Genomic sequences of *Streptococcus agalactiae* with high-level gentamicin resistance, collected in the BSAC bacteraemia surveillance

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Running head: Highly gentamicin resistant *S. agalactiae*

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Background. Like other streptococci, *Streptococcus agalactiae* typically has intrinsic low-level aminoglycoside resistance. High-level gentamicin resistance was seen in two of 1125 isolates collected in the British Society for Antimicrobial Chemotherapy (BSAC) Bacteraemia Surveillance Programme between 2001 and 2014. These organisms, both isolated in 2014, were characterised. **Methods**. Identifications were by latex agglutination, MICs by BSAC agar dilution, and sequencing by Illumina methodology. **Results**. Gentamicin MICs were >1024 mg/L versus a species mode of 8 mg/L; both isolates also were unusually ciprofloxacin resistant with MICs of 64 mg/L versus a species mode of 1 mg/L. They were distinct by sequence, but both belonged to the ST19 clone, which occurs globally. Both had *aac6'-aph2''*, carried by different transposons, explaining their gentamicin resistance, and had *gyrA*[81:S-L];*parC*[79:S-Y], accounting for ciprofloxacin resistance. **Conclusion**. These are the first multiresistant *S. agalactiae* with the bifunctional AAC(6')-APH(2") enzyme to be reported in the UK for over 10 years. Despite belonging to the same clonal complex, the two isolates and their resistance transposons were distinct. Both retained full susceptibility to penicillin, but any penicillin-gentamicin synergy is likely to be lost.

Introduction

Streptococcus agalactiae (Group B streptococcus, GBS) is an important agent of neonatal sepsis. In 2010, the UK incidence was 0.41 cases /1000 live births, with a growing number of adult septicaemias also recorded.[1] Most clinical isolates belong to clonal complexes (CCs) 1,10,17,19, 23 and, among these, CC17 is considered to have increased virulence potential.[2] Antibiotic resistance is not a major issue in the species. Penicillin remains the treatment of choice and resistance is extremely rare, though strains with reduced susceptibility are a growing problem in Japan.[3] Tetracycline resistance is frequent,[2] but is of limited clinical relevance, given that tetracyclines are contra-indicated in neonates and their expectant mothers, who form the largest patient groups. Like all streptococci, GBS have low-level intrinsic resistance to gentamicin, reflecting poor uptake; high-level resistance, mediated by the bifunctional AAC(6')-APH(2") enzyme that is prevalent in enterococci and staphylococci, has been reported on a few occasions in GBS in the UK[4] and France,[5] with some recent proliferation in Argentina,[6] but is considered still to be exceptional.

Between 2001 and 2014, the BSAC Bacteraemia Surveillance Programme examined 1125 *S. agalactiae* from over 70 UK and Irish sites. In 2014, for the first time, we identified two isolates of this species with high-level gentamicin resistance. They were referred from separate hospitals, and both also were unusually resistant to ciprofloxacin. These genomes of these organisms were sequenced to elucidate their relatedness and the genetic bases of their resistance phenotypes.

Materials and methods

Isolates and susceptibility testing

The collection strategy for the BSAC Bacteraemia Surveillance Programme has been described[7]. Until 2008, 25 UK and Irish diagnostic laboratories contributed up to 10 consecutive β-haemolytic streptococci per annum; subsequently the number of laboratories was increased to 40 and the number of isolates per site reduced to seven. There is some turnover of collection laboratories, and 70 sites have participated during the period

reviewed (2001-14). On receipt by PHE, β-haemolytic streptococci are identified using the Streptococcal Grouping Latex kit (Pro-Lab Diagnostics, Bromborough, UK) and MICs are determined by BSAC agar dilution.[8] The two highly-gentamicin-resistant isolates were collected in 2014 at separate hospitals 70 miles (112 km) apart: BSB14107 was from a 28-year-old woman and was isolated in the January; BSB14238 was from a 33-year-old man and was isolated in the April. We do not have clinical details for the patients.

Sequencing

Isolates were sequenced at PHE's Genomic Service Delivery Unit on an Illumina HiSeq 2500 platform using the Nextera XT sample preparation method. In-silico MLST was performed using the mapping-based tool MOST,[9] with reference sequences downloaded from the *S. agalactiae* MLST database.[10] Antimicrobial resistance genes were detected using a locally-curated database of resistance determinants and the in-house algorithm 'GeneFinder'.[11] Short reads were assembled into contigs using SPAdes[12] and those carrying the gentamicin resistance determinant were identified by BLAST. Coding sequences were determined with Glimmer[13] with functions inferred from homology searches with BLAST. The genetic relatedness of the isolates was assessed by Single Nucleotide Polymorphisms (SNP) analysis, as previously described.[14]

Results and Discussion

Susceptibility

Among 3218 β-haemolytic streptococci collected from bacteraemic patients by the BSAC Surveillance Programme from 2001-2014, 1125 were identified as *S agalactiae*. MIC distributions for these are shown in Table 1. Isolates BSB14107 and BSB14238 were the only two with high-level resistance to gentamicin and were among a small minority (7/1125) with high-level ciprofloxacin resistance. Both also were highly resistant to tetracycline, though this trait was highly prevalent in the whole collection, with MICs $\geq$16 mg/L for 77.5%

of the isolates. Both retained normal susceptibility to β-lactams and vancomycin (Table 1). BSB14107 was highly resistant to both erythromycin and clindamycin (MICs >128 mg/L); BSB14238 had low-level resistance to erythromycin (MIC 2 mg/L), but remained fully susceptible to clindamycin (MIC 0.12 mg/L).

Sequencing and strain characterisation

Based on in-silico MLST, using whole genome sequences, both resistant isolates belonged to the ST19 clone, which occurs internationally. Nevertheless, their sequences differed by at least 934 SNPs, indicating them to be distinct. The phenotypes of both isolates (Table 1) could be explained by the acquired resistance genes or mutations identified. Both carried *aac(6')-aph(2'')*, explaining high-level gentamicin resistance; both also had chromosomal *gyrA*[81:S-L];*parC*[79:S-Y] mutations, explaining high-level ciprofloxacin resistance. Furthermore, both had *tet*(M), accounting for tetracycline resistance; this was embedded in the same position in Tn916-like elements in both strains and was unlinked to *aac(6')-aph(2'')*. BSB14107 additionally had *mef*(E)*, erm*(B)*, msr*(D)*, lsa*(E) and *lnu*(B), all of which contributed to high-level macrolide and lincosamide resistance, as observed in this strain. BSB14238 had *erm*(TR), explaining low-level macrolide resistance; it also carried *lnu*(C), but this gene does not reliably cause clindamycin resistance in streptococci[15] and so is not discordant with observed susceptibility.

The *aac(6')-aph(2'')* gene was transposon-borne in both isolates, but the genetic environments (Figure 1) differed between the two organisms. In BSB14107 the arrangement most closely resembled that previously described from *S. agalactiae* SGB76[16] though this strain did not carry *aac(6')-aph(2'').* Specifically*, aac(6')-aph(2")* was located on the same contig as a known mobile element carrying *aadE* and *lnu*(B) along with the spectinomycin adenyltransferase determinant, *spc*; however, no direct association with known mobile elements was apparent for *aac(6')-aph2"* itself (figure 1a). In BSB14238, *aac(6')-aph(2")* was linked to the insertion element IS*256*, as previously described in (i) Tn*3706* from *S. agalactiae* isolated in France in 1987[17] and (ii) Tn*4001* from

staphylococci.[18] However, only the upstream copy of IS*256* was confirmed; downstream of *aac(6')-aph(2")*we found only the end of the putative second IS*256* copy; failure to detect the entire element probably reflects problems inherent in resolving repeats in short-read assemblies.

In summary, *S. agalactiae* with high-level gentamicin resistance mediated by the bifunctional AAC(6')-APH(2") enzyme have (re-)emerged in the UK. In is unknown whether the isolates were imported or if they acquired their resistance transposons locally. Isolates with this mechanism were reported in the country in 2002,[4] but have not, to our knowledge, been recorded subsequently. Despite belonging to ST19 and sharing exceptional fluoroquinolone resistance, the present isolates and their resistance transposons were distinct from each other: that in BSB14107 was distinct from any previously found in GBS.

Despite the organisms' multiresistance, and their evident ability to cause infection in the source patients, their wider clinical significance is uncertain. UK guidelines[19] advocate empirical penicillin plus gentamicin for neonatal sepsis, and similar regimens are widely used international. The general view in the UK is that the penicillin covers against GBS whilst the gentamicin covers against Enterobacteriaceae, which are the other likely pathogens in the setting. If this view is correct, high-level gentamicin resistance GBS will be of little significance. A counter view is that gentamicin may potentiate penicillins in *S. agalactiae* bacteraemia, as in streptococcal endocarditis. In this case high-level resistance would be predicated to abrogate this synergy, potentially impacting upon outcomes. There is, however, scant clinical evidence to support this latter, view and a recent in vitro analysis found that gentamicin only gave a small acceleration of penicillin-mediated killing, without convincing synergy.[20] A separate and less debatable risk is that GBS may become a vector of transposons encoding AAC(6')-APH(2"), facilitating its widening dissemination.

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Transparency declarations

DML: Advisory Boards or ad-hoc consultancy Accelerate, Achaogen, Adenium, Allecra, AstraZeneca, Auspherix, Basilea, BioVersys, Cubist, Centauri, Discuva, Meiji, Pfizer, Roche, Shionogi, Tetraphase, VenatoRx, Wockhardt, Zambon, Zealand. Paid lectures – AstraZeneca, Beckman-Coulter, Cepheid, Merck Nordic and Wockhardt. Relevant shareholdings– Dechra, GSK, Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio value. **APMcG**: Speakers' bureau for Astellas, Grant investigator for Cubist/Merck, The Medicine Company, Bayer Healthcare, Achaogen and Tetraphase. **All others:** No personal interests, however, PHE's AMRHAI Reference Unit has received financial support for conference attendance, lectures, research projects or contracted evaluations from numerous sources, including: Accelerate Diagnostics, Achaogen Inc, Allecra Therapeutics, Amplex, AstraZeneca UK Ltd, Basilea Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, The BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department of Health, Enigma Diagnostics, Food Standards Agency, GlaxoSmithKline Services Ltd, Henry Stewart Talks, IHMA Ltd., Kalidex Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme Corp., Meiji Seika Pharmo Co., Ltd, Mobidiag, Momentum Biosciences Ltd, Nordic Pharma Ltd, Norgine Pharmaceuticals,

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References

1. Lamagni TL, Keshishian C, Efstratiou A K *et al.* Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. *Clin Infect Dis* 2013; **57:** 682-8.

2. Da Cunha V, Davies MR, Douarre PE *et al. Streptococcus agalactiae* clones infecting humans were selected and fixed through the extensive use of tetracycline. *Nat Commun* 2014; **5:** 4544.

3. Seki T, Kimura K, Reid ME, Miyazaki A *et al.* High isolation rate of MDR group B streptococci with reduced penicillin susceptibility in Japan. *J Antimicrob Chemother* 2015; **70:** 2725-8.

4. Liddy H, Holliman R. Group B Streptococcus highly resistant to gentamicin. *J Antimicrob Chemother* 2002; **50:** 142-3.

5. Hays C, Louis M, Plainvert C *et al.* Changing epidemiology of Group B Streptococcus susceptibility to fluoroquinolones and aminoglycosides in France. *Antimicrob Agents Chemother* 2016; **60:** 7424-30.

6. Villar HE, Jugo MB. Emergence of high-level resistance to gentamicin and streptomycin in Streptococcus agalactiae in Buenos Aires, Argentina *Rev Esp Quimioter* 2013; **26:** 112-5.

7. Reynolds R, Hope R, Williams L. BSAC Working Parties on Resistance Surveillance. Survey, laboratory and statistical methods for the BSAC Resistance Surveillance Programmes. *J Antimicrob Chemother* 2008; **62** Suppl 2: ii15-28.

8. Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. A guide to sensitivity testing. *J Antimicrob Chemother* 1991; **27** Suppl D: 1-50.

9. PHE Bioinformatics 'MOST' https://github.com/phe-bioinformatics/MOST

10. *S. agalactiae* MLST database https://pubmlst.org/sagalactiae

11. Doumith M, Godbole G, Ashton P *et al.* Detection of the plasmid-mediated *mcr-1* gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J Antimicrob Chemother* 2016; **71:** 2300-5.

12. Center for Algorithmic Biotechnology. http://cab.spbu.ru/software/spades/

13. Glimmer. http://www.cs.jhu.edu/~genomics/Glimmer/

14. Turton JF, Wright L, Underwood A *et al.* High-resolution analysis by whole-genome sequencing of an international lineage (Sequence Type 111) of *Pseudomonas aeruginosa* associated with metallo-carbapenemases in the United Kingdom. *J Clin Microbiol* 2015; **53:** 2622-31.

15. Achard A Villers C, Pichereau V *et al.* New *lnu(C)* gene conferring resistance to lincomycin by nucleotidylation in *Streptococcus agalactiae* UCN36. *Antimicrob Agents Chemother* 2005; **49**: 2716-9.

16. Montilla A, Zavala A, Cáceres Cáceres R et al. Genetic environment of the lnu(B) gene in a *Streptococcus agalactiae* clinical isolate *Antimicrob Agents Chemother* 2014; **58:** 5636-7.

17. Horaud T, de Céspèdes G, Trieu-Cuot P. Chromosomal gentamicin resistance transposon Tn3706 in Streptococcus agalactiae B128. Antimicrob Agents Chemother. 1996 May;40(5):1085-90

18. Lyon BR, May JW, Skurray RA. *Tn*4001: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. *Mol Gen Genet* 1984; **193:** 554-6.

19. National Institute for Health and Care Excellence. Neonatal infection (early onset): Antibiotics for Prevention and Treatment. Clinical guideline CG149. August 2012 https://www.nice.org.uk/guidance/cg149/chapter/1-guidance#antibiotics-for-suspected-infection-2.

20. Ruppen C, Lupo A, Decosterd L et al. Is penicillin plus gentamicin synergistic against clinical group B Streptococcus isolates?: An in vitro study. *Front Microbiol* 2016; **7:** 1680