Identification of bacteria and potential sources in neonates at risk of infection delivered by Caesarean and vaginal birth

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Neonatal gastric aspirates (NGA) are routinely screened in UK hospitals to investigate fetal/neonatal infections associated with cases of adverse pregnancy outcome (APO). The aim of this study was to describe and compare the microbiology of NGA from Caesarean and vaginal deliveries using molecular methods, and to evaluate other possible clinical and non-clinical variables that may have determined the presence of the bacteria in the samples. The value of using NGA and molecular methods to investigate potential pathogens associated with the risk of early infection was also evaluated. Bacteria were identified by a combined molecular approach on the basis of the 16S rRNA gene using both clone analysis and denaturing gradient gel electrophoresis. A total of 43 and 34 different species were identified in the vaginal (n=121) and Caesarean (n=119) deliveries, respectively; 26 of the species observed (51 %) were common to both modalities, although usually less prevalent in the Caesarean cases. Multivariate analysis confirmed an association between infection and prolonged rupture of membranes in vaginal deliveries (odds ratio =5.7, 95 % confidence interval=1.1–29.0). Various associations between infection and given variables were also shown, including labour, intrapartum antibiotic prophylaxis, and time and place of sample collection. The molecular methods allowed identification of a range of bacteria and potential sources not previously observed in NGA, including possible genito-urinary, gastrointestinal and oral pathogens. NGA represents a valuable sample for investigating potential pathogens associated with APO and the risk of early infection in neonates using molecular methods.

INTRODUCTION

Adverse pregnancy outcomes (APOs) denote those complications presented during pregnancy or immediately after birth that may have caused the termination of the pregnancy and compromise the well-being of the fetus, neonate and/or the mother. Complications occur in 11 % of pregnancies in the UK (ONS, 2005), many of these have unknown causes, which represent a huge concern to both society and the health-care system (Petrou, 2003). Intra-amniotic infection is an important and frequent cause of APO. It has been associated with 25–40 % cases of preterm birth, which accounts for 75 % of perinatal mortality and morbidity (McCormick, 1985). Also, women with microbial invasion of the amniotic cavity are more likely to present spontaneous rupture of membranes (ROM), develop chorioamnionitis and present with an adverse perinatal outcome (Romero et al., 2006). Furthermore,
fetal bacteraemia has been detected in 30% of cases with preterm ROM and positive amniotic fluid cultures (Carroll et al., 1996), with a high number of neonates requiring special care within neonatal intensive care units.

Bacteria identified in neonatal gastric aspirates (NGA) may be predictive of those pathogens possibly associated with the occurrence of an APO and early onset neonatal infection, as positive detection of bacteria in NGA has shown to correlate 100% with the presence of chorioamnionitis and/or presentation of preterm ROM in preterm infants (Miralles et al., 2005). These fluids are routinely cultured in UK hospitals as the minimum standard operating procedure to investigate fetal/neonatal infections in cases of APO, such as preterm birth or low birth weight; and in newborns that are considered at risk of early infection. This is mainly based upon non-specific clinical features, such as respiratory distress, unstable temperature and maternal fever, or due to other factors such as congenital anomalies, the use of invasive procedures and devices during birth, women with previous group B streptococcal infection and prolonged ROM (HPA, 2004).

There is still a limited knowledge as regards associated pathogen(s) in APO and their origin, which significantly limits prevention and provision of treatment. For example, evaluation of NGA as a tool to predict neonatal sepsis has been of mixed success (Evans et al., 1988; Leibovich et al., 1987; Puri et al., 1995). One limitation is related to the culture techniques routinely used because of the large number of uncultivable bacteria, as well as the very specific environmental or nutritional requirements to grow fastidious species (Aas et al., 2005). Therefore, a more detailed and sensitive analysis is required to better evaluate the potential use of NGA for clinical investigation and further research.

In theory, NGA should contain bacteria to which the fetus has been exposed to in utero (swallowed amniotic fluid). However, a number of micro-organisms present in the maternal vagina and skin (Dominguez-Bello et al., 2016) and sensitive analysis is required to better evaluate the microbiota in mothers and neonates, and may have influenced the bacterial content.

Hence, the aims of this study were: (i) to describe and compare bacterial prevalence in samples of NGA between neonates at risk of early infection that were born by Caesarean and vaginal birth, (ii) to determine if other clinical and/or non-clinical variables may have contributed to the presence of bacteria or specific bacteria in the samples, (iii) to evaluate the value of using NGA and molecular methods to investigate the source of potential pathogens associated with the occurrence of APO and the risk of early infection in neonates.

**METHODS**

**Samples.** A total of 240 samples of NGA were analysed in this study: 121 were obtained from vaginal deliveries and 119 from Caesarean sections. This study was approved by the Outer North East London Research Ethics Committee (REC reference no. 08/H0701/61). Gastric aspirates are routinely taken at the maternity and neonatal wards, Royal London Hospital, Barts and The London NHS Trust, under the Standard operating procedure for the investigation of NGA and infection screen swabs from neonates (reference no. BSOIP 234.1) from newborns that present with a clinical manifestation of possible neonatal sepsis or are at risk of developing an infection (HPA, 2004). These fluids are obtained through aspiration: inserting a sterile nasogastric tube into the stomach of the newborn during the first hours post-delivery and before feeding.

**DNA isolation.** Due to the high viscosity of the samples, NGA received pre-treatment with a sputum liquefying agent as suggested previously (Jones et al., 2010). Genomic DNA was extracted by using the ArchivePure DNA yeast and Gram −/+ purification kit (Flowgen Bioscience) following the manufacturer’s recommendations. DNA was then stored at 4 °C until the amplification step was performed.

**Cloning analysis.** Bacterial 16S rRNA genes were amplified from the genomic DNA using modified universal primers as suggested by Frank et al. (2008). This set of primers amplified the majority of the 16S rRNA gene, approximately 1500 bp in length. The amplification protocol was performed as described previously (Gafan et al., 2004). The amplicons generated by PCR were cloned into One Shot chemically competent TOP10 Escherichia coli using a TOPO TA cloning kit (Invitrogen) as recommended by the manufacturer. All PCR products were then purified using the QiAquick PCR purification kit (Qiagen) for subsequent sequencing. The number of clones analysed in this study was based upon the richness as seen in 10 initial clones. It was observed that those samples identifying up to three species in 10 clones did not show any significant increase when raising the number of clones analysed to 50. However, richer samples required a higher number of clones until no significant change was observed (data not shown).

**Denaturing gradient gel electrophoresis (DGGE).** A shorter fragment of the 16S rRNA gene (~235 bp) was amplified. Touchdown PCR and methods to perform DGGE have been explained elsewhere (Gafan & Spratt, 2005). The observed bands were excised using a sterile scalpel and eluted in water at 4 °C for 24 h as described by Ampe et al. (1999). Eluted DNA (1 µl) from each DGGE band was reamplified. PCR products were then purified and sequenced.

**Sequencing.** All sequencing was carried out at the Genome Centre, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, with an ABI 3700 automated DNA sequencer, producing sequences of 800 bp (from cloning) and 150 bp (from DGGE) in length. The sequences of the 16S rRNA gene fragments were analysed by comparison using the Ribosomal Database Project (Cole et al., 2007) and the Human Oral Microbiome Database (www.homd.org) (Chen et al., 2010) based upon ≥ 97% similarity with the closest known relative (Patel, 2001).

**Statistical analysis.** Two-sided Fisher’s exact tests were used to assess the associations between detection of bacteria and the binomial variables. The Mann–Whitney U test and the Independent t-test were also used for quantitative data. Multivariate logistic regression models were constructed to determine the effect of the covariates on the detection of bacteria after adjusting for important variables. A P-value of less than 0.05 was taken to indicate statistical significance.
RESULTS

Samples analysed in this study were obtained as surplus to the NGA routinely sent to the Department of Medical Microbiology, Royal London Hospital, Barts and The London NHS Trust, during the period August 2007 to February 2009. Samples obtained from 121 (50 %) vaginal deliveries and 119 (50 %) Caesareans were collected the following day from the Department of Medical Microbiology. Demographical and clinical data were obtained from the Standardized Electronic Neonatal Database (www.neonatal.org.uk/send) and from the maternity notes (Table 1). Also characteristics of the sample collection as well as Gram stain and culture results, were obtained from the Department of Medical Microbiology database (Tables 1, 2 and 3).

Demographical and clinical data in the vaginal and Caesarean groups were generally homogeneous with some exceptions inherent to Caesarean delivery, such as higher maternal age and multiple deliveries. Also, a higher number of women from black ethnic origin who delivered by Caesarean was observed. The time for collection of the sample was also significantly higher in vaginal deliveries. When comparing bacteria positive using Gram stain and culture, the only significant difference was observed for Gram-positive rods, which were of higher prevalence in vaginal deliveries (this possibly refers to Lactobacillus spp. and Gardnerella vaginalis as observed with the molecular methods).

The samples of NGA were analysed for bacterial content using two different molecular techniques based upon the small-subunit (16S) rRNA bacterial gene. With the clone analysis-PCR, 77 of 240 (32 %) samples were positive. All the samples were subjected to cloning; however, due to a technique-sensitive protocol and limited amount of the samples, bacteria were identified only from 52 of the

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Table 1. Participant’s demographic and clinical data arranged by mode of delivery

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>Total [n=240]</th>
<th>Mode of delivery</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaginal [n=121]</td>
<td>Caesarean [n=119]</td>
<td></td>
</tr>
<tr>
<td>Neonate's gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n) (%)</td>
<td>104 (43)</td>
<td>52 (43)</td>
<td>52 (44)</td>
</tr>
<tr>
<td>Male (n) (%)</td>
<td>135 (56)</td>
<td>69 (57)</td>
<td>66 (55)</td>
</tr>
<tr>
<td>Mean age ± SD (range)</td>
<td>29 ± 6 (17–46)</td>
<td>27 ± 5 (17–42)</td>
<td>30 ± 6 (17–46)</td>
</tr>
<tr>
<td>Maternal age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal ethnic origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian–Bangladeshi/Indian/Pakistani (n) (%)</td>
<td>117 (49)</td>
<td>61 (50)</td>
<td>56 (47)</td>
</tr>
<tr>
<td>Asian–other (n) (%)</td>
<td>16 (7)</td>
<td>9 (7)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>Black African/Caribbean/other (n) (%)</td>
<td>30 (13)</td>
<td>8 (7)</td>
<td>22 (18)</td>
</tr>
<tr>
<td>White British/Irish/other (n) (%)</td>
<td>68 (28)</td>
<td>39 (32)</td>
<td>29 (24)</td>
</tr>
<tr>
<td>Other–mixed (n) (%)</td>
<td>8 (3)</td>
<td>4 (3)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Clinical characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple deliveries (n) (%)</td>
<td>19 (8)</td>
<td>4 (3)</td>
<td>15 (13)</td>
</tr>
<tr>
<td>Primiparous (n) (%)</td>
<td>118 (54)</td>
<td>53 (49)</td>
<td>65 (60)</td>
</tr>
<tr>
<td>Previous complications (n) (%)</td>
<td>63 (56)</td>
<td>28 (51)</td>
<td>35 (61)</td>
</tr>
<tr>
<td>In labour (n) (%)</td>
<td></td>
<td></td>
<td>57 (53)</td>
</tr>
<tr>
<td>Ruptured membranes (n) (%)</td>
<td></td>
<td></td>
<td>46 (51)</td>
</tr>
<tr>
<td>Prolonged ROM (&gt;24 h) (n) (%)</td>
<td>39 (22)</td>
<td>25 (28)</td>
<td>14 (16)</td>
</tr>
<tr>
<td>Preterm birth (n) (%)</td>
<td>140 (58)</td>
<td>64 (53)</td>
<td>76 (64)</td>
</tr>
<tr>
<td>Mean gestational age (weeks) ± SD</td>
<td>35 ± 5</td>
<td>35 ± 5</td>
<td>35 ± 4</td>
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<tr>
<td>Low birth weight (n) (%)</td>
<td>131 (55)</td>
<td>60 (50)</td>
<td>71 (60)</td>
</tr>
<tr>
<td>Mean birth weight (g) ± SD</td>
<td>2322 ± 962</td>
<td>2397 ± 948</td>
<td>2245 ± 974</td>
</tr>
<tr>
<td>IAP (n) (%)</td>
<td>35 (17)</td>
<td>15 (15)</td>
<td>20 (19)</td>
</tr>
<tr>
<td>Smoking during pregnancy (n) (%)</td>
<td>16 (8)</td>
<td>10 (11)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>Sample collection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At the maternal ward (no neonatal ward)</td>
<td>58 (24)</td>
<td>32 (27)</td>
<td>26 (22)</td>
</tr>
<tr>
<td>Median time for collection (h) (range)</td>
<td>4.2 (0.2–23.9)</td>
<td>4.7 (0.2–23.9)</td>
<td>3.7 (0.3–23.1)</td>
</tr>
<tr>
<td>Samples collected within 12 h of birth (n) (%)</td>
<td>190 (79)</td>
<td>92 (76)</td>
<td>98 (83)</td>
</tr>
</tbody>
</table>

[¶] Data not known for n number of cases; –, data only analysed for Caesarean cases.
*One Caesarean case where the neonate’s gender was unknown.
†P value determined using an independent t-test.
‡P value determined using Fisher’s exact test.
§P value determined using the Mann–Whitney U test.
PCR-positive samples (68%) successfully. It is possible that some inhibitors might have been present in the samples or were acquired during one of the steps involved in cloning. A total of 932 clones (10 to 47 clones per sample) were selected and sequenced for identification purposes. Forty-two species were detected using this approach. With the DGGE analysis, 227 samples were investigated. Bands \( n = 372 \) were cut from the polyacrylamide gels for sequencing. A total of 92 (41%) of these were positive, detecting a total of 37 species in the 88 positive samples (96%) that were successfully characterized with this technique.

The results obtained from both techniques were analysed as a combined molecular approach in order to determine the microbiology of NGA. A total of 59% (142/240) of the samples were negative for bacterial detection with the molecular techniques used. From the 98 (41%) positive samples, 80 (82%) samples were characterized and the total number of species identified was 51. The mean (± SD) number of species per sample characterized was 3.7 ± 3.0 for the positive samples, containing up to 15 species in one of the samples. The most prevalent species were significantly higher in vaginal deliveries compared to Caesarean deliveries.

### Table 2. Gram staining results related to the samples of NGA analysed in this study

<table>
<thead>
<tr>
<th>Gram stain result*</th>
<th>Total (n) (%) [n=240]</th>
<th>Mode of delivery</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaginal [n=121]</td>
<td>Caesarean [n=119]</td>
</tr>
<tr>
<td>Total positive (n) (%)</td>
<td>62 (26)</td>
<td>38 (31)</td>
<td>24 (20)</td>
</tr>
<tr>
<td>G(+) cocci (total)</td>
<td>43 (18)</td>
<td>27 (22)</td>
<td>16 (13)</td>
</tr>
<tr>
<td>G(+) cocci pairs</td>
<td>5 (2)</td>
<td>3 (2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>G(+) cocci chains</td>
<td>10 (4)</td>
<td>6 (5)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>G(+) cocci clusters</td>
<td>4 (2)</td>
<td>4 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>G(+) rods</td>
<td>14 (6)</td>
<td>12 (10)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>G(−) cocci</td>
<td>3 (1)</td>
<td>1 (1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>G(−) rods</td>
<td>20 (8)</td>
<td>13 (11)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>G(−) bacillus</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

*Data obtained from the Department of Medical Microbiology database, Royal London Hospital, Barts and The London NHS Trust.
†P value determined using Fisher’s exact test.

### Table 3. Culture results related to the samples of NGA analysed in this study

<table>
<thead>
<tr>
<th>Culture result*</th>
<th>Total (n) (%) [n=240]</th>
<th>Mode of delivery</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaginal [n=121]</td>
<td>Caesarean [n=119]</td>
</tr>
<tr>
<td>Total positive (n) (%)</td>
<td>60 (25)</td>
<td>38 (31)</td>
<td>22 (18)</td>
</tr>
<tr>
<td>‘Skin flora’</td>
<td>11 (5)</td>
<td>5 (4)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>GBS</td>
<td>9 (4)</td>
<td>4 (3)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>(\alpha)-Haemolytic streptococcus (including viridans streptococcus)</td>
<td>7 (3)</td>
<td>2 (2)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Coagulase-negative staphylococcus</td>
<td>7 (3)</td>
<td>6 (5)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>‘Anaerobes’</td>
<td>6 (3)</td>
<td>4 (3)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Coliform group bacteria</td>
<td>5 (2)</td>
<td>5 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E. coli</td>
<td>4 (2)</td>
<td>3 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>4 (2)</td>
<td>4 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>‘Mouth flora’</td>
<td>3 (1)</td>
<td>3 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Streptococcus spp. (Streptococcus bovis)</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>2 (1)</td>
<td>2 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>2 (1)</td>
<td>2 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>1 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gardnerella vaginalis</td>
<td>1 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Yeast</td>
<td>1 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Data obtained from the Department of Medical Microbiology database, Royal London Hospital, Barts and The London NHS Trust.
sections (Table 4). The percentages of the specific bacteria per group were calculated out of the percentage of samples able to be characterized; however, it was not possible to carry this out with the statistical analysis where all the samples were included.

Mode of delivery has previously been shown to determine different patterns of initial colonizers in neonates; therefore, the prevalence of the bacteria in Caesarean and vaginal deliveries was analysed separately in order to evaluate other possible clinical and non-clinical variables. Detection of general bacteria and Caesarean delivery did not show any significant association as determined with the molecular approach; although associations were close to significance for those with ruptured membranes at delivery \( (P=0.073) \), prolonged ROM \( (P=0.058) \), those who received IAP \( (P=0.059) \) and women who smoked during pregnancy \( (P=0.062) \). However, significant associations were observed for detection with Gram staining and culture techniques as shown by the Fisher’s exact test (Table 5). On analysing specific bacteria, few associations were observed. Presence of the \textit{Streptococcus mitis} group, was significantly associated with IAP \( (P=0.025) \), \textit{Streptococcus agalactiae} (group B streptococci, GBS) was significantly higher in the cases of Caesarean patients who were in labour \( (P=0.028) \), and \textit{Pseudomonas} spp. were associated with samples collected at more than 12 h post-delivery \( (P=0.015) \). Nevertheless, none of these associations remained in the multivariate analyses for Gram staining, culture or molecular detection in newborns delivered by Caesarean section.

In contrast, a different pattern of associations was observed in the vaginal deliveries. Presence of general and specific bacteria showed several associations with clinical and non-clinical variables using the bivariate analysis (Table 6). No associations were observed between positive Gram staining and culture analyses and the variables investigated in this group. Table 7 summarizes the results of the multivariate logistic regression; infection was significantly greater for prolonged ROM in cases delivered vaginally.

**DISCUSSION**

In accordance with the UK’s National Standard Methods, samples of NGA are investigated using minimal microbiology standard procedures to aid treatment and prognosis. Gram staining allows a rapid detection of bacteria; while microbial culture permits the growth and isolation of micro-organisms, particularly focusing on a range of bacteria historically associated with neonatal sepsis (HPA, 2004). Traditionally, cultured isolates have been limited to a few species including \textit{E. coli}, \textit{Haemophilus influenzae}, \textit{Listeria monocytogenes}, GBS, and species of the genera \textit{Klebsiella}, \textit{Pseudomonas} and \textit{Corynebacteria}; and usually only one single micro-organism per sample was detected (Borderon et al., 1994). However, Jones et al. (2010) recently demonstrated that culture analysis of NGA is only positive when bacterial numbers exceeded \( 4.50 \times 10^5 \) c.f.u. ml\(^{-1}\). Routine analysis may thus underestimate the number and richness (diversity) of positive samples. In the current study, we have detected bacteria in 41 % of the samples using PCR-based methods, each containing up to 11 different species, compared to only 25–26 % with routine detection and up to 2 species reported per sample.

Using molecular methods, Oue et al. (2009) have identified 20 species in samples of NGA \( (n=42) \). Those given were also observed in this study; however, we also identified another 31 species using the combined approach. Interestingly, many of these bacteria have not been previously detected in NGA. A combination of methods including a recombinant DNA technique (clone analysis) and a profiling DGGE demonstrated improved bacterial detection. The procedures required vary considerably for each method, which may well explain the detection variations between both techniques (Table 4). In any case, the predominant bacteria were identified by both methods while the differences were observed with the less prevalent species.

Due to the invasiveness of the procedure, no healthy neonates are normally sampled for this specimen. In theory, gastric fluids in the healthy fetus should be sterile since it is thought to develop in a bacteria-free environment and bacterial colonization of the amniotic cavity is considered a pathological finding (Romero et al., 2007). Nevertheless, bacteria acquired at the time of delivery and through other sources of contaminations may be present in the samples. It was recently demonstrated that the initial microbiota in healthy neonates reflects the mother’s vaginal bacteria if delivered vaginally and the skin microbiota in Caesarean sections (Dominguez-Bello et al., 2010). However, in the aforementioned study (Dominguez-Bello et al., 2010) the samples were obtained from relatively superficial sites (skin, oral mucosa and nasopharyngeal aspirate) and before removing the vernix caseosa, which would explain the observations. Conversely, our data demonstrated that a high percentage of samples obtained from the vaginal delivery group were negative (49 %, 59/121) even when using highly sensitive molecular techniques. Furthermore, the microbiota our study has identified in the NGA resembles that described elsewhere in studies of the amniotic fluid (Han et al., 2009; Kotecha et al., 2004; Miralles et al., 2005) rather than solely of a vaginal/skin origin. Therefore, this supports the assumption that NGA contains swallowed amniotic fluid.

Interestingly, in the amniotic fluid studies, an association between intra-amniotic infection and clinical events, such as early onset neonatal sepsis, chorioamnionitis and funisitis, was demonstrated, which supports the potential value of NGA as an alternative to investigate those infections associated with APO, as suggested by Miralles et al. (2005). However, the bacterial complexity observed in the samples suggests the possibility that bacteria in general and specific bacteria may have originated from other sources of clinical and non-clinical origin or a combination of these.

Although the results indicate that the predominant bacteria have probably originated from the birth canal during
<table>
<thead>
<tr>
<th>Species</th>
<th>Total (n) (%) [n=240]</th>
<th>Mode of delivery</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaginal [n=121]</td>
<td>Caesarean [n=119]</td>
</tr>
<tr>
<td>Total PCR detection positive (n) (%)</td>
<td>98 (41)</td>
<td>62 (51)</td>
<td>36 (30)</td>
</tr>
<tr>
<td>No. of samples with bacteria characterized (n) (%)*</td>
<td>80 (82)</td>
<td>51 (82)</td>
<td>29 (81)</td>
</tr>
<tr>
<td>Total samples included in study (n)†</td>
<td>196‡</td>
<td>100‡</td>
<td>96‡</td>
</tr>
<tr>
<td>*S. pneumoniae/S. mitis/S. oralis/S. infantis§</td>
<td></td>
<td>27 (14)</td>
<td>19 (19)</td>
</tr>
<tr>
<td>U. urealyticum/Ureaplasma parvum§</td>
<td></td>
<td>18 (9)</td>
<td>15 (15)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>14 (7)</td>
<td>13 (13)</td>
</tr>
<tr>
<td>Lactobacillus iners</td>
<td></td>
<td>13 (7)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>Gardnerella vaginalis</td>
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<td>10 (5)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Sneathia sanguinegens/Sneathia amnionii§</td>
<td></td>
<td>9 (5)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa/Pseudomonas otitidis§</td>
<td></td>
<td>8 (4)</td>
<td>6 (6)</td>
</tr>
<tr>
<td>Lactobacillus crispatus</td>
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<td>5 (5)</td>
</tr>
<tr>
<td>Streptococcus sanguinis/Streptococcus gordoni§</td>
<td></td>
<td>7 (4)</td>
<td>5 (5)</td>
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<tr>
<td>Staphylococcus epidermidis</td>
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<td>6 (6)</td>
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<tr>
<td>Peptoniphilus spp.</td>
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<td>6 (3)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Veillonella montpellierensis (Veillonella atypica/</td>
<td>5 (3)</td>
<td>3 (3)</td>
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<tr>
<td>Veillonella parvula)§</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td></td>
<td>5 (3)</td>
<td>3 (3)</td>
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<tr>
<td>A. christensenii</td>
<td></td>
<td>4 (2)</td>
<td>4 (4)</td>
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<tr>
<td>Streptococcus salivarius/Streptococcus vestibularis§</td>
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<td>4 (2)</td>
<td>3 (3)</td>
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<tr>
<td>Brevundimonas diminuta</td>
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<td>2 (2)</td>
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<tr>
<td>H. influenzae</td>
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<td>4 (2)</td>
<td>2 (2)</td>
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<tr>
<td>Prevotella bivia#</td>
<td></td>
<td>3 (2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>S. siniensis/S. parasanguinis§#</td>
<td>3 (2)</td>
<td>2 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius</td>
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<td>2 (2)</td>
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<tr>
<td>Spingomonas spp.</td>
<td></td>
<td>3 (2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Streptococcus constellatus/Streptococcus intermedius/</td>
<td>3 (2)</td>
<td>1 (1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Streptococcus anginosus§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulicatella elegans</td>
<td></td>
<td>2 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Veillonella parvula/Veillonella dispar§</td>
<td></td>
<td>2 (1)</td>
<td>1 (1)</td>
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<tr>
<td>Spingopyxis alaksensis</td>
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<tr>
<td>Massilia sp.#</td>
<td></td>
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<td>1 (1)</td>
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<tr>
<td>Moraxella osloensis</td>
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<tr>
<td>Propionibacterium acnes§</td>
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<td>2 (2)</td>
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<tr>
<td>Prevotella sp.</td>
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<td>2 (2)</td>
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<tr>
<td>F. nucleatum</td>
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<td>2 (1)</td>
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<tr>
<td>Hyphomicrobium zavarzini</td>
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<tr>
<td>Acinetobacter baumannii#</td>
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<td></td>
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<tr>
<td>Corynebacterium riegelii</td>
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<td>1 (1)</td>
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<tr>
<td>Bacteroides sp.¶</td>
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<td>1 (1)</td>
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<tr>
<td>Staphylococcus sp.¶</td>
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<tr>
<td>Enterococcus faecalis¶</td>
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<tr>
<td>Lactobacillus gasseri#</td>
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<tr>
<td>Anaerococcus vaginalis¶</td>
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<td>1 (1)</td>
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<tr>
<td>Faecalibacterium prausnitzii¶</td>
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<td>1 (1)</td>
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<tr>
<td>Megasphaera sp.#</td>
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<td>1 (1)</td>
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<tr>
<td>Fusobacterium necrophorum¶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caldimonas taiwanensis¶</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Silanimonas lentis¶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium sp.¶</td>
<td></td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Atopobium minutum¶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens¶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusobacterium periodonticum¶</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Klebsiella pneumoniae#</td>
<td></td>
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<p>| | | | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
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Table 4. cont.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total (n (%)) [n=240]</th>
<th>Mode of delivery</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaginal [n=121]</td>
<td>Caesarean [n=119]</td>
</tr>
<tr>
<td>Proteus mirabilis†</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Mycoplasma hominis†</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

*Percentage of the samples that could be characterized in this study was calculated out of the number of PCR-positive samples.
†Total number of samples studied was calculated as the percentage of samples characterized out of the total number of samples.
‡Percentage of specific bacteria was calculated out of the total number of samples characterized.
§Species considered in the same group due to difficulty of differentiation by the 16S rRNA gene approach.
¶Species detected with both molecular techniques.
§Species identified with cloning analysis only.
#Species identified with DGGE approach only.

vaginal delivery and as a result of a prolonged ROM (odds ratio=5.7), most of the prevalent species described in vaginal deliveries are also observed in Caesarean sections, exceptions were Staphylococcus epidermidis and Aerococcus christensenii, frequent colonizers of the female genito-urinary tract (Collins et al., 1999). This observation supports the ascending route as the most common pathway of intrauterine infection in which micro-organisms from the vaginal/cervical tract gain access to the intrauterine cavity (Romero et al., 2006); for example bacterial vaginosis has frequently been associated with APO (Kimberlin & Andrews, 1998). Another explanation, however, may be provided by previous work wherein it was demonstrated that a suction-like effect during uterine contractions may introduce vaginal fluid into the amniotic cavity (Zervomanolakis et al., 2007). Gram staining results also support the hypothesis since there was a significant association between the presence of bacteria and Caesarean patients in labour.

Our data confirm that prolonged ROM (in vaginal and Caesarean deliveries) and ruptured membranes (in Caesarean deliveries) determine the presence of bacteria in the neonates as observed with both the Gram stain and molecular data. It is well known that ROM in labour or not in labour can result in increased rates of maternal and fetal infection (Bryant-Greenwood & Millar, 2000; Romero et al., 1991). Administration of IAP has been instituted as a routine hospital procedure since the mid-1990s, particularly to decrease the incidence of early onset group B streptococcal sepsicaemia (Ohlsson & Shah, 2009; Schrag et al., 2000). In this study, no cases of elective Caesarean received IAP; all the cases were emergency Caesareans. The presence of S. agalactiae was close to significance with IAP in Caesarean (P=0.086) and also in vaginal deliveries. The routine administration of IAP in women with a history of infection with GBS may be an explanation as it is not known if the bacteria are viable; also, IAP may disrupt the perinatal microflora, allowing antibiotic-resistant GBS strains and other pathogenic bacteria to proliferate.

Streptococcus pneumoniae/Streptococcus mitis/Streptococcus oralis/Streptococcus infantis were the most prevalent bacteria (27%) in the samples and were associated with IAP in Caesarean sections. Although viridans streptococci (S. mitis and S. oralis) are of low pathogenicity, S. mitis has been reported to cause early onset neonatal infection and neonatal death as a result of its resistance to antibiotics (Adams & Faix, 1994). High prevalence of the S. mitis group in perinatal infections has not been reported before; however, S. pneumoniae is one of the major bacterial pathogens worldwide causing bacteraemia and community-acquired infections in infants (Selva et al., 2010). Unfortunately, members of the S. mitis group are genetically closely related to each other and we were not able to differentiate them in this study.

Ureaplasma urealyticum has been implicated in several forms of APO (Waites et al., 2005). However, carriage in the women's genito-urinary tract does not necessarily trigger an APO (Govender et al., 2009), nor does the treatment of the infection alter the occurrence of prematurity (Romero et al., 1989). High prevalence of U. urealyticum in NGA from vaginal deliveries indicates this pathogen is likely to be derived from the birth canal during delivery as is present at low prevalence in the Caesarean cases; although, it is also possible it was acquired from contamination during the collection of the sample since U. urealyticum, as well as Lactobacillus spp., Pseudomonas spp., Gardnerella vaginalis and Sneathia spp., were present at significantly higher levels in vaginal deliveries collected on the maternal wards. This may be due to the difficulty in achieving sterile conditions in the maternal wards and also because they usually take a longer time for collection. Similarly, an association was shown between Pseudomonas spp. and Caesarean samples collected at more than 12 h post-delivery (P=0.015). Therefore, greater care during collection and transport of the samples should be advised.

The most common pathogens recognized as causing nosocomial bloodstream infection in neonatal intensive care units (E. coli, Staphylococcus spp., Klebsiella and Pseudomonas spp.) were also identified in the samples. E. coli was associated with prolonged ROM in vaginal deliveries. This is one of the most common bacterial
Table 5. Associations between clinical characteristics of the pregnancy and Gram stain, culture and molecular results in samples delivered by Caesarean section

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Gram stain</th>
<th>Caesarean deliveries (n=119)</th>
<th>Culture</th>
<th>Molecular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n=24)</td>
<td>Negative (n=95)</td>
<td>P values*</td>
<td>Positive (n=22)</td>
</tr>
<tr>
<td>In labour (n) (%)</td>
<td>16 (80)</td>
<td>41 (47)</td>
<td>0.012†</td>
<td>11 (65)</td>
</tr>
<tr>
<td>Ruptured membranes (n) (%)</td>
<td>14 (74)</td>
<td>32 (45)</td>
<td>0.038†</td>
<td>9 (53)</td>
</tr>
<tr>
<td>Prolonged ROM (24 h) (%)</td>
<td>5 (21)</td>
<td>9 (10)</td>
<td>0.154</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Preterm birth (n) (%)</td>
<td>6 (25)</td>
<td>70 (74)</td>
<td>&lt;0.001†‡</td>
<td>8 (37)</td>
</tr>
<tr>
<td>Low birth weight (n) (%)</td>
<td>8 (33)</td>
<td>63 (66)</td>
<td>0.005†‡</td>
<td>10 (46)</td>
</tr>
</tbody>
</table>

*P values determined using Fisher’s exact test.
†Significant difference as determined by P <0.05.
‡Prevalence associated with no observation of the clinical variable.

Although no associations were observed with preterm birth, low birth weight or previous complications identified in the samples, the potential involvement of the bacteria in the complications is indicated. It is likely that the bacteria causing the infections in the vagina may have originated from the maternal site harboring a complex community (e.g., the mouth and/or nose), and may be transmitted to the fetus and/or neonate during delivery.

Conclusions

The presence of certain bacterial species in the samples collected from women in labour and Caesarean deliveries suggests a possible association with the complications observed. Further studies are necessary to investigate this potential pathogen's involvement in APO.

Another interesting observation was the association between periodontal disease and APO. It has been suggested that an association exists between periodontal disease and APO, although not significant in the Caesarean group. However, an association between periodontal disease and Caesarean deliveries has also been observed in a multi-factorial analysis.

Significant association was observed between Lactobacillus spp. and neonates delivered at term and presenting a birth weight >2500 g. Although not significant in the Caesarean group, further studies are necessary to investigate this potential pathogen's involvement in APO.
Table 6. Associations between molecular detection of general and specific bacteria in NGA and clinical characteristics of the pregnancy in vaginal deliveries

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Vaginal deliveries (n=121)</th>
<th>Molecular</th>
<th>Positive (n=62)</th>
<th>Negative (n=59)</th>
<th>P value*</th>
<th>Streptococcus mitis</th>
<th>Ureaplasma spp.</th>
<th>Lactobacillus iners</th>
<th>Lactobacillus crispatus</th>
<th>Escherichia coli</th>
<th>Streptococcus agalactiae</th>
<th>Pseudomonas spp.</th>
<th>Gardnerella vaginalis</th>
<th>Sneathia spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous complications (n (%)*[66])</td>
<td>13 (50)</td>
<td>15 (52)</td>
<td>1.000</td>
<td>0.469</td>
<td>1.000</td>
<td>0.611</td>
<td>0.611</td>
<td>–</td>
<td>0.023†‡</td>
<td>1.000</td>
<td>0.111</td>
<td>0.491</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Prolonged ROM (&gt;24 h) (n (%))</td>
<td>18 (29)</td>
<td>7 (12)</td>
<td>0.025†</td>
<td>0.204</td>
<td>0.213</td>
<td>0.313</td>
<td>0.313</td>
<td>–</td>
<td>0.017†</td>
<td>0.556</td>
<td>1.000</td>
<td>0.281</td>
<td>0.344</td>
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</tr>
<tr>
<td>Preterm birth (n (%))</td>
<td>32 (52)</td>
<td>32 (54)</td>
<td>0.856</td>
<td>0.328</td>
<td>0.593</td>
<td>0.020†‡</td>
<td>0.188</td>
<td>0.021†‡</td>
<td>0.379</td>
<td>0.342</td>
<td>1.000</td>
<td>0.146</td>
<td>0.721</td>
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<tr>
<td>Low birth weight (n (%))</td>
<td>28 (45)</td>
<td>32 (54)</td>
<td>0.365</td>
<td>0.133</td>
<td>0.789</td>
<td>0.008†‡</td>
<td>0.095</td>
<td>0.057</td>
<td>0.075</td>
<td>0.619</td>
<td>1.000</td>
<td>0.061</td>
<td>0.491</td>
<td></td>
</tr>
<tr>
<td>IAP (n (%)*[39])</td>
<td>6 (13)</td>
<td>9 (18)</td>
<td>0.582</td>
<td>0.688</td>
<td>0.604</td>
<td>0.572</td>
<td>0.491</td>
<td>1.000</td>
<td>1.000</td>
<td>0.060</td>
<td>0.060</td>
<td>0.153</td>
<td>0.586</td>
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</tr>
<tr>
<td>Samples collected at the maternal ward (n (%)*[1])</td>
<td>22 (36)</td>
<td>10 (17)</td>
<td>0.038†</td>
<td>0.582</td>
<td>0.004†‡</td>
<td>0.002†‡</td>
<td>0.022†‡</td>
<td>0.018†‡</td>
<td>0.105</td>
<td>0.289</td>
<td>0.031†</td>
<td>&lt;0.001†</td>
<td>0.031†</td>
<td></td>
</tr>
</tbody>
</table>

*P values determined using Fisher’s exact test.
†Significant difference as determined by P<0.005.
‡Prevalence associated with no observation of the clinical variable.

[1] Data not known for n number of cases; (%), percentage within group (molecular).
vagina, the gut or the oral cavity), and that more than one single pathogen may translocate. As observed, DGGE profiling may provide a very useful tool for potential clinical use due to its sensitivity in detecting the most prevalent bacteria, ease of use and relatively low cost. In this study, several clinical variables (mode of delivery, prolonged ROM, labour and IAP) have been shown to influence the presence of bacteria in neonates; however, bacteria possibly acquired during collection are also found in the samples. Under a more strict collection protocol, the study of NGA should provide a new insight into the investigation of the species of bacteria possibly associated with APO. Specifically, the effect of antibiotic prophylaxis and antibiotic resistance in clinical trials should be evaluated.

ACKNOWLEDGEMENTS

We would like to thank the UNESCO-L’OREAL partnership for supporting this study through the International Fellowship awarded to C.G-M. Consumables for this study were supported with departmental funds. Special thanks to the staff of the hospital’s laboratory, maternity and neonatal wards at Barts and The London NHS Trust for their support, especially to Dr Mark Wilks and Miss Anita Sanghi.

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