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

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

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Abstract:

Streptococcus agalactiae (group B *Streptococcus*, GBS) is a leading cause of sepsis in neonates. The rate of invasive GBS disease in non-pregnant adults also continues to climb. Aminoglycosides alone have little or no effect on GBS, but synergistic killing with penicillin has been shown *in vitro*. High-level gentamicin resistance (HLGR) in GBS isolates, however, leads to loss of a synergistic effect. We therefore performed a multicentre study to determine the frequency of HLGR GBS isolates and to elucidate the molecular mechanisms leading to gentamicin resistance. From eight centres in four countries, 1128 invasive and colonizing GBS isolates were pooled and investigated for the presence of HLGR. We identified two strains that displayed HLGR (BSU1203 and BSU452), both of which carried the *aacA-aphD* gene, typically conferring HLGR. Though, only one strain (BSU1203) also carried the previously described chromosomal gentamicin resistance transposon, designated Tn*3706*. In the other strain (BSU452), plasmid purification and subsequent DNA sequencing resulted in the detection of plasmid pIP501 carrying a remnant of a Tn*3* family transposon. Its ability to confer HLGR was proven by transfer into an *Enterococcus faecalis* isolate. Conversely, loss of HLGR was documented after curing both GBS BSU452 and the transformed *E. faecalis* strain from the plasmid. This is the first report showing a plasmid mediated HLGR in GBS. Thus, in our clinical GBS isolates HLGR is mediated both chromosomally and extrachromosomally.

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.

Materials and Methods

GBS isolates

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

Definition and identification of HLGR in GBS

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

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

Bacterial strains

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

Genetic basis of HLGR

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

Germany), with 35 cycles of amplification steps consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. PCR products were sequenced on an ABI PRISM 310 Genetic Analyzer, using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). To identify HLGR resistance gene, we performed multiplex PCR as described by Vakulenko *et al*.(19). For the detection of Tn*3706* specific nucleotide sequences, we used PCR with the primers O1, O2 and O3, as described by Horaud *et al* (20). The primers used for this study are presented in Table 3. Detection of open reading frames (ORFs) was carried out by using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence comparison was performed by using the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequences were submitted to GenBank under the accession number (KP698941).

Transfer and mobilization of the genetic element conferring HLGR

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

Results

GBS isolates with HLGR

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

PCR detection of genes conferring HLGR in GBS

To investigate the resistance determinants in HLGR GBS strains, we performed PCRs that were specific for the *aac(6')-Ie-aph(2")-Ia* gene and the flanking IS*256* element (19). The *aac(6')-Ie-aph(2")-Ia* gene was readily detected in both strains, yielding the expected product of 348 bp (19).

Detection of an old transposon and a novel element

A PCR with a primer set annealing on the structural gentamicin resistance gene and the IS*256* sequence, located downstream of this gene, showed the expected 369-bp product for strain GBS BSU1203 (19), indicating the presence of the previously described chromosomal transposon Tn*3706*. For further characterization of the resistance determinant, PCRs that were specific for insertion sequence elements of Tn*3706* were performed, as published previously (20). These PCRs were positive in GBS strain BSU1203 and matched those previously described in HLGR GBS strain B128 (20), confirming the presence of Tn*3706*. In GBS strain BSU452 the *aac(6')-Ie-aph(2")-Ia* gene was found, but none of the PCR reactions specific for the transposon structures of Tn*5281,* Tn*4001* or Tn*3706* (20), yielded a product, suggesting the presence of a novel HLGR resistance determinant in this strain.

Characterization of a novel mobile genetic element conferring HLGR in GBS

To identify the genetic structure of GBS strain BSU452 carrying the *aac(6')-Ie-aph(2")-Ia* gene, we performed an inverse PCR on a plasmid preparation of BSU452. Primers annealing 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 IS*1216*. This structure
163 displayed high similarities to a Tn*3* 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 IS*256* 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

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

Discussion

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

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

Investigations on the genetic basis for HLGR in *E. faecalis* led to the identification of the *aacA-aphD* gene (28). It is typically found on the composite transposon Tn*5281*. The transposon resembles Tn*4001* in *Staphylococcus aureus* and is characterized by the presence of two IS*256* copies flanking the transposon structure. The *aacA-aphD* gene, later designated *aac(6')- aph(2")*, encodes a bifunctional enzyme with an acetyltransferase and a phosphotransferase function. The enzyme catalyzes inactivation of the vast majority of aminoglycosides with the exception of streptomycin. In most *Enterococcus* spp. with HLGR, the transposon harbouring the *aac(6')- aph(2")* gene is found on a plasmid (29). Truncated forms of Tn*4001* are typically located on plasmid DNA (30). Intact Tn*4001* transposons can also be located on chromosomal DNA. In the previously described HLGR GBS strain B128, the *aacA-aphD* gene was found on a Tn*4001* derivative (designated Tn*3706*), located on chromosomal DNA (8). In one of our strains (BSU1203), the finding of transposon Tn*3706* conferring HLGR is in agreement with the previously published findings about HLGR GBS strain B128 (8, 22, 23)). Horaud *et al*. (23) described that its transposition from *E. faecalis* occurred on GBS plasmid pIP501. However, after conjugative transfer between GBS strains, the hybrid replicons pIP501::Tn*3706* were found to be structurally unstable. This observation indicated that streptococcal pIP501-like plasmids do not constitute appropriate delivery vectors for the dissemination of Tn*3706* among GBS, and therefore, HLGR is found relatively rarely among GBS (23). Although these arguments speak against a high potential for spread, the persistence of HLGR in GBS128 and BSU1203 indicates that Tn*3706* can be stably integrated into the chromosome.

In GBS BSU452, we identified a different mobile genetic element. The genes surrounding the *aac(6')- aph(2")* gene did not display the structures of transposon Tn*4001*, or any of the closely related derivatives or its truncated forms. We detected plasmid pIP501, which is a

conjugative plasmid that often carries multiresistance genes. It has previously been described in *S. agalactiae* in association with HLGR and belongs to the Inc18 group of plasmids (25). Tn3 family transposons are commonly associated with Inc18 plasmids and often confer antibiotic resistance in *Enterococcus* spp. (31). They are, however, typically associated with glycopeptide and macrolide resistance (32) and not HLGR. Investigators have previously reported the presence of an IS*1216* transposase on Tn*3*-like remnants,(33) as we found in our GBS BSU452 strain; however, IS*1216* is typically associated with tetracycline resistance in streptococcal species (34). To the best of our knowledge, the detection of the *aacA-aphD* gene on a Tn*3*-like transposon and the presence of IS*216* in association with HLGR is a novel finding. It has been reported neither for enterococci nor for GBS.

The resistance determinant in GBS BSU452 shows close homologies to parts of the enterococcal resistance plasmid pTEF1 of the *E. faecalis* strain V583 (35), suggesting that it may have been transferred through horizontal gene transfer. This is, however, speculative for GBS strain BSU452, since the presence of a HLGR Tn*3*-like transposon in GBS has not been previously described. Nevertheless, horizontal gene transfer of resistance genes from *Enterococcus* spp. to other gram-positive bacteria by mobile genetic elements is a well-described mechanism in the spread of antibiotic resistance (31, 32). Horizontal gene transfer has recently been suggested for the acquisition of vancomycin resistance genes in GBS (36). GBS strain BSU452 was isolated from the sputum of a cystic fibrosis patient, but there was no evidence of enterococcal colonization. Considering that patients with cystic fibrosis are often treated with antibiotics (including aminoglycosides), and their microbiome in the respiratory tract is different from that of untreated healthy patients, it is possible that horizontal gene transfer to GBS originated from the selected flora. However this hypothesis cannot be proven in our case and remains speculation. Though, a plasmid-borne HLGR has high potential for further spread in a GBS population, the concern of this phenomenon cannot 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

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

Ref[a]	n[a]	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[b]	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[c]	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	- [d]	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 *Streptococcus* infections	Isolation of GBS from blood, CSF and other sterile body fluids	- [e]	1998 – 2013	Bern, Switzerland	0
Total	1128						2

GBS, Group B *Streptococcus*; HLGR, high-level gentamicin resistance; Ref, reference; n, number of GBS isolates; EOD, early-onset disease.

[a] GBS isolates investigated and published in a context other than the presence of HLGR. The number of GBS isolates investigated for HLGR may vary from the number in the source publication for technical reasons.

[b] For this study, only GBS isolates with serotype III were available.

[c] GBS isolation occurred during a routine visit or during a visit due to an exacerbation of clinical symptoms.

[d] Prospective collection during routine diagnostic microbiology laboratory analysis. GBS isolates were investigated for this study.

[e] Collection of invasive GBS isolates (all age groups) during routine diagnostic microbiology laboratory analysis. GBS isolates were investigated for this study.

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

Species	Strain	Description	MIC gentamicin	*aac(6')-Ie-aph(2")-Ia* gene	Transposon
GBS	BSU1203	wild-type strain	≥1024 mg/L	yes	Tn*3706*
GBS	BSU452	wild-type strain	512 mg/L	yes	Tn*3*-like
E. faecalis	BSU386	wild-type strain	12 mg/L	no	no
E. faecalis	BSU580	BSU386 + pIP501^{BSU452}	≥1024 mg/L	yes	Tn*3*-like
E. faecalis	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.

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

Name	Sequence (5'- 3')	Target gene
Inverse primer HLGR1	CTT CAT CTT CCC AAG GCT CTG	*aac(6')- aph(2")*
Inverse primer HLGR2	GCC AGA ACA TGA ATT ACA CGA GG	*aac(6')- aph(2")*
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	

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Figure 1. Plasmid preparations of GBS and *E. faecalis* strains.

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