Genital carriage of the genus Haemophilus in pregnancy: species distribution and antibiotic susceptibility

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Recent reports have hypothesized that colonization of the maternal genital tract with non-capsulated Haemophilus influenzae could result in neonatal invasive disease. In this study, genital carriage of the genus Haemophilus was investigated in 510 pregnant women attending an Italian hospital for routine controls. Overall, vaginal carriage of the genus Haemophilus was 9.0% (46/510). A high colonization rate with Haemophilus parainfluenzae (37/510, 7.3%) was found; other species, such as Haemophilus pittmaniae (7/510, 1.4%) and Haemophilus haemolyticus (2/510, 0.4%), were detected for the first time in the genital flora by 16S rRNA gene sequencing. Notably, no Haemophilus influenzae was identified, in agreement with previous investigations indicating that this species is rarely isolated from the genito-urinary tract of pregnant women. No antibiotic resistance was detected in Haemophilus pittmaniae and Haemophilus haemolyticus, but quite a high degree of ampicillin (10/37, 27%) and ciprofloxacin (3/37, 8.1%) resistance was observed in Haemophilus parainfluenzae. Five ampicillin-resistant isolates were β-lactamase producers, whereas five isolates exhibited a β-lactamase-negative ampicillin-resistant (BLNAR) phenotype. Sequencing of penicillin-binding protein 3 revealed that Val511Ala, Asn526Ser, Ala530Ser and Thr574Ala changes were associated with BLNAR phenotypes. Two ciprofloxacin-resistant isolates carried substitutions in both GyrA (Ser84Phe and Asp88Tyr) and ParC (Ser84Tyr and Met198Leu); the other ciprofloxacin-resistant isolate had substitutions in ParC only (Ser138Thr and Met198Leu). In conclusion, 10% of pregnant women carried a species of Haemophilus in their genital tract. The emergence of non-β-lactamase-mediated resistance in genital Haemophilus parainfluenzae is a matter of concern because of the risk of mother-to-baby transmission.

INTRODUCTION

Species of the genus Haemophilus form part of the indigenous microflora in the upper respiratory and urogenital tracts (Harper & Tilse, 1991). Within the genus Haemophilus, Haemophilus influenzae is the most important human pathogen able to cause severe diseases in both children and adults, whilst Haemophilus parainfluenzae is an uncommon agent of human infections (Cardines et al., 2009; Frankard et al., 2004; Jordens & Slack, 1995). No studies on the presence/distribution of the different species of Haemophilus in the genital flora of pregnant women have been published recently. Two studies dating back to the 1980s and 1990s reported very low Haemophilus influenzae colonization rates (0.18 and 0.3%, respectively) in the reproductive tract of asymptomatic pregnant women (Albritton et al., 1982; Schönsheyder et al., 1991). Other reports observed higher carriage rates (from 8 to 15%) for Haemophilus species, but the genital swabs were obtained from non-pregnant women often with symptoms suggestive of genital infections (Martel et al. 1989; Sturm, 1986). The topic of asymptomatic carriage of the Haemophilus species in pregnant women deserves special consideration because of possible vertical transmission of bacteria from mother to baby, resulting in neonatal sepsis (Rele et al., 2006; Takala et al., 1991). In particular, in the era of Haemophilus influenzae type b conjugate vaccines, neonatal invasive disease due to non-vaccine preventable non-capsulated Haemophilus influenzae has been associated with sepsis in the mother and an ascending infection from the
Genital carriage of Haemophilus in pregnancy

maternal genital tract (possibly colonized by non-capsulated H. influenzae) has been suggested recently (Ladhani et al., 2010; Van Eldere et al., 2014). As neonatal non-capsulated H. influenzae invasive infections now account for ~5% of all neonatal invasive bacterial infections, investigations on the carrier state of pregnant women are needed (Heath et al., 2001). H. parainfluenzae diseases have occasionally been reported in adults and neonates where they are possibly acquired through the maternal genital tract (Govind et al., 2012; Rele et al., 2006). Notably, new species of Haemophilus have been described recently, and an update of the knowledge on the pathogenic and/or communal roles played by the different species within this genus has been proposed (Norskov-Lauritsen, 2014).

In this study, the carriage rate of the genus Haemophilus in the genital tract of 510 pregnant women was investigated. According to our results, no H. influenzae was detected, whereas H. parainfluenzae was the most frequently detected species. As the emergence of β-lactam resistance mediated by TEM-derived extended-spectrum β-lactamas (ESBLs; TEM-15, TEM-34 and TEM-182) and/or associated with altered penicillin-binding protein (PBP) 3 has been described recently in H. parainfluenzae, the antimicrobial susceptibility profiles and the resistance mechanisms were investigated (García-Cobos et al., 2013; Tinguely et al., 2013; Tristram et al., 2008).

METHODS

Study population and bacterial isolates. From October 2012 to October 2013, 510 pregnant women attending the Cà Granda Hospital Maggiore Policlinico (Milan, Italy) for routine controls were enrolled in this study. The mean and median ages of the women were 38.9 and 33.5 years, respectively (range 14.7–45.8 years). The vaginal samples were taken between weeks 17 and 40 of pregnancy (mean 35.4 weeks and median 36.0 weeks). The exclusion criteria were high-risk pregnancy, vaginal infection, ongoing antibiotic therapy and having performed vaginal lavage. The vaginal swabs, conserved in sterile transport medium, were sent to the laboratory for bacterial identification.

Haemophilus species were selectively detected on chocolate blood agar plates supplemented with bacitracin, vancomycin and amphotericin B (Becton Dickinson), and preliminary identification at the species level was by conventional methods, including requirement for factor V and X. All Haemophilus species isolates were confirmed by PCR and sequencing of the full-length 16S rRNA gene (Bäckman et al., 1999). The reference 16S rRNA gene sequences were GenBank accession number FQ312002.1 for H. parainfluenzae strain T3T1, GenBank accession number GU561423.1 for Haemophilus haemolyticus and GenBank accession number AJ290755.2 for Haemophilus pittmaniae strain HK85.

Antimicrobial susceptibility testing. The MICs of ampicillin, amoxicillin–clavulanic acid, cefotaxime, meropenem and ciprofloxacin were determined by Etest (bioMérieux) following the manufacturer’s recommendations; the interpretative breakpoints were based on European Committee on Antimicrobial Susceptibility Testing criteria (http://www.eucast.org/clinicalbreakpoints/) for H. influenzae. The β-lactamase-negative ampicillin-resistant (BLNAR) reference strain H. influenzae ATCC 49247 was used as control. The β-lactamase activity was screened by using the Nitrocefin stick test (Oxoid).

Characterization of genes associated with antimicrobial resistance in H. parainfluenzae. In all H. parainfluenzae isolates of this study, substitutions in PBP3 were determined by PCR and sequencing of the encoding b3tL gene, as described previously (García-Cobos et al., 2013). The deduced amino acid sequence of the PBP3 transpeptidase region was aligned in comparison with the corresponding sequence from H. parainfluenzae T3T1. In all β-lactamase-positive isolates, the presence of the blα TEM gene and its variants was investigated by PCR and sequencing, as previously reported (García-Cobos et al., 2013). In all ciprofloxacin-resistant isolates, the quinolone resistance-determining regions (QRDRs) of the gyrA and parC genes were examined by PCR and sequencing (Law et al., 2010).

Ethics. The Ethics Committee of the Istituto Superiore di Sanità (Rome, Italy) and the Ethics Committee of Hospital Maggiore Policlinico (Milan, Italy) both approved this study. Participating women provided written informed consent.

RESULTS

Detection of Haemophilus species in pregnant women

Overall, 46/510 (9.0%) pregnant women enrolled in this study carried a Haemophilus species in their genital tract. Of the 46 women, 37 (37/510, 7.3%) carried H. parainfluenzae, seven (7/510, 1.4%) carried H. pittmaniae and two (2/510, 0.4%) carried H. haemolyticus. No other Haemophilus species was detected. The two H. haemolyticus isolates were found to be faintly haemolytic on horse blood agar.

The age of the 46 carrier women ranged from 15 to 42 years; mean and median ages were 32.5 and 34.0 years, respectively. The period of pregnancy of the 46 women ranged from 25 to 38 weeks (mean and median 35.4 and 36.0 weeks, respectively).

Species identification was confirmed by 16S rRNA gene sequence analysis for all 46 Haemophilus species isolates.

Antimicrobial susceptibility phenotype

Antimicrobial susceptibility results of the 37 H. parainfluenzae isolates are shown in Table 1. Resistance to ampicillin was the most frequently detected. Amongst 37 isolates, 10 (27.0%) were resistant to ampicillin; of these, five (5/37, 13.5%) were found to be β-lactamase producers (MIC 6–256 μg ml⁻1) and were classified as β-lactamase-positive ampicillin-resistant (BLPAR) isolates, whilst the remaining five isolates (5/37, 13.5%) showed a BLNAR phenotype (MIC 1.5–2 μg ml⁻1), according to definitions previously reported for H. influenzae (Markowitz, 1980). One BLNAR isolate (Hp372) showed resistance to amoxicillin–clavulanic acid with an MIC of 3 μg ml⁻1, the remaining four BLNAR isolates maintained susceptibility to this antimicrobial agent; although the MIC values of three isolates (Hp95, Hp420 and...
Five isolates were susceptible to ampicillin (MIC 0.38 μg ml⁻¹) and 0.75 μg ml⁻¹ for both isolates), amoxicillin–clavulanic acid (MIC 0.38 μg ml⁻¹), cefotaxime (MIC 0.008 and 0.094 μg ml⁻¹), meropenem (MIC 0.064 and 0.094 μg ml⁻¹) and ciprofloxacin (MIC 0.012 and 0.016 μg ml⁻¹).

Hp483) were at the susceptibility breakpoint (resistance breakpoint >2 μg ml⁻¹). One isolate (Hp43) was resistant to cefotaxime. No resistance to meropenem was detected. Three strains were resistant to ciprofloxacin (MIC 2–32 μg ml⁻¹); of these, one had co-resistance to ampicillin.

The seven H. pittmaniae were susceptible to all antimicrobial agents tested, using the interpretative breakpoints for H. influenzae (Table 2). Similarly, the two H. haemolyticus isolates were susceptible to ampicillin (MIC 0.38 μg ml⁻¹ for both isolates), amoxicillin–clavulanic acid (MIC 0.38 and 0.75 μg ml⁻¹), cefotaxime (MIC 0.008 and 0.094 μg ml⁻¹), meropenem (MIC 0.064 and 0.094 μg ml⁻¹) and ciprofloxacin (MIC 0.012 and 0.016 μg ml⁻¹).

H. parainfluenzae resistance genotype

All five BLPAR isolates carried the TEM-1 β-lactamase. To identify associations between β-lactam resistance phenotypes and ftsI mutation patterns, ftsI gene sequencing was performed on all 37 H. parainfluenzae isolates, including both susceptible and resistant isolates. Of the 37 isolates, 33 (33/37, 89.2%) had substitutions in the deduced amino acid sequences of their PBP3, independent of the ampicillin susceptibility phenotype, corresponding to 10 different substitution patterns (Tables 3 and 4). Changes at PBP3 aa 343 and 488 were found frequently in both ampicillin-susceptible and -resistant isolates, suggesting that these changes had no or little impact on resistance, as also reported by other authors (García-Cobos et al., 2013) (Table 3 and 4). Val436Asp, Val488Ile and Glu398Asp substitutions were rarely detected only in susceptible isolates (Table 4). Of the five BLPAR isolates defined according to the phenotype, three isolates (Hp316, Hp372 and Hp483) had the previously described essential Asn526Ser substitution in combination with the Ala530Ser change (Table 3) (Tristram et al., 2007; Ubukata et al., 2001). The remaining two isolates exhibited (in addition to the common substitutions at 343 and 488) the Thr574Ala substitution alone (one isolate, Hp95) or the Val511Ala plus Thr574Ala changes (one isolate, Hp420). Of note, the single isolate (Hp372) resistant to amoxicillin–clavulanic acid showed the pattern characterized by the double substitution Asn526Ser and Ala530Ser, as well as one (Hp483) of the three isolates with reduced susceptibility to the same antibiotic. The remaining two isolates (Hp95 and Hp420) with increased amoxicillin–clavulanic acid MIC (2 μg ml⁻¹) had the Thr574Ala plus the common Ala343Val substitutions and the Thr574Ala plus Val511Ala changes, respectively. Of the five BLPAR isolates, only one (isolate Hp291) had a PBP3 substitution, Thr574Ala (in addition to the common Ala343Val and Val488Ile). Finally, the isolate (Hp43) resistant to cefotaxime displayed the additional Asp540Ala substitution that was also found in a fully susceptible isolate (Hp30).

The analysis of the QRDRs of gyrA and parC for the three ciprofloxacin-resistant isolates revealed that the two isolates (Hp208 and Hp260) showing a high MIC value (32 μg ml⁻¹) exhibited the same substitutions in both GyrA and ParC. In particular, a double substitution in GyrA was detected at Ser84 and Asp88 (Ser84Phe and

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC (μg ml⁻¹)</th>
<th>Susceptibility category</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MIC₉₀</td>
<td>MIC₉₀</td>
</tr>
<tr>
<td>Amoxicillin–clavulanic acid</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.016</td>
<td>0.032</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.064</td>
<td>0.25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.032</td>
<td>0.047</td>
</tr>
</tbody>
</table>

*Five isolates were β-lactamase producers; five isolates were BLNAR.
Asp88Tyr); a substitution in ParC occurred at Ser84 (Ser84Tyr), and, outside the QRDR, another substitution was detected at position 198 (Met198Leu). The remaining ciprofloxacin-resistant isolate (Hp474) with MIC 2 \( \text{mg ml}^{-1} \) had no changes in GyrA, but a double substitution in ParC, outside the QRDR, was found at Ser138 and Met198 (Ser138Thr and Met198Leu).

**DISCUSSION**

In this study, we investigated the genital carriage of all species of *Haemophilus* in pregnant women. No presence of *H. influenzae* was found, but there was an unexpected quite high carriage of *H. parainfluenzae*. Moreover, and interestingly, two uncommon species, *H. pittmaniae* and *H. haemolyticus*, were identified, although at a lower rate.

### Table 3. Deduced amino acid substitutions in the transpeptidase region of PBP3 from 11 *H. parainfluenzae* isolates resistant to \( \beta \)-lactams

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (( \mu \text{g ml}^{-1} )) *</th>
<th>AMP*</th>
<th>AMC</th>
<th>CTX</th>
<th>( \beta )-Lactamase production</th>
<th>( \text{bla}_{\text{TEM}} ) gene</th>
<th>PBP3 amino acid substitutions†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ampicillin-resistant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLNAR‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp95</td>
<td>1.5</td>
<td>2</td>
<td>0.094</td>
<td>No</td>
<td>–</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp316</td>
<td>1.5</td>
<td>1.5</td>
<td>0.032</td>
<td>No</td>
<td>–</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td>Hp372</td>
<td>2</td>
<td>3</td>
<td>0.016</td>
<td>No</td>
<td>–</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td>Hp420</td>
<td>1.5</td>
<td>2</td>
<td>0.023</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td>V511A</td>
</tr>
<tr>
<td>Hp483</td>
<td>2</td>
<td>2</td>
<td>0.032</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>BLPAR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp162</td>
<td>≥256</td>
<td>1</td>
<td>0.016</td>
<td>Yes</td>
<td>( \text{bla}_{\text{TEM-1}} )</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td>Hp208</td>
<td>6</td>
<td>0.25</td>
<td>0.016</td>
<td>Yes</td>
<td>( \text{bla}_{\text{TEM-1}} )</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td>Hp247</td>
<td>12</td>
<td>0.5</td>
<td>0.016</td>
<td>Yes</td>
<td>( \text{bla}_{\text{TEM-1}} )</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td>Hp291</td>
<td>≥256</td>
<td>1</td>
<td>0.016</td>
<td>Yes</td>
<td>( \text{bla}_{\text{TEM-1}} )</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp431</td>
<td>≥256</td>
<td>0.75</td>
<td>0.016</td>
<td>Yes</td>
<td>( \text{bla}_{\text{TEM-1}} )</td>
<td>A343V</td>
<td>V488I</td>
</tr>
<tr>
<td><strong>Cefotaxime-resistant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp43</td>
<td>0.38</td>
<td>1</td>
<td>1.5</td>
<td>No</td>
<td>–</td>
<td>A343V</td>
<td>V488I</td>
</tr>
</tbody>
</table>

*AMP, ampicillin; AMC, amoxicillin–clavulanic acid; CTX, cefotaxime. Italics indicate resistant phenotype.
†Numbering based on *H. parainfluenzae* T3T1 sequence.
‡According to the phenotype: ampicillin MIC \( >1 \text{ mg ml}^{-1} \).

### Table 4. Deduced amino acid substitutions in the transpeptidase region of PBP3 from 26 *H. parainfluenzae* isolates susceptible to \( \beta \)-lactams

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (( \mu \text{g ml}^{-1} )) *</th>
<th>AMP*</th>
<th>AMC</th>
<th>CTX</th>
<th>PBP3 amino acid substitutions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp1</td>
<td>0.19</td>
<td>0.125</td>
<td>0.023</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp30</td>
<td>0.25</td>
<td>0.19</td>
<td>0.016</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp37</td>
<td>0.19</td>
<td>0.5</td>
<td>0.016</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp169</td>
<td>0.19</td>
<td>0.125</td>
<td>0.016</td>
<td>A343V</td>
<td>–</td>
</tr>
<tr>
<td>Hp47, Hp159, Hp182, Hp193, Hp308, Hp408, Hp418, Hp446, Hp455, Hp146, Hp261, Hp289, Hp296, Hp441, Hp474</td>
<td>0.19–0.75, 0.25–1.5</td>
<td>0.016–0.094</td>
<td>A343V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hp477</td>
<td>0.19–0.75</td>
<td>0.38–1</td>
<td>0.016–0.094</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hp446, Hp455, Hp474</td>
<td>0.094–1, 0.125–1.5</td>
<td>0.016–0.023</td>
<td>A343V</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hp433, Hp484</td>
<td>0.064–0.19, 0.094–0.25</td>
<td>0.016–0.047</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*AMP, ampicillin; AMC, amoxicillin–clavulanic acid; CTX, cefotaxime.
†Numbering based on *H. parainfluenzae* T3T1 sequence.
The absence of *H. influenzae* is in line with some previous studies indicating that genito-urinary tract colonization with *H. influenzae* is infrequent (Albritton *et al.*, 1982; Schönhheyder *et al.*, 1991). However, an increase in neonatal sepsis due to non-capsulated *H. influenzae* has recently been observed and vertical transmission from mother to baby has been hypothesized (Ladhani *et al.*, 2010; Van Eldere *et al.*, 2014). Our finding suggested that, in the era of *H. influenzae* type b conjugate vaccines, genital carriage of non-vaccine preventable strains, such as non-capsulated *H. influenzae*, has not increased. However, as a high case-to-carrier ratio has been reported for *H. influenzae* in women with preterm rupture of membranes and pregnancy has recently been found to be associated with a greater risk of invasive *H. influenzae* infection (possibly resulting in neonatal sepsis), our negative result should not lead to an underestimate of this issue (Collins *et al.*, 2014; Berndsen *et al.*, 2012).

Of note, we did not detect any *Haemophilus* isolate belonging to the ‘cryptic genospecies of *H. influenzae* biotype IV’ (also referred to as *Haemophilus quentini*), although this genospecies has been found previously to be involved in urogenital, neonatal and mother/infant infections (Quentin *et al.*, 1989; Van Eldere *et al.*, 2014). Even though traditional biochemical tests are not able to discriminate so-called *H. quentini* from other closely related *Haemophilus* species such as *H. haemolyticus* (Glover *et al.*, 2011; Mak *et al.*, 2005), the two *H. haemolyticus* isolates herein detected were identified definitively by employing 16S rRNA gene sequence analysis (Claridge, 2004). Future research including larger populations of pregnant women will confirm or not the presence of so-called *H. quentini* in the genital flora.

Several previous investigations characterized *H. parainfluenzae* isolates from the genital mucosa and/or involved in urogenital disease (Rele *et al.*, 2006; Sturm, 1986), but only one genital carriage study was published reporting a colonization rate of 2% for both *H. parainfluenzae* and *H. influenzae* in non-pregnant women (Houang *et al.*, 1989). In our study, the proportion of women carrying *H. parainfluenzae* was significantly higher and it reached the lower value previously reported for maternal colonization with group B streptococci in Europe (ranging from 6.5 to 36% in Southern Europe and Scandinavia, respectively) (Barcătei *et al.*, 2008). Interestingly, the cohort of pregnant women herein was also enrolled for investigating group B Streptococci carriage, in an independent survey; therefore we were able to cross data between the two colonization studies. Overall, 15.5% (79/510) of the pregnant women were found to possess group B streptococci in their genital tract (L. Daprai and M. L. Garlaschi, personal communication). Group B streptococci were detected in 11 of the 37 (29.7%), in one of the seven (14.3%) and in two of the two (100%) women carrying *H. parainfluenzae*, *H. pittmaniae* and *H. haemolyticus*, respectively, indicating that co-colonization by *Haemophilus* species and group B streptococci can occur.

*H. pittmaniae* is a new species (first described in 2005) making up part of the normal flora of the human oral mucous membranes or other body fluids, whilst *H. haemolyticus* is an old species often under-recognized due to the failure of the current phenotypic methods to differentiate *H. influenzae* from non-haemolytic variants of *H. haemolyticus* (Murphy *et al.*, 2007; Norskov-Lauritsen *et al.*, 2005; Norskov-Lauritsen, 2014). In the present study, we were able to identify these species by employing 16S rRNA gene sequence analysis. To the best of our knowledge, this is the first report describing *H. pittmaniae* and *H. haemolyticus* as part of the female genital flora. According to the literature, all three *Haemophilus* species we detected in the genital tract of pregnant women are regarded as opportunistic pathogens, although with limited pathogenicity compared with *H. influenzae*. Cases of invasive disease including neonatal sepsis due to *H. parainfluenzae* and *H. haemolyticus* are well documented, suggesting that the presence of such micro-organisms in the lower genital tract of pregnant women should be considered with attention, especially when they possess antibiogram resistance traits (Govind *et al.*, 2012; Morton *et al.*, 2012).

In *H. parainfluenzae*, resistance to ampicillin due to β-lactamase production has long been known (Scheifele & Fussell, 1981), but TEM-derived ESBLs have been described recently (García-Cobos *et al.*, 2013; Tristram *et al.*, 2008). In this study, almost one third of the *H. parainfluenzae* isolates were ampicillin resistant. No resistance due to the production of TEM-derived ESBL was detected, but a rising emergence of non-β-lactamase-mediated resistance was found. This type of resistance has been demonstrated to be associated with modifications in PBP3 in *H. influenzae* (Ubukata *et al.*, 2001).

As, until now, the magnitude of PBP3 mutations reported in the literature for *H. parainfluenzae* has been limited (García-Cobos *et al.*, 2013; Tinguey *et al.*, 2013; Tristram *et al.*, 2008), we decided to investigate possible associations between β-lactam susceptibility phenotypes and PBP3 changes, analysing the *fisl* gene in both susceptible and resistant isolates. According to our results, BLNR phenotypes were associated with the following substitutions: Val511Ala, Asn526Ser, Ala530Ser and Thr574Ala, which were all found only in resistant isolates. The exact role of such substitutions in affecting β-lactam susceptibility in *H. parainfluenzae* has yet to be determined, and further PBP3 sequences and investigations are required. However, the Asn526 substitution is the most common BLNR-associated substitution in *H. influenzae* (Asn526Lys) (Tristram *et al.*, 2007; Ubukata *et al.*, 2001) and it has also been described previously in *H. parainfluenzae*, where the Asn has been found to be replaced by either Ser or His (García-Cobos *et al.*, 2013; Tristram *et al.*, 2008). In *H. influenzae*, definition of the BLNR genotype includes the presence of either an Asn526 or an Arg517 substitution (Ubukata *et al.*, 2001). However, the Arg517 substitution was not found in either our BLNR isolates...
or in previously described *H. parainfluenzae* isolates (García-Cobos et al., 2013; Tristem et al., 2008). The Val511Ala, Ala530Ser (both near the conserved KTG motif) and Thr574Ala changes have been reported previously in *H. parainfluenzae* linked to resistance or reduced susceptibility to β-lactams, although often in combination with Asn526Ser, and rarely in *H. influenzae* (Barbosa et al., 2011; García-Cobos et al., 2013; Kishii et al., 2010; Tinguely et al., 2013). Obviously, in our *H. parainfluenzae* isolates the possible presence of additional resistance mechanisms affecting susceptibility to β-lactams, such as overexpression of the AcrAB efflux pump or alterations in outer membrane permeability, cannot be ruled out (Kaczmarek et al., 2004). These additional mechanisms could be involved in the cefotaxime resistance exhibited by the isolate Hp43 possessing Asp540Ala, but this change was also found in a cefotaxime-susceptible isolate herein analysed. However, the Asp540Ala substitution has not been found in a cefotaxime-susceptible isolate herein analysed. For isolate Hp474, exhibiting a low level of resistance, the ftsl gene from different *Hae- mophilus* species has recently been documented, suggesting a role of the inter-species recombination in dissemination of PBP3-associated resistance, the presence of modified PBP3 in *H. parainfluenzae* could contribute to a potential increase in β-lactam resistance in other *Haemophilus* species that share the same ecological niche (Witherden et al., 2014).

Previous studies have demonstrated that, in *H. parainfluen- zae*, resistance to fluoroquinolones is mainly associated with mutations in the QRDRs of gyrA and parC (Law et al., 2010; Rodriguez-Martinez et al., 2011; Tinguely et al., 2013). The mutation pattern we found in highly resistant isolates to ciprofloxacin has been reported previously in *H. parainfluenzae*, although Ser84 in ParC was herein replaced by Tyr instead of Phe (Law et al., 2010; Rodríguez-Martínez et al., 2011; Tinguely et al., 2013). For isolate Hp474, exhibiting a low level of resistance, the role of the double substitution identified in ParC (outside of the QRDR) is uncertain, although such substitutions have already been observed in resistant *H. parainfluenzae* (Rodriguez-Martínez et al., 2011).

To conclude, this study demonstrates that ~10% of pregnant women carried a *Haemophilus* species in their genital tract, but *H. influenzae* was not detected. Colonization with *H. parainfluenzae* was observed at an unexpected high rate, whilst other rare species, such as *H. pittmanii* and *H. haemolyticus*, were detected for the first time amongst the genital flora. Genital *H. parainfluenzae* frequently possess antibiotic resistance traits. Several PBP3 substitutions were found to be associated with resistance or reduced susceptibility to β-lactams in our isolates. The emergence of non-β-lactamase-mediated resistance is a matter of concern in view of the potential vertical transmission of antibiotic-resistant *H. parainfluenzae* from mother to neonates at birth. Further studies are required to evaluate whether maternal colonization with *Haemophilus* species during pregnancy actually increases the risk of neonatal infections.

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**REFERENCES**


