Distribution of pilus islands of group B streptococcus associated with maternal colonization and invasive disease in South Africa

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Group B streptococcus (GBS) is a leading cause of neonatal sepsis. Sortase-dependent pilus-like structures have been identified on the surface of GBS, and have been found to be important in the adhesion and attachment of GBS to host cells. Three pilus island alleles, PI-1, PI-2a and PI-2b, have been described, and their proteins are being explored as vaccine candidates. The pilus islands from 541 colonization isolates and 284 invasive isolates were characterized by PCR. All isolates carried at least one pilus island, and they were identified alone or in combinations at the following overall frequencies: PI-2a, 29.8%; PI-2b, 0.2%; PI-1 + PI-2a, 24.8%; and PI-1 + PI-2b, 45.1%. A combination of PI-1 + PI-2a (28.7 vs 17.6%) was more common among colonizing compared with invasive isolates. Conversely, a combination of PI-1 + PI-2b (37.2 vs 60.2%) was more frequently associated with invasive disease compared to colonization. There was a strong association between pilus islands when adjusted for serotype distribution, PI-2a was identified in 92.6% of colonizing and 90.0% of invasive serotype Ia isolates, whereas serotype III was associated with co-expression of a PI-1 and PI-2b among 84.6% of colonizing and 96.5% of invasive isolates. Based on this homogeneity of pilus island distribution, a pilus-based vaccine developed for Europe and the USA will have similar coverage in South Africa.

INTRODUCTION

Group B streptococcus (GBS) is a commensal bacterium of both the genito-urinary and gastrointestinal tract, and a leading cause of neonatal invasive disease (Ho et al., 2006; Qinlan et al., 2000; Schuchat, 1998). Development of a GBS vaccine requires identification and characterization of antigens from GBS isolates from geographically diverse settings to determine the optimal vaccine formulation candidate antigens (Lauer et al., 2005; Maione et al., 2005). The roles of external structures or surface molecules on GBS biology and pathogenesis, and their potential as vaccine candidates, are being explored (Baker & Edwards, 2003; Larsson et al., 1999; Maione et al., 2005; Margarit et al., 2009).

Genome-wide analysis of eight GBS strains led to the identification of genomic regions whose genes encode pilus structures that extend beyond the bacterial cell-wall peptidoglycan and the capsular polysaccharide (Dramsi et al., 2006; Lauer et al., 2005; Rosini et al., 2006). Pili are formed from polymerized proteins, which have an N-terminal conserved signal peptide and a C-terminal LPXTG motif; the LPXTG domain enabling covalent attachment of the pilus proteins to the cell-wall peptidoglycan (Lalioui et al., 2005; Nobbs et al., 2008; Ton-That & Schneewind, 2004). GBS pili have been described as contributing to GBS adhesiveness and supporting the transcytoses of the bacteria through differentiated human epithelial cells (Pezzicoli et al., 2008).

Three pilus islands (PI-1, PI-2a and PI-2b) have been described (Lauer et al., 2005; Rosini et al., 2006). A published report of isolates collected from the USA and Europe suggested that all GBS isolates have either PI-2a or PI-2b present, and that many isolates carry the additional PI-1 (Margarit et al., 2009). Identification of these surface exposed antigens that are conserved in the majority of GBS strains has raised optimism that a pilus-based vaccine can be developed as an alternative to or complement GBS polysaccharide-protein conjugate vaccines (Margarit et al., 2009). The aim of this study was to determine the prevalence of pilus islands in GBS isolates associated with maternal vaginal colonization and invasive disease in infants from an African setting.

Abbreviations: 95% CI, 95% confidence interval; GBS, group B streptococcus; HREC, Human Research Ethics Committee; PoPS, prevention of perinatal sepsis.
**METHODS**

**Bacterial isolates.** The study involved characterization of 541 vaginal-colonizing GBS isolates identified in women during labour who had participated in a study on ‘Prevention of Perinatal Sepsis (PoPS)’ between 2005 and 2007 as described previously (Cutland et al., 2009). In addition, 284 invasive GBS isolates including 222 (78.2 %) from blood and 62 (21.8 %) from cerebrospinal fluid were obtained from 282 infants with invasive disease hospitalized at Chris-Hani Baragwanath Hospital (CHBH), Soweto, South Africa, between 2004 and 2008. Of the invasive isolates 137 (48.2 %) were obtained from infants less than 7 days old (early onset), 108 (38.0 %) were from infants between 7 and 90 days old (late onset) and 39 (13.7 %) were from infants older than 90 days. All the isolates had been archived in skim milk, tryptone, glycerol and glucose broth (STGG) at −70 °C at the Respiratory and Meningeal Pathogens Research Unit (RMPRU), Johannesburg, South Africa. Serotyping of the isolates was determined as described previously (Madzivhandila et al., 2011). GBS reference strains 2603 V/R (PI-1 and PI-2a) and COH1 (PI-2b) were obtained from the American Type Culture Collection (ATCC).

**Primer and probe design.** Nucleotide sequences of complete and incomplete GBS genomes were obtained from the open genome resources (OGER) database (http://oger.tu-bs.de) (Klein et al., 2009) and additional sequences of pilus island genes were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/). Sequence identity measurements and alignments were performed with BioEdit software version 5.0.9. Ancillary protein 1 (AP1) was selected as a real-time PCR target to allow identification and discrimination between the pilus islands. Isolates that tested negative for all the AP1 genes, or isolates from which PI-2a and PI-2b could not be detected, were reamplified by conventional PCR with a second set of primers representing conserved regions of AP2. Primers and Taq-Man probes were designed with the Primer Express 3.0 software (Applied Biosystems). Primer and probe sequences and reaction concentrations are listed in Table 1.

**PCR.** Frozen bacterial isolates were subcultured on CNA (colistin nalidixic acid) agar and incubated overnight at 37 °C in 5 % CO2. One GBS colony was suspended in 300 μl nuclease-free distilled H2O and the suspension was heated at 95 °C for 10 min. The tubes were centrifuged at 9000 g for 1 min to pellet the cell debris. Four microlitres of the supernatant was added to each PCR. PCRs were run on an AB 7500 instrument (Applied Biosystems) in a 25 μl reaction volume with TaqMan universal PCR master mix without AmpErase UNG (Applied Biosystems). The detection of PI-2b was performed as a singleplex reaction, and PI-1 and PI-2a were detected in duplex. AP2 was detected by standard PCR and PCR products were detected by agarose gel electrophoresis.

**Statistical analysis.** Data were analysed with GraphPad Prism version 4.01 and STATA version 8.0. Distribution of pilus islands in maternal colonizing isolates and invasive isolates was determined by a two-tailed Fisher’s exact test. A logistic regression model was used to test for the association between pilus islands in relation to serotype and the disease onset. A X2 test was used to compare pilus island distribution in infants aged younger than 7 days, between 7 and 90 days, and older than 90 days. A P value of <0.05 was considered significant.

**Ethical considerations.** Analysis of the GBS isolates for this study was approved by the Human Research Ethics Committee (HREC) on Human Subjects at the University of the Witwatersrand (HREC reference no. M080366). Signed informed consent for collection of the colonization isolates was obtained from study participants of the PoPS study (HREC reference no. 030207) and approval to archive neonatal sepsis isolates from a surveillance study (HREC reference no. M03-10-07).

## RESULTS

The real-time PCR assay was able to detect the AP1 gene for PI-2a and PI-2b in 49 and 36 % of isolates, respectively. Additional screening with PCR assays for the AP2 gene of PI-2a and PI-2b negative isolates, revealed that all isolates harboured either PI-2a or PI-2b in 55 and 45 % of isolates, respectively. PI-1 was found in 70 % of all isolates, and was associated with PI-2a 45 % of the time. In contrast, the

### Table 1. Oligonucleotide sequences of the primers and probes for detection AP1 and BP

<table>
<thead>
<tr>
<th>Gene/pilus protein</th>
<th>Sequence (5’→3’)*</th>
<th>Primer concn (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PI-1AP1</strong></td>
<td>Forward GGTGAAACCCAAGATACCAATCA</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Reverse CTTTGCTAATGGTGTAAGCATTG</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Probe 6-FAM CACTTGAAAAGATTAATTG-MGB</td>
<td>250</td>
</tr>
<tr>
<td><strong>PI-2aAP1</strong></td>
<td>Forward ACTGGGTCCAAAGAGCTT</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Reverse GATGGCCCAAATTTTCAAGG</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Probe VIC-AGGTTTCTCGTTATT-MGB</td>
<td>250</td>
</tr>
<tr>
<td><strong>PI-2bAP1</strong></td>
<td>Forward TCTATGCAAGTGGCAAAAGGTGA</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Reverse TCGGCCCTCGGTTTGA</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Probe NED-CATATCCTGGTCATATG-MGB</td>
<td>250</td>
</tr>
<tr>
<td><strong>PI-1AP2</strong></td>
<td>Forward CCGGGCTCATCATCGGAGCTC</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse ACACGAGTCGCTGCTGCCTCCGA</td>
<td>300</td>
</tr>
<tr>
<td><strong>PI-2aAP2</strong></td>
<td>Forward ATGGTGTCTCCTCTGCTGCTT</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse TCGGCCGCTCGTTTTTCAAGGG</td>
<td>300</td>
</tr>
<tr>
<td><strong>PI-2bAP2</strong></td>
<td>Forward ACCGGGCTGAAGTATGTGGCAG</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse GCCCAACCTCGTTGCCGATTAACGG</td>
<td>300</td>
</tr>
</tbody>
</table>

6-FAM, 6-Carboxyfluorescein; MGB, minor groove binder.

*FAM, NED and VIC are fluorescent dyes (Applied Biosystems).
majority (99.5%) of PI-2b positive isolates were associated with PI-1 (Table 2).

Pilus islands in colonization isolates were identified alone or in combinations at the following overall hierarchical frequency: PI-1 + PI-2b (n=201; 37.2%), PI-2a (n=183; 33.8%), PI-1 + PI-2a (n=155; 28.7%) and PI-2b (n=2; 0.4%) (Fig. 1). More than one type of pilus island was identified in 356 (65.9%) of colonizing isolates. Pilus islands in invasive disease isolates were identified at the following overall frequency PI-1 + PI-2b (n=171; 60.2%), PI-2a (n=63; 22.2%), PI-1 + PI-2a (n=50; 17.6%). More than one type of pilus island was identified in 221 (77.8%) of invasive disease isolates (Fig. 1).

There were significant differences when the pilus island distributions were stratified by colonization or invasive disease. A combination of PI-1 and PI-2b was more common among invasive disease isolates (171/284; 60.2%) than colonizing isolates (201/541; 37.2%; P=0.0005). However when adjusted for serotype distribution, these differences were not significant.

When the pilus island distribution in invasive isolates was stratified according to age of onset, the differences were significant in infants aged younger than 7 days, and those between 7 and 90 days. A combination of PI-1 and PI-2b was significantly carried by infants aged between 7 and 90 days (54.7 vs 80.6%; P<0.0001), whilst PI-2a alone or in combination with PI-1 were significantly associated with infants aged younger than 7 days (31.4 vs 17.6% P=0.02 and 13.9 vs 1.9% P=0.0008, respectively) (Fig. 1). The distribution of pilus islands alone or in combination from infants older than 90 days were as follows PI-2a (33.3 %), PI-1 + PI-2a (38.5 %), PI-1 + PI-2b (28.2 %) (Fig. 1).

When the differences in pilus island distribution were adjusted with respect to the differences in the serotype distribution, there were significant associations between the capsular serotype and pilus island combinations

### Table 2. Pilus island distribution among invasive disease and colonization GBS isolates grouped by serotype

<table>
<thead>
<tr>
<th>Serotype</th>
<th>PI-2a</th>
<th>PI-2b</th>
<th>PI-1 + PI-2a</th>
<th>PI-1 + PI-2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>ID</td>
<td>Col</td>
<td>ID</td>
<td>Col</td>
</tr>
<tr>
<td>Ia</td>
<td>151</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ib</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>14</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NT</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>183</td>
<td>63</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Col, Colonization isolates; ID, Invasive disease isolates; NT, nontypable.

Fig. 1. Group B streptococcal pilus islands grouped by source of isolation.
irrespective of whether isolates were from a colonization or invasive group (Table 2). The majority of colonization (84.2%, *P*<0.0001) and invasive disease (90.7% *P*<0.0001) isolates representing capsular serotype IIIa carried a combination of PI-1 and PI-2b. Similarly PI-2a alone was frequently carried by 92.6 and 90.9% of colonization and invasive disease isolates representing serotype Ia, respectively. In isolates that carried a combination of PI-1 and PI-2a, serotype Ib, II and V accounted for 71.6% of colonization and 56.0% of invasive disease isolates. All but one non-typable colonization isolate carried a combination of PI-1 and PI-2a (Table 2).

When pilus island distribution was adjusted with respect to serotype and the disease onset, serotype was the dominant predictor in the distribution of PI-2a alone [adjusted odds ratio 0.38; 95% confidence interval (95%CI) 0.3–0.5; *P*<0.0001] or combination of PI-1 and PI-2b (adjusted odds ratio 2.59; 95%CI 2.00–3.26; *P*<0.0001). A combination of PI and PI-2a showed no significant association with serotype when adjusted with respect to serotype and the disease onset (adjusted odds ratio 1.25; 95%CI 0.94–1.66; *P*=0.12).

**DISCUSSION**

This is, to the best of our knowledge, the first study describing the prevalence of pilus islands in isolates from vaginally colonized mothers, and infants with invasive disease, collected outside of Europe and the USA (Margarit et al., 2009; Martins et al., 2010). There are currently no published data on the pilus island distribution from sub-Saharan Africa where GBS remains the most frequently isolated pathogens in neonates (Berkley et al., 2005; Gray et al., 2007; Madhi et al., 2003; Milledge et al., 2005). Our data were consistent with previous studies from industrialized countries in that all isolates were associated with the presence of either PI-2a or PI-2b. PI-1 and PI-2b were almost always present together, and the presence of PI-1 in PI-2a harbouring isolates was largely dependent on serotype, with the majority of serotype Ib, II and V isolates harbouring these two pilus islands together, whereas the majority of type Ia isolates were associated PI-2a on its own (Margarit et al., 2009; Martins et al., 2010). This is, to the best of our knowledge, the first study from a developing country wherein a substantial population of colonization and invasive disease isolates has been analysed, allowing better comparisons between strains from invasive disease and maternal colonization.

Although there were differences in the pilus island distribution between colonization and invasive disease isolates, and with respect to the age of onset of invasive disease, when the groups were adjusted for differences in serotype distribution within these groups there were no significant differences in the pilus island distribution. This can be attributed to the stable relationship between serotype and pilus island combinations, and suggests that the difference in pilus island distribution between colonizing and invasive disease isolates was due to the difference in the relative invasive potential of different serotypes (Madzivhandila et al., 2011). This is similar to what has been reported by Margarit et al. (2009) where it was shown that the relationship between pilus island and serotype was conserved irrespective of whether isolates were from a colonizing or invasive group.

Our PCR, which targeted conserved regions of AP1, failed to detect either of the PI-2 islands in 15% of all isolates. High levels of sequence variability have been reported in PI-2a and to a lesser extent in PI-2b by Margarit et al. (2009), and we demonstrated that screening for additional target genes, such as AP2 for the detection of PI-2, is essential to maximize the sensitivity of detection. Despite the high levels of variability that showed variant-specific protection in PI-2a backbone protein immunized mice, which raised questions on the prospects of this target, a recent publication by Nuccitelli et al. (2011) applied a structural vaccinology approach to design a synthetic protein with multivalent activity.

Since a vaccine composed of pilus island components has the potential to cover all serotypes, there is a need for in depth research into the global epidemiology of GBS pilus islands to ensure that vaccines in development are broadly protective across all geographical locations. Our data on pilus island distribution are comparable to the data in the published study by Margarit et al. (2009), and the similarities are sufficient to confirm that the serotype and associated pilus island distribution in South Africa is similar to that reported in the USA and Europe, and based on this homogeneity of pilus island distribution, a pilus-based vaccine developed for these industrialized countries will have similar coverage in South Africa. What remains to be determined is whether pressure from such a vaccine can result in the emergence of strains that lack pilus islands as the dominant virulent types as has been witnessed for other pathogens, such as *Streptococcus pneumoniae* (Bogaert et al., 2005; Singleton et al., 2007).

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