Molecular epidemiology of group B streptococcal meningitis in children beyond the neonatal period from Angola

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Streptococcus agalactiae is a major pathogen of neonates and immunocompromised adults. Prior studies have demonstrated that, beyond the neonatal period, S. agalactiae rarely causes invasive infections in children. However, during 2004–2005, S. agalactiae was the causative agent of 60 meningitis episodes in children aged 3 months to 12 years from Angola. To identify and study the specific causative genetic lineages of S. agalactiae childhood meningitis, which lack characterization to date, we conducted an extensive molecular analysis of the recovered isolates (n=21). This constitutes what we believe to be the first molecular study of the population structure of invasive S. agalactiae isolates from Africa. A low genetic diversity was observed among the isolates, where the majority belonged to clonal complex (CC) 17 presenting the capsular subtype III-2 (86 % of cases) and marked by the intron group II GBSi1, which has previously been observed to be associated with neonatal hosts. The predominance of single-locus variants of sequence type (ST) 17 suggested the local diversification of this hypervirulent clone, which displayed novel alleles of the fbsB and sip virulence genes. The absence of the scpB–lmb region in two S. agalactiae isolates with the Ia/ST23 genotype is more typical of cattle than human isolates. Globally, these data provide novel information about the enhanced invasiveness of the CC17 genetic lineage in older children and suggest the local diversification of this clone, which may be related to the future emergence of a novel epidemic clone in Angola.

INTRODUCTION

Streptococcus agalactiae, group B Streptococcus (GBS), is the leading cause of neonatal invasive infections in industrialized countries, and ten polysaccharide capsule types (serotypes) (Ia, Ib and II–IX) have been identified (Schrag et al., 2000; Slotved et al., 2007). GBS disease in newborns is classified as early-onset disease (EOD) or late-onset disease (LOD), depending on the age of the infant at the time of disease manifestation. EOD (<7 days of age) represents the majority of cases and is associated with transmission from colonized mothers to the newborn through aspiration of infected amniotic fluid or passage through the birth canal, regularly manifesting as pneumonia and bacteraemia (Liu & Nizet, 2004; Schrag et al., 2000; Trager et al., 1996). LOD (7–89 days of age) is characterized by bloodstream infection with a high incidence of meningeal involvement. The source of causative GBS for LOD is still not completely understood, but community or nosocomial acquisition as well as vertical transmission and prematurity may be implicated (Gagneur et al., 2009; Lin et al., 2003; Mullaney, 2001; Schrag et al., 2000). The vast majority of LOD episodes are caused by a homogeneous capsular type III genetic clone, defined by multilocus sequence typing (MLST) as sequence type (ST) 17 (Gherardi et al., 2007; Jones et al., 2003; Manning et al., 2009; Tazi et al., 2010). Dissimilarities in the pathogenic potential between carriage and invasive isolates have raised the question of whether the latter possess unique biological features that would favour crossing of the blood–brain barrier to cause meningitis. More recent studies have shown that the highly virulent clone ST17 presents an exclusive
protein pattern, such as BibA, FbsB and CspA variants, which seem to be crucial for disease pathogenesis (Brochet et al., 2006; Springman et al., 2009; Tazi et al., 2010).

The population structure and virulence traits of invasive GBS have been elucidated in recent studies from Europe (Gherardi et al., 2007; Luan et al., 2005; Jones et al., 2003, 2006) and North America (Bohsnack et al., 2008; Manning et al., 2009). In contrast, the few studies performed with African isolates have been restricted to capsular typing of invasive GBS (Gray et al., 2007; Madhi et al., 2003) or MLST data on GBS from maternal carriage (Brochet et al., 2009).

GBS could represent a serious public health problem in Angola, as it constitutes a significant cause of bacterial meningitis and this country has the second highest mortality rate for under fives in the world (220 deaths per 1000 live births; WHO, 2010). Unfortunately, no GBS screening programmes during pregnancy along with intrapartum antibiotic prophylaxis are available, which increases the risk of vertical transmission and, consequently, the probability of EOD or LOD.

In the present study, we analysed the phenotypic and genonic characteristics of GBS isolates responsible for meningitis in Angolan children beyond the neonatal period. To our knowledge, this is the first study that combines several molecular methods for the characterization of invasive African GBS isolated from children belonging to an age group for which this meningitis aetiological agent is uncommon (Kim, 2010; Sáez-Llorens & McCracken, 2003; Tzanakaki & Mastrantonio, 2007).

**METHODS**

**Study population and bacterial isolates.** The Paediatric Hospital of Luanda is a reference hospital in Angola, and contains the only laboratory in the whole country with the skills to diagnose bacterial meningitis. This laboratory was established in 2002 in collaboration with the Portuguese National Institute of Health in response to the Angolan bacterial meningitis endemic situation (Bernardino et al., 2003). Patients attending this hospital belong to a low socioeconomic group and come from all 18 provinces of Angola, either independently or transferred from other hospitals. We analysed 21 GBS isolates responsible for meningitis in children aged 91 days to 12 years from a total of 60 cases of GBS meningitis diagnosed at the Paediatric Hospital of Luanda during the years 2004 (n=33) and 2005 (n=27). Due to hospital constraints, namely regarding its ability for long-term storage of biological material, only 21 of the 60 GBS isolates were kept at −80 °C, and only those 21 were sent to the Portuguese National Institute of Health for further characterisation.

**GBS identification and antimicrobial susceptibility profile.** GBS isolates were obtained from cerebrospinal fluid cultures and confirmed at the species level, as described previously (Florindo et al., 2010; Pelkonen et al., 2009). Antimicrobial susceptibility testing (penicillin G, erythromycin, clindamycin and vancomycin) was executed by Etest according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2009), and the presence of macrolide resistance-associated genes (ermTR, ermB and mefA) was analysed by PCR amplification, as described elsewhere (Gyax et al., 2006; Sutcliffe et al., 1996).

**Capsular genotyping, PFGE and MLST.** Capsular genotyping was carried out by PCR and DNA sequencing of the cpsD-cpsE-cpsF region, as documented previously (Florindo et al., 2010). Genomic DNA was digested with Smal and the fragments were resolved by PFGE as described elsewhere (Rato et al., 2008). Cluster analysis was performed using BioNumerics software (Applied MATHs) to create UPGMA dendrograms. The Dice similarity coefficient of the Smal restriction PFGE profiles was used with optimization and position tolerance settings of 0 and 1 %, respectively. Distinct PFGE types were assigned based on a similarity coefficient of <80 % (Rato et al., 2008). Clones were defined as clusters of isolates (three or more) when they presented a dendrogram profile similarity of ≥80 %. For the MLST method (Jones et al., 2003), PCR fragments (~500 bp) of seven housekeeping loci were amplified and sequenced. Alleles of all loci were examined on an MLST database (http://pubmlst.org/sagalactiae/) and the combination provided an allelic profile or ST. Clonal complexes (CCs) comprising isolates sharing six or seven identical alleles were defined.

**Alpha-like protein (Alp) family.** The molecular characterization included the study of a major antigen, the Alp family, which was analysed by multiplex PCR for direct identification of the alpha-C, rib, epsilon and alp2-alp4 genes (Gherardi et al., 2007).

**Detection of mobile genetic elements (MGEs).** The presence of two MGEs, IS1548 and GBS1, within the scpB-lmb intergenic region was evaluated by PCR, as described previously (Al Safadi et al., 2010). In the absence of MGEs, the presence of the flanking genes (scpB and lmb) was verified.

**Allelic variation in bibA (gbs2018), fbsB and sip.** The genetic polymorphisms of three virulence genes, bibA (encoding a surface protein), fbsB (encoding the fibrinogen-binding protein B) and sip (encoding a surface immunogenic protein), were investigated by PCR and DNA sequencing (Brochet et al., 2006; Springman et al., 2009).

**RESULTS AND DISCUSSION**

**Antibiotic susceptibility profiles**

Antibiotic susceptibility testing revealed that all isolates were fully susceptible to penicillin G, as revealed by their MIC values (range 0.047–0.064 μg ml⁻¹), which indicated that the empiric antibiotic therapy [dose regimen: penicillin G (100 000 U kg⁻¹ i.v. every 6 h) plus chloramphenicol (25 mg kg⁻¹ i.v. every 6 h)] that is applied at the Paediatric Hospital of Luanda whenever there is a suspicion of bacterial meningitis was effective against GBS. Moreover, no resistance was detected for vancomycin (MIC range 0.38–1 μg ml⁻¹), clindamycin (MIC range 0.19–0.25 μg ml⁻¹) or erythromycin (MIC=0.25 μg ml⁻¹), with the exception of a single isolate presenting intermediate erythromycin resistance (MIC=0.5 μg ml⁻¹); however, none of the most common antimicrobial resistance genes were detected in this isolate. The low frequency or absence of macrolide resistance in invasive GBS has also been observed by other authors (de Azavedo et al., 2001; Gherardi et al., 2007; Zhao et al., 2008), which could suggest that invasive isolates are less likely to carry resistance determinants. We speculate that invasive isolates, mostly confined to sterile anatomical sites, have less contact with commensal or pathogenic microbiota, and thus are less prone to horizontal genetic transfer phenomena.
Population structure of the GBS isolates

Capsular genotyping of the 21 GBS isolates revealed two cps genotypes, Ia and III-2, where the latter was predominant (86%) and carried the rib gene (Fig. 1). As no data are available on the GBS colonization rate and genotype distribution in Angola, we cannot draw conclusions about the predominance of these two capsular clones among invasive GBS isolates responsible for meningitis, as observed by others (Bohnsack et al., 2008; Gherardi et al., 2007; Gray et al., 2007; Luan et al., 2005; Manning et al., 2009; Tazi et al., 2010; Zhao et al., 2008).

The discriminatory power of the PFGE method was higher than that of the capsular typing and allowed the identification of 13 different DNA band profiles corresponding to six PFGE types (named A–F) distributed into two major clonal clusters (I and II), three singletons (D–F) and a group of two isolates belonging to PFGE type C (Fig. 1). Isolates from cluster II with the same PFGE profile (sharing 100% similarity) belonged to different STs (ST17 or ST109). Seven STs were identified among the 21 isolates using MLST. Three isolates, all exhibiting capsular genotype Ia, were ST23, and the remaining 18 isolates displayed the capsular genotype III-2 and were ST17 or single-locus variants (SLVs) of this ST. Within these SLVs, ST450 and ST451 corresponded to novel STs, described for the first time in this study to our knowledge. The absence of other clones with the ability to cause meningitis, such as III/CC19 and V/CC1 as reported by others (Gherardi et al., 2007; Jones et al., 2003; Manning et al., 2009; Tazi et al., 2010), may reflect local genotype distribution characteristics and/or the limited number of isolates available in the current study.

The predominance of the CC17 lineage supports the previously reported association between CC17 and neonatal infections (Gherardi et al., 2007; Jones et al., 2003; Manning et al., 2009; Tazi et al., 2010), although the children enrolled in those studies belonged to a different age group (up to 3 months of age). In contrast to other studies (Gherardi et al., 2007; Manning et al., 2009; Tazi et al., 2010), analysis of the CC17 lineage showed an atypical distribution of STs within this lineage, where the majority (77.8%) of the CC17 isolates were SLVs of ST17, suggesting a local diversification of this clone. Moreover, the identification of PFGE and MLST genetic variants among the CC17 isolates corroborated previous studies describing the relative homogeneity of this genetic lineage (Brochet et al., 2006; Gherardi et al., 2007; Rolland et al., 1999; Springman et al., 2009). This limited diversity indicates that CC17 has emerged recently from the core population, reflecting a distinct genome architecture with putative implications in host tropism and virulence (Manning et al., 2009; Sørensen et al., 2010; Tazi et al., 2010), where the presence of MGEs may be relevant, as demonstrated by the upregulation of the lmb gene by IS1548 (Al Safadi et al., 2010).

Genomic organization of the scpB–lmb region

In line with the results presented above, we screened for the presence of two MGEs, IS1548 and GBSi1, situated between the scpB and lmb genes, and studied the genetic
polymorphism of three virulence-associated genes. All isolates belonging to genotype III-2/CC17 carried GBSi1 within the scpB–lmb intergenic region (Fig. 1), which is considered a marker of the CC17 genetic lineage (Al Safadi et al., 2010; Luan et al., 2005; Zhao et al., 2009). The absence of GBSi1 or IS1548 was observed in one of the three Ia/ST23 isolates, whereas the other two isolates lacked the scpB and lmb genes, suggesting that they may have originated either directly or indirectly from cattle, as these genes are usually absent in bovine isolates (Al Safadi et al., 2010; Brochet et al., 2006; Franken et al., 2001). The possibility of other sources for GBS acquisition, namely from the community or cattle (Manning et al., 2010), was further supported by the fact that none of the 21 invasive isolates were recovered from newborns, which contradicted the usual GBS pathogenesis. Nevertheless, data from a previous study in Angola reported that a relevant number of children attending the Paediatric Hospital of Luanda with signs of meningitis died without a laboratory diagnosis (123/717 in 2004) (Pelkonen et al., 2009), suggesting that GBS vertical transmission is probably underestimated in Angola. In addition, the lack of clinical data precluded the establishment of any association between GBS meningitis in older children and the presence of predisposing conditions for GBS infection (such as human immunodeficiency virus infection, malaria or severe malnutrition), which was verified in 72.7% of South African children infected with GBS after the neonatal period (Madhi et al., 2003).

**Allelic variation in bibA, fbsB and sip**

The relationship between the allelic variation of virulence-associated genes and MLST genetic lineages (Fig. 1) partially contrasted with the literature data (Brochet et al., 2006; Springman et al., 2009). Indeed, our findings regarding the sip and fbsB genes revealed: (i) a sip3a allele, described here for what we believe to be the first time for the CC17 lineage; (ii) a novel minor variant of sip2 found only in ST287 isolates (sip2;1; GenBank accession no. HQ267706); (iii) the sip2 allele in one ST23 (CC23) isolate (previously considered to be exclusive to CC17 isolates); and (iv) a novel allelic variant of fbsB2b shared by all CC17 isolates (fbsB2b;1; GenBank accession no. HQ267707).

CC17 and CC23 Angolan isolates presented particular genetic signatures involving St, cps genotype, MGEs and surface protein genes, CC17/III-2/GBSi1/rib/gbs2018-3 and ST23/Ia/ΔMGE/epsilon/gbs2018-1, which was in accordance with studies carried out in other countries (Al Safadi et al., 2010; Brochet et al., 2006; Gherardi et al., 2007; Springman et al., 2009). In addition, the presence of the scpB and lmb genes in 19 out of 21 isolates highlights the hypothesis that the scpB–lmb region may be related to colonization or other mechanisms of human GBS infection (Al Safadi et al., 2010). In contrast, the detection of sip2 and sip3a alleles in genotypes Ia/ST23 and III-2/ST174, respectively (Fig. 1), indicates the occurrence of recombination events among distant lineages. These findings suggest that the putative existence of an exclusive set of surface proteins in CC17 isolates as epidemiological markers of this highly virulent lineage (Brochet et al., 2006; Springman et al., 2009) should be viewed with caution.

In conclusion, the predominance of CC17 causing episodes of meningitis in older children from Angola could suggest an adaptation of this lineage to childhood infection, as it rarely causes bacteraemia or meningitis in the adult population (Jones et al., 2003, 2006; Luan et al., 2005; Tazi et al., 2010); however, vertical transmission and some clinical predisposing conditions cannot be excluded. Thus, further epidemiological studies are required to elucidate the course of GBS infection in neonatal and post-neonatal cases of meningitis, as well as the putative cattle origin of GBS, as suggested from our data. Finally, the use of both colonizing and invasive circulating clones in further studies is mandatory, as they may contain specific implications for the design of a universal GBS vaccine.

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**REFERENCES**


