415 <sup>e</sup> Collection of invasive GBS isolates (all age groups) during routine diagnostic microbiology laboratory analysis. GBS isolates were investigated for  
416 this study.

417

418



419 **Table 2.** Bacterial strains and their corresponding genetic elements conferring HLGR

Species	Strain	Description	MIC gentamicin	<i>aac(6')-Ie-aph(2'')-Ia</i> gene	Transposon
GBS	BSU1203	wild-type strain	≥1024 mg/L	yes	Tn3706
GBS	BSU452	wild-type strain	512 mg/L	yes	Tn3-like
<i>E. faecalis</i>	BSU386	wild-type strain	12 mg/L	no	no
<i>E. faecalis</i>	BSU580	BSU386 + pIP501 <sup>BSU452</sup>	≥1024 mg/L	yes	Tn3-like
<i>E. faecalis</i>	BSU720	BSU580 cured	12 mg/L	no	no
GBS	BSU729	BSU452 cured	24 mg/L	no	no

420 GBS, Group B *Streptococcus*; HLGR, high-level gentamicin resistance.

421

422

423

424

425 **Table 3.** Primers used for PCR and DNA sequencing

Name	Sequence (5' - 3')	Target gene
Inverse primer	CTT CAT CTT CCC AAG GCT CTG	<i>aac(6')</i> - <i>aph(2'')</i>
HLGR1		
Inverse primer	GCC AGA ACA TGA ATT ACA CGA GG	<i>aac(6')</i> - <i>aph(2'')</i>
HLGR2		
369 Vakulenko PCR	CAGGAATTTATCGAAAATGGTAGAAAAG	
369 Vakulenko PCR	CACAATCGACTAAAGAGTACCAATC	
348 Vakulenko PCR	CAGAGCCTTGGGAAGATGAAG	
348 Vakulenko PCR	CCTCGTGTAATTCATGTTCTGGC	
Primer O1	GGACCTACATGATGAATGGA	
Primer O2	CCTTTACAGAATATTCAATAATGC	
Primer O3	GTATAG CAATATGCAAATCC	
pip501-for	TCGCTCAATCACTACCAAGC	
pip501-rev	CTTGAACGAGTAAAGCCCTT	

426

427

428

429

430

431

432

433

434

435

436

437 **Figure 1.** Plasmid preparations of GBS and *E. faecalis* strains.  
438 Shown are plasmid preparations of *E. faecalis* and GBS strains separated by agarose gel  
439 electrophoresis (0.8 % gel). 1: *E. faecalis* strain BSU386 (wild-type strain without HLGR). 2:  
440 *E. faecalis* strain BSU 580 (wild-type strain BSU386 after transformation with plasmid  
441 preparation from *S. agalactiae* strain BSU452, displaying HLGR). 3: *E. faecalis* strain  
442 BSU720 (*E. faecalis* strain BSU580 after plasmid curing and loss of HLGR). 4: *S. agalactiae*  
443 strain BSU452 (patient isolate displaying HLGR). 5: *S. agalactiae* strain BSU729 (*S.*  
444 *agalactiae* strain BSU452 after plasmid curing and loss of HLGR). M: molecular size marker.  
445

