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Levels of periodontal pathogens in neonatal gastric aspirates and possible maternal sites of origin

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Keywords: adverse pregnancy outcomes; neonatal infection; oral cavity; quantitative polymerase chain reaction; vagina
Accepted 4 May 2011
DOI: 10.1111/j.2041-1014.2011.00616.x

SUMMARY

Maternal periodontal infection has been recognized as a risk factor for preterm and low birthweight infants. It is suspected that pathogens causing periodontal disease may translocate to the amniotic cavity and so contribute to triggering an adverse pregnancy outcome. This study aimed to determine levels and proportions of periodontal bacteria in neonatal gastric aspirates obtained from complicated pregnancies and the respective maternal oral and vaginal samples using a quantitative polymerase chain reaction approach, and also to determine the origin of the neonate's bacteria by sequence comparisons between the three sites. *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* were not observed in the neonates or in the women's vaginas. Interestingly, *Porphyromonas gingivalis* was identified in the neonates in two samples (2.98E+02 and 1.75E+02 cells ml^{-1}) and in association with *Fusobacterium nucleatum*, which was observed at high prevalence (10%) and at high levels reaching up to 2.32E+03 cells ml^{-1}. Although *F. nucleatum* was also present in the vaginal samples, the results demonstrated that the neonatal strains were more likely to originate from the mother's oral cavity than to be vaginal strains.

INTRODUCTION

Adverse pregnancy outcomes denote those complications presenting during pregnancy or immediately after birth that may cause the termination of the pregnancy and compromise the wellbeing of the fetus/neonate or the mother. Maternal periodontal infection has shown association with adverse pregnancy outcome, specifically preterm birth and low birthweight, in several observational and interventional studies (Offenbacher *et al.*, 1996; Jeffcoat *et al.*, 2011) but other studies have not found the same associations (Davenport *et al.*, 2002; Michalowicz *et al.*, 2006) so this hypothesis remains controversial. The strongest evidence supporting the association is provided by clinical findings, in which possible oral bacteria have been detected in the amniotic environment of women who presented with an adverse pregnancy outcome, suggesting the possibility of an

oral–uterine translocation (Han *et al.*, 2006). Animal studies have confirmed that oral bacteria are able to translocate via the bloodstream, cross the placental membranes, and specifically invade the amniotic cavity resulting in preterm delivery, fetal growth restriction and fetal death (Han *et al.*, 2004; Fardini *et al.*, 2010).

Microorganisms implicated in periodontal disease possess numerous virulence factors that may allow them to survive, translocate and colonize distant non-oral sites. *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* have been strongly associated with severity of periodontal disease, disease progression, and unsuccessful therapy as defined by the World Workshop on Clinical Periodontics (1996). Their selection criteria also evaluated their capability to disseminate in the circulation and contribute to systemic diseases. *Aggregatibacter actinomycetemcomitans* has been implicated in the aetiology of infective endocarditis, and translocation could occur because of its ability to attach to collagen (Tang *et al.*, 2008). *Porphyromonas gingivalis* has been implicated in several cardiovascular diseases and is able to inactivate toll-like receptors by the release of gingipains, which inhibit the host cell recognition process (Kishimoto *et al.*, 2006). Although *T. forsythia* has not been previously associated with non-oral infections, its enhanced pathogenicity in periodontal disease in the presence of *P. gingivalis* and *Fusobacterium nucleatum* suggests that it should be considered (Sharma, 2010). *Fusobacterium nucleatum*, one of the most prevalent species in the oral cavity in health and disease, is also the most common oral species isolated from amniotic fluid in cases of preterm labour with intact membranes, which accounts for the particular interest this microorganism has received in several studies investigating its possible contribution to adverse pregnancy outcome (Bearfield *et al.*, 2002; Liu *et al.*, 2007).

Nevertheless, the presence of potential oral bacteria in a woman's amniotic cavity does not necessarily indicate that the oral cavity is the source. It is possible that the bacteria observed may have originated from other body sites. Generally, the oral cavity and the vagina harbour similar microbiota. Species such as *Prevotella*, *Veillonella*, *Lactobacillus*, *Leptotrichia*/*Sneathia* and *Streptococcus* are common to both sites. To date, no species of the genera *Aggregatibacter (Actinobacillus)*, *Porphyromonas* or *Tannerella* have been reported as common inhabitants of the female genitourinary tract in humans; even though, *Porphyromonas* spp. have been identified in the vagina (Hill *et al.*, 2005). However, there is no report of any species-specific and species-sensitive techniques to investigate the presence of these pathogens in the genitourinary tract of women.

On the other hand, *F. nucleatum* has been reported as an inhabitant of the vagina (Hillier *et al.*, 1993). The maternal vagina could therefore represent a reservoir for this so-called oral pathogen. The origin of *F. nucleatum* in amniotic fluid is still not clear. A previous study suggested that *F. nucleatum* subspecies found in the amniotic fluid from women with intact membranes (*F. nucleatum* subsp. *vincentii*, subsp. *nucleatum* and subsp. *polymorphum*) match more closely the strains present in subgingival sites (Hill, 1993); whereas *F. nucleatum* subsp. *animalis* has been reported as commonly prevalent in the human gut (Strauss *et al.*, 2008). However, Han *et al.* (2010) recently demonstrated a match between the subgingival species *F. nucleatum* subsp. *animalis* and a strain identified from the fetus in a case of term stillbirth; the same strain was not found in the vagina, rectum or supragingival sample of the same woman. There is a need to further investigate the origin of *F. nucleatum* and other periodontal pathogens in association with adverse pregnancy outcomes.

In this study, we investigated samples of neonatal gastric aspirates (NGA) (containing swallowed amniotic fluid) obtained from complicated pregnancies. These samples have previously been demonstrated to elucidate those pathogens possibly associated with adverse pregnancy outcome because positive bacterial detection correlates at the 100% level with the presence of chorioamnionitis and presentation of preterm rupture of membranes in preterm infants (Miralles *et al.*, 2005). We have previously described the bacterial content in these samples using a combination of broad-range end-point polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis approaches identifying various potential pathogens from possible genitourinary, gastrointestinal and oral origin (Gonzales-Marin *et al.*, submitted for publication). Interestingly, *F. nucleatum* was observed in two (2%) samples of NGA as well as exclusively oral species such as *Granulicatella elegans* and *Streptococcus sinensis*. *Aggregatibacter*

actinomycetemcomitans, *P. gingivalis*, *T. forsythia* and *F. nucleatum* were included in the analyses.

The use of a quantitative-PCR analysis ensured a higher sensitivity to detect even those species present at very low levels. In addition, relative levels of bacteria would provide an estimate of the clinical relevance regarding their presence in the samples. Women's oral and vaginal samples were investigated as the origin of the bacteria. Finally, similarities and differences in species and subspecies of *Fusobacterium* found at the three sites were investigated.

METHODS

Samples of neonatal gastric aspirate

Gastric aspirates are routinely taken at the 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' (Ref. no. BSOP 23i4.1) from newborns that present with a clinical manifestation of possible neonatal sepsis (respiratory distress, unstable temperature and cardiovascular depression) or are at risk of developing an infection (preterm birth, low birthweight, preterm rupture of membranes, maternal fever, etc.) (Health Protection Agency, 2004). These aspirates are obtained by inserting a sterile nasogastric tube into the stomach of the newborns during the first hours after delivery and before feeding. In theory, they should contain bacteria to which the fetus has been exposed *in utero* (swallowed amniotic fluid), and transient microorganisms acquired during delivery (Gonzales-Marin *et al.*, submitted for publication).

Maternal samples

A representative group of women, whose children were required to provide an NGA sample as part of their routine care, were invited to participate in the study. Ethical approval was obtained from the Outer North East London Research Ethics Committee (REC Ref. No. 08/H0701/61) for collection of all samples. The participants were requested to provide a sample from their oral cavity and a lower vaginal swab. The oral samples consisted of a pool of saliva, supragingival and subgingival plaque, and tongue surface scraping. A sample of saliva was obtained by asking the women to hold, and pass around the mouth, a sterile oral swab (Salimetrics Europe Ltd, Newmarket, UK) for 1–2 min. A supragingival plaque sample was collected from the gingival margins of three or four teeth using a sterile toothpick. A full-mouth periodontal examination at six sites per tooth was performed to determine the periodontal status and to identify those sites with deep pockets from which the subgingival samples were obtained using a paper-point technique (Jervøe-Storm *et al.*, 2007). The tongue scraping sample was obtained with a nylon bristle brush (Cytology Brush, Cytosoft™; CardinalHealth, Dublin, Ireland) making a few strokes on the tongue's dorsal surface. Finally, lower vaginal swabs were self-collected by the mothers (Strauss *et al.*, 2005).

Growth of bacterial strains

Reference strains were grown in this study for the construction of the standards for quantitative PCR (Table 1). All the bacterial strains were grown in an anaerobic chamber (MACS 1000; Don Whitley Scientific Ltd, Shipley, UK) with an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide on blood agar solid medium supplemented with 5% defibrinated horse blood (TCS Biosciences Ltd, Botolph Clayden, UK) at 37°C.

DNA isolation

Genomic DNA from the clinical samples and reference strains was extracted using the ArchivePure DNA Purification Kit for Yeast and Gram –/+ (Flowgen Bioscience, Nottingham, UK) following the manufacturer's instructions. Because of the high viscosity of the NGA samples, these samples received pretreatment with dithiothreitol (Mucolyse, Pro-Lab Diagnostics, Round Rock, TX) as detailed elsewhere (Jones *et al.*, 2010).

Quantitative PCR analyses

The quantitative analyses were performed separately for each bacterial species using genomic DNA extracted from the samples. Levels of *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia* and general bacterial load were determined using the SYBR Green I method. Multiwell 96-well plates were used, each well containing 4 μl of the 2 × LightCycler® FastStart DNA Mastermix (Roche Diagnostic,

Table 1 Primers used for bacteria quantification and conditions for each set of primers

		Primers				qPCR conditions	
Bacterial target	Strain[1]	Primer set[2]	Sequences	Reference	Fragment length (bp)	Annealing temp. (°C)	Extension time (s)
General bacteria	NCTC 10562	357F	5′-CTCCTACGGGAGGCAGCAG-3′	Lane (1991); Weisburg *et al.* (1991)	200	58	10
		518R	5′-ATTACCGCGCTGGTGG-3′	Muyzer *et al.* (1993)			
F. nucleatum	NCTC 10562	Fn1F	5′-GACAGAGCTTTGCGTCC-3′	Nagano *et al.* (2007)	609	57	25
		Fn2R	5′-TGGGCGCTGAGGTTCGAC-3′				
A. actinomycetem-comitans	ATCC 33384	AaF	5′-ATTGGGGTTTAGCCCTGGTG-3′	Tran and Rudney (1999)	360	61	15
		C11R	5′-ACGTCATCCCCACCTTCCTC-3′	Loy *et al.* (2008)			
P. gingivalis	DSM 20709	Pg2F	5′-GCGTATGCAACTTGCCTTAC-3′	Wahlfors *et al.* (1995)	518	59	21
		Pg2R	5′-GTTTCAACGGCAGGCTGAAC-3′				
T. forsythia	ATCC 43037	Tf2F	5′-GCGTATGTAACCTGCCCGCA-3′	Sakamoto *et al.* (2001)	240	62	11
		357R	5′-CTGCTGCCTCCCGTAGGAG-3′	Lane (1991); Weisburg *et al.* (1991)			

[1]Strains used for construction of standards; [2]Species-specific or a combination of a specific and a universal primer were used; all primers target a fragment in the 16S rRNA gene.

Mannheim, Germany), 5 μM of each forward and reverse primer used for each specific amplification (Table 1), 2 μl of the template and de-ionized water to a final reaction volume of 10 μl. All multiwell plates were sealed, centrifuged and then amplified in a LightCycler LC480, with activation of polymerase (95°C for 5 min), followed by 45 cycles of 10 s at 95°C and 6 s at the T_A determined for each set of primers (Table 1) as recommended by Sambrook *et al.* (1989). This was followed by an extension step that was based on the expected fragment length and was calculated as 1 s for every 25 bp (Table 1). Double-stranded product was measured at 76°C. Melting curve analysis was finally performed immediately after the amplification as follows: 30 s at 95°C, 30 s at 65°C and acquisition at 99°C.

Standard curves for specific bacteria were constructed using DNA extracted from known concentrations of the bacteria grown in pure culture (10^2 to 10^7 cells ml^{-1}). However, the levels of general bacteria could not be precisely calculated because of the diversity of the samples (unknown numbers of species and their relative abundance) and the differences in numbers of *rrn* operons. Therefore, to reduce miscalculations, a pure culture of *F. nucleatum* containing five copies of the gene was used. Five was determined as the average copy number in the range of bacteria observed in NGA (Gonzales-Marin *et al.*, submitted for publication), which vary between 1 and 10 (Lee *et al.*, 2009). Positive controls (known dilution of the strain) and negative controls (water) were included in all tests. Fluorescence curves were analysed against the standards and the absolute amounts were determined using LightCycler® 480 software v. 1.5 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Relative amounts were calculated as the percentage of specific bacteria out of the total bacterial load.

Data collection and statistical analyses

Demographical and clinical data were obtained from the Standarized Electronic Neonatal Database and from the maternity notes. The women were also asked to complete a validated questionnaire (Davenport *et al.*, 2002). All the statistical analyses and graphs were produced using the IBM®SPSS® Statistical 19 software (SPSS, Chicago, IL). Boxplots where constructed using interquartile range and outliers. The non-parametric Mann–Whitney *U*-test was used to compare quantitative levels of bacteria with the binomial demographical/clinical

variables. The two-sided Fisher's exact test was used to compare prevalence of bacteria (using a set detection limit) with the variables. Independent Student's *t*-test was used with parametric data to compare prevalence of bacteria with variables presented as quantitative data (i.e. gestational age and birthweight). Finally, multivariate analysis was performed to control for the effect of important variables upon the occurrence of bacteria in the samples. In all the analyses, a P-value < 0.05 was considered to indicate significance.

Sequencing and identification of *Fusobacterium* species/subspecies

Identification of the subspecies of *F. nucleatum* was performed using species-specific primers in an end-point PCR as detailed previously (Gafan *et al.*, 2004). The products were sequenced directly using the forward primer. All sequencing was carried out at the Genome Centre, Barts and The London with an ABI 3700 automated DNA sequencer producing sequences about 800 bp long. The sequence were identified using the Human Oral Microbiome Database (Dewhirst *et al.*, 2008). The quality of the sequences was checked using the CHROMAS software (http://www.technelysium.com.au/chromas.html). Sequences were aligned using MULTALIGN (Corpet, 1998; http://multalin.toulouse.inra.fr/multalin/). Other bacteria identified in this study followed the same protocol.

RESULTS

Demographical and clinical data

A total of 188 samples of NGA analysed in this study were obtained from the Microbiology Department, Royal London Hospital, Barts and The London NHS Trust on the following day after collection in the hospital. Nineteen women, constituting a representative subset of the neonatal group were included in the

Table 2 Demographical and clinical characteristics, and level of bacteria in the neonatal and maternal sites

		Maternal	
	Neonatal gastric aspirates	Oral cavity	Low vaginal swab
Total number of samples analysed	188	19	13
Demographical data			
Neonate's gender			
Female, *n* (%)	78 (42)[1]	6 (32)	5 (38)
Male, *n* (%)	109 (58)[1]	13 (68)	8 (62)
Maternal age; mean (SD) range	28 (5.7) 17–46[2]	28 (5.9) 18–37	27 (6.5) 18–37
Maternal ethnic origin			
Asian-Bangladeshi/Indian/Pakistani, *n* (%)	95 (51)[1]	7 (37)	2 (15)
Asian-other, *n* (%)	14 (7)[1]	–	–
Black African/Caribbean/other, *n* (%)	22 (12)[1]	1 (5)	1 (8)
White British/Irish/other, *n* (%)	50 (27)[1]	11 (58)	10 (77)
Other-mixed, *n* (%)	6 (3)[1]	–	–
Clinical characteristics			
Multiple delivery, *n* (%)	17 (9)	1 (5)	1 (8)
Vaginal delivery, *n* (%)	91 (49)[3]	10 (53)	8 (62)
Caesarean delivery, *n* (%)	95 (51)[3]	9 (47)	5 (38)
Primiparous, *n* (%)	90 (53)[4]	10 (56)[1]	8 (67)[1]
Previous complications, *n* (%)	49 (34)[5]	7 (41)[2]	4 (36)[2]
Gestational age (weeks), mean (SD)	35 (4.6)	35 (24–41)	34 (24–41)
Preterm birth, *n* (%)	105 (56)	11 (58)	9 (69)
Birthweight (g), mean (SD)	2397 (938)	2360 (700–3848)	2200 (700–3770)
Low birthweight, *n* (%)	95 (51)[1]	9 (47)	6 (46)
Prolonged ROM (>24 h), *n* (%)	25 (13)[6]	3 (16)[9]	3 (23)[9]
Intrapartum antibiotics, *n* (%)	27 (14)[7]	4 (25)[3]	2 (20)[3]
Smoking during pregnancy, *n* (%)	11 (6)[8]	2 (11)[1]	2 (15)

Data not known for: [1]one case, [2]two cases, [3]three cases, [4]19 cases, [5]43 cases, [6]49 cases, [7]33 cases, [8]40 cases, and [9]four cases.

Table 3 Data from women's periodontal examination and questionnaires

Periodontal status	Total (%)(N = 19)
Presence of pockets >4 mm, *n* (%)	13 (68)
Number of sites with pockets >4 mm, median (range)	2 (0–18)
Data obtained with questionnaire	
Drinking habits, *n* (%)	1 (5)
Smoking during pregnancy/smoker, *n* (%)	2 (11)
Infections/antibiotic during pregnancy, *n* (%)	3 (16)
Regular dental care, *n* (%)	10 (53)
Antenatal care, *n* (%)	6 (32)
Stress during pregnancy, *n* (%)	7 (37)
Married or living together, *n* (%)	17 (89)
Father's age[1], mean (range)	31 (20–40)
Education: college/university, *n* (%)	13 (72)[1]
Sexual relations during pregnancy, *n* (%)	16 (89)[1]
Oro-genital contact, *n* (%)	11 (61)[1]

[1]Data not known for one case.

study; 13 (68%) of them also agreed to provide a vaginal swab. General neonatal and maternal data are summarized in Table 2. Results from the periodontal examination and clinical variables collected using the questionnaires are detailed in Table 3. In most of the cases, the woman presented relatively few localized periodontal pockets (between 4 and 6 mm). In only one of the cases the number of sites with pockets reached 18 (10%) distributed around eight different teeth. Presence of pockets in at least one site and number of pockets were therefore included in this report.

Absolute amounts of general and specific bacteria in NGA

The number of samples analysed per bacteria targeted varied mainly because of the availability of the sample. One hundred and sixty-five samples were analysed for general bacteria, 163 for *F. nucleatum*, 101 for *A. actinomycetemcomitans*, 145 for *P. gingivalis* and 99 for *T. forsythia.* Absolute levels of the bacteria in the samples are shown in Fig. 1A. The average number of general bacteria in NGA was 1.04E+05 cells ml^{-1}. In general, *A. actinomycetemcomitans* (mean = 1.77E+01) and *T. forsythia* (mean = 5.52E−01) were observed at very low levels in the samples evaluated. These levels may represent the background signal of the reaction because similar levels were also observed in the controls. *Porphyromonas gingivalis* was also present at low levels (mean = 9.33E+00); however, two of the samples produced high levels (2.98E+02 and 1.75E+02). Interestingly, the sample that contained the highest level of *P. gingivalis* also presented similar levels of

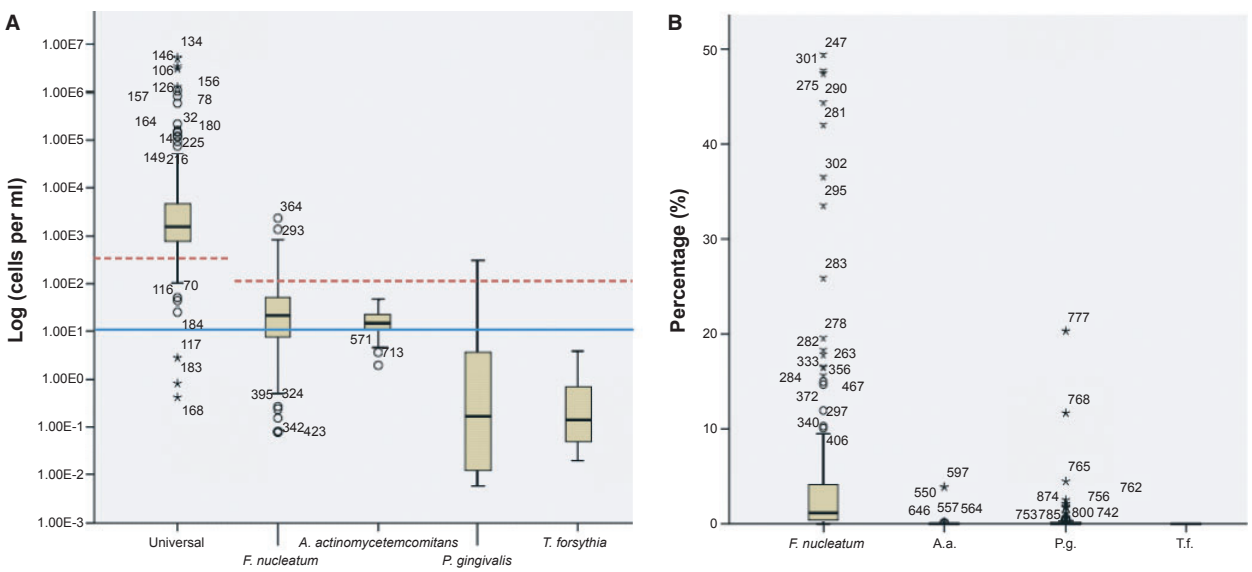

Figure 1 Box-plots representing (A) absolute and (B) relative levels of universal and specific bacteria in neonatal gastric aspirates as determined using quantitative polymerase chain reaction (qPCR) analysis. Blue line, detection limit used to separate positive samples from possible non-specific product identified by q-PCR SYBR-Green method; dashed red line, detection limit set at 500 cells ml^{-1} for general bacteria and 100 cells ml^{-1} for specific bacteria observed when q-PCR results with positive results were compared with the end-point PCR. Numbers represent order of each sample in the list used to construct the graphs (allocated by the IBM SPSS v.19.0 software).

F. nucleatum (1.51E+02). Overall, *F. nucleatum* was identified at high levels (mean = 1.09E+01) reaching up to 2.32E+03 cells ml^{-1} and more than 100 cells ml^{-1} in 26 samples (16%). It could be observed that the end-point PCR, as performed for the species/subspecies analysis of *F. nucleatum*, was usually positive when samples contained more than 100 cells ml^{-1} (with a sensitivity of 90% and a specificity of 91%). Bacterial identity was confirmed using direct sequencing of the end-point PCR products for *F. nucleatum* and *P. gingivalis*.

Relative levels of potential oral bacteria in NGA

In all the cases, levels of general bacteria were higher than the levels of each specific bacterium tested. Percentage of *F. nucleatum* ranged from <0.01 to 49.5% (mean = 1.2) of the total bacterial load. *Porphyromonas gingivalis* present at high levels was also observed at a high proportion in the two samples representing 11.7% and 20.3%; the percentage of *P. gingivalis* in all the remainder of the samples analysed ranged from <0.01% (in 48 samples) to 4.5% (mean = < 0.01). One of the samples of NGA contained a relatively high percentage of *A. actinomycetemcomitans*, representing 3.9% of the total bacterial load, compared with the rest of the samples, in which relative amounts were not higher than 0.2% (mean = 0.01). Percentages of *T. forsythia*, however, were always <0.01% (Fig. 1B).

Quantitative analyses of periodontal bacteria in the maternal oral cavity and vagina

The average level of general bacteria in the samples from the women's oral cavities was 1.65E+06 cells ml^{-1}; whereas the vaginal samples contained an average of 6.00E+05 cells ml^{-1}. Levels and proportions of *F. nucleatum* in the oral cavity (mean = 2.44E+05; 9.6%) were high compared with the equivalent in the vagina (mean = 9.53E+02; 0.7%), in which only seven (53%) presented levels higher than 100 cells ml^{-1}. Absolute levels of *F. nucleatum* between samples of individual cases are compared in Table 4. Using 10 cells ml^{-1} as a detection limit to avoid the false positives inherent in the SYBR-Green based detection chemistry, *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* were present in five (28%), seven (37%) and 19 (100%) of the oral samples respectively (mean = 5.83E+02, 2.03E+04 and 8.41E+04, respectively). Only *A. actinomycetemcomitans* was observed in the vagina in five samples (39%) but at low levels that could not be amplified for identification, and representing <0.01% of the total load in all these cases (Fig. 2).

Table 4 Absolute and relative amount of *Fusobacterium* spp. comparing levels between the neonatal and maternal sites from each individual case

	NGA		Oral cavity		Vagina	
Case ID	Absolute amount (cells ml^{-1})	Relative amount (%)	Absolute amount (cells ml^{-1})	Relative amount (%)	Absolute amount (cells ml^{-1})	Relative amount (%)
1	2.32E+03[1]	n/a	1.22E+05[1]	17.0	8.35E+02[1]	3.7
4	4.10E+01	2.0	2.14E+04[1]	2.4	3.55E+02[1,2]	0.0
5	1.51E+02[1]	10.3	4.46E+03[1]	0.2	4.73E+01[2]	0.0
10	6.24E+02[1]	36.6	1.67E+05[1]	4.5	1.13E+03[1,2]	0.0
16	1.74E+00	3.5	1.89E+03[1]	0.4	9.25E+02[1,n/a]	3.3
53	4.51E+01	0.4	1.59E+05[1]	7.0	8.65E+00[n/a]	0.2
54	2.07E+01	1.8	2.66E+06[1]	24.4	6.43E+02[1]	0.8
55	4.66E+01[2]	0.0	2.71E+05[1]	10.6	1.74E+02[1]	0.3
62	6.65E+00[2]	0.4	3.97E+05[1]	15.1	8.18E+03[1]	0.1
65	6.46E+00	15.0	3.06E+05[1]	23.7	2.05E+01[2]	0.0
162	1.34E+02[1,n/a]	5.3	2.35E+05	32.4	5.19E+01	0.0

[1]Samples considered positive (>100 cells ml^{-1}).

[2]Sample negative with Fusobacteria-specific primer in an end-point PCR technique; [n/a]sample not available for end-point PCR.

NGA, neonatal gastric aspirates; n/a, sample not available.

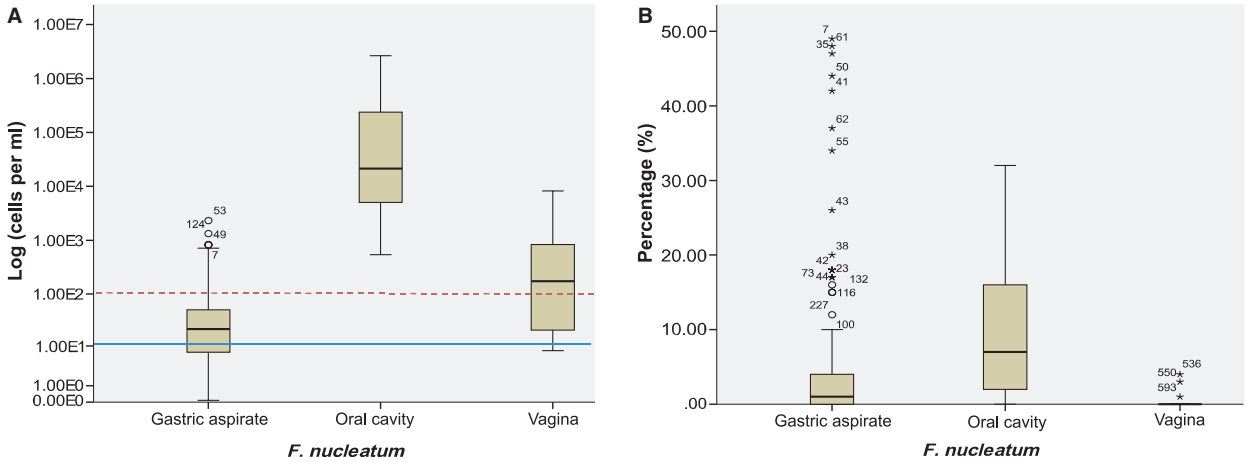

Figure 2 Diagrams representing comparisons between the (A) absolute and (B) relative amounts of *Fusobacterium nucleatum* between the neonatal and maternal samples. Blue line, detection limit used to separate positive samples from possible non-specific product identity by quantitative polymerase chain reaction (qPCR) SYBR-Green method; dashed red line, levels over the detection limit (100 cells ml^{-1}) were usually positive when compared with the qPCR results with positive results with the end-point PCR.

Statistical analyses

General bacterial levels in NGA showed association with vaginal delivery ($P = 0.037$). Also, absolute and relative levels of *F. nucleatum* in NGA showed association with twin delivery ($P = 0.019$); and relative amounts were associated with women presenting with preterm rupture of membranes ($P = 0.001$). It could be observed that those neonates born with a low birthweight (<2500 g) and from a non-white maternal's ethnic origin presented higher than 100 cells ml^{-1} levels of *F. nucleatum* even though association was not quite significant ($P = 0.065$ and $P = 0.062$, respectively). Nonetheless, a multivariate analysis did not confirm any of the associations. Alternatively, using a detection limit of 500 cells ml^{-1} for general bacteria (based on 88% sensitivity/17% specificity against the end-point PCR), an inverse association with gestational age ($P = 0.001$) and birthweight ($P = 0.006$) was observed. Interestingly, this association was maintained for gestational age (odds ratio = 1.2, 95% confidence intervals 1.0–1.3) after adjusting for other covariates.

In the oral cavity, higher amounts of general bacteria were associated with white maternal ethnic origin ($P = 0.048$) and smoking ($P = 0.035$), while low levels of general bacteria in the vagina were associated with preterm rupture of membranes ($P = 0.039$). *Fusobacterium nucleatum* in the oral cavity was also associated with smoking ($P = 0.025$). The percentage of *A. actinomycetemcomitans* in the oral cavity showed an association with non-white maternal origin ($P = 0.015$); particularly women of an Asian/Bangladeshi origin. Finally, high levels and percentages of *A. actinomycetemcomitans* in the oral cavity were associated with sexual relations during pregnancy ($P = 0.026$ and $P = 0.010$, respectively) but the small number of samples did not allow further analysis.

It could also be observed that women who presented with periodontal pockets (regardless of the number of sites affected) tended to have higher levels of general bacteria ($P = 0.023$), and specifically of *F. nucleatum* ($P = 0.027$), in the samples of NGA. Interestingly, no sample of NGA contained *F. nucleatum* when women did not present with periodontal pockets; this group also presented the lowest levels of *F. nucleatum* in their oral cavity; whereas the prevalence in the vagina was mixed.

Fusobacterium species/subspecies in the neonatal and maternal sites

Table 5 summarizes the species of Fusobacteria identified by direct sequencing in the three sites analysed. Usually, the species/subspecies with the highest similarity was selected to identify the taxa. However, some samples identified more than one species with the same percentage of nucleotide matches, in which case both were included in the table. This was only observed in two samples of

Table 5 Detection and identification of *Fusobacterium* spp. and subspecies in the neonatal and maternal sites using direct sequencing

Fusobacterium spp./subspecies	NGA	Oral cavity	Vagina
Number of samples (*N*)	201	19	13
Fusobacterium spp. primers, positive *N* (%)[1]	18 (9)	19 (100)	5 (38)
F. necrophorum	1 (6)	–	1 (20)
F. periodonticum	–	3 (16)	–
F. naviforme	2 (11)	–	–
F. nucleatum	17 (94)	16 (84)	4 (80)
F. nucleatum subsp. *polymorphum*	13 (72)	6 (32)	–
F. nucleatum subsp. *animalis*	2 (11)	1 (5)	3 (60)
F. nucleatum subsp. *vincentii*	3 (18)	7 (37)	–
F. nucleatum subsp. *nucleatum*	–	3 (16)	1 (20)

[1]Segment 1 represent the total number of samples. Segment 2 and 3 are a break down of the percentages of *Fusobacterium* spp. and of *F. nucleatum* respectively.
NGA, neonatal gastric aspirates.

NGA and one sample from the oral cavity. Similarity was in most of the cases >97% as previously suggested (Patel, 2001), except for two samples from the vagina.

The results were also evaluated to compare the species and subspecies between samples of each individual case (Table 6); in which case, all matches >97% were included. In summary, eight samples provided results for the species/subspecies of *Fusobacterium* amplified in the neonatal sample and at least one maternal sample. In four cases, bacteria from the vaginal samples could not be amplified; this may be either because of the small number of bacteria in the sample (cases 5 and 65) or because of low percentage of the total load (cases 5, 10 and 65). A 100% sequence match between the samples was not observed in any case; but sample 1 and 54 demonstrated fewer mismatches with their corresponding oral sample compared with their vaginal counterpart (Fig. 3).

DISCUSSION

We have previously described the bacterial content in NGA using a broad-range end-point PCR approach (Gonzales-Marin *et al.*, submitted for publication). Fifty-one different species were identified in 240 neonatal samples. A likely source of the bacteria was determined as being the vagina during delivery, as also suggested by Dominguez-Bello *et al.* (2010). However, the use of a more sensitive technique, the quantitative PCR analysis, allowed a better understanding of the origin of the bacteria. For example, using a detection limit of 500 cells ml^{-1}, it could be inferred that high levels of bacteria in the samples of NGA were most likely to have been acquired during labour through a 'suction-like' effect, as suggested by Zervomanolakis *et al.* (2007), rather than from the birth canal. An association with increase in gestational age at delivery and a lack of association with vaginal delivery observed in this study when a detection limit was included supports this theory.

In general, even though the main pathogens associated with periodontal disease may possess the mechanisms and virulence factors to translocate haematogenously; they were not commonly found in the samples of NGA. Absence of *A. actinomycetemcomitans* and *T. forsythia* in the neonatal samples was demonstrated. On the other hand, *F. nucleatum* was observed at high prevalence and in high levels, whereas *P. gingivalis* was detected in a relatively high percentage in only two samples (one preterm

Table 6 *Fusobacterium* spp. identified in neonatal and maternal sites and compared by cases

Participant's ID	NGA	Oral cavity	Vagina
1	Fnp 99.5%; Fnv 99.1%	Fnv 99.8%; Fnp 99.6%	Fnn 94.3%
4	Fnp 99.3%; Fnv 99.1%	*F. periodonticum* 100%	Negative
5	Fnv, *F. naviforme* 99.5%; Fnn 99.3%	Fnv 99.8%; Fnp 99.6%	Negative
10	Fnp 99.5%; Fnv 99.3%	Fnv 99.5%; *F. naviforme* 99.3%	Negative
16	*F. necrophorum* 97.4%	Fnn 99.6%	Not available
53	Fnp 97.9%; Fnv, Fnn 97.7%	Fnv 99.6%; Fnp 99.5%	Not available
54	Fnp 98.1%; Fnn, Fnv 98%	Fnn, Fnp 99.3%; Fnv 99.1%	Fna 99.6%; Fnn, Fnv, *F. naviforme* 99.2%
65	Fna, Fnv, *F. naviforme* 99%; Fnn 98.7%	Fnp 100%; Fnv 99.5%	Negative

NGA, neonatal gastric aspirates; Fnp, *Fusobacterium nucleatum* subsp. *polymorphum*; Fna, *F. nucleatum* subsp. *animalis*; Fnv, *F. nucleatum* subsp. *vincentii*; Fnn, *F. nucleatum* subsp. *nucleatum*.

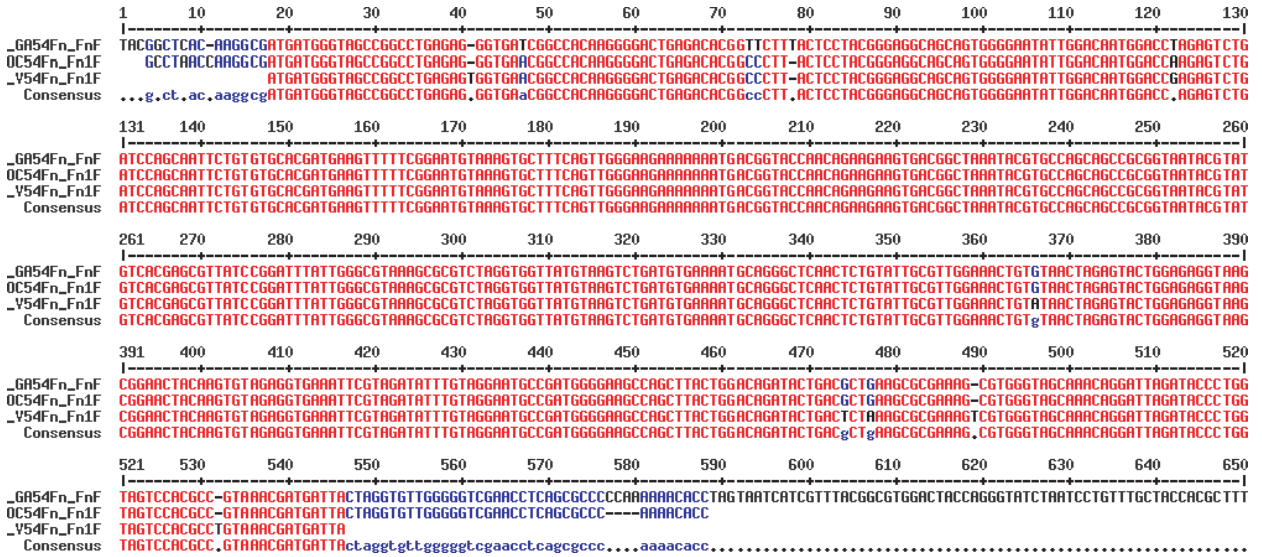

Figure 3 Diagram representing the sequence alignments of a fragment of the 16S rRNA gene of the *Fusobacterium* spp. amplified from the NGA and their respective counterpart in the maternal oral cavity and vagina from CASE 54. *Sequence starts at approximately the 212th base pair of the *Escherichia coli* 16S rRNA gene sequence position (primer FnF1).

infant delivered vaginally at 36 weeks, and one infant delivered by caesarean section at term). Interestingly, the sample that contained the highest level of *P. gingivalis* (vaginal delivery), also presented similar levels of *F. nucleatum* (1.51E+02 cells ml^{-1}). This may indicate that *F. nucleatum* may possess the required mechanisms, not only to translocate haematogenously as extensively demonstrated before; but to serve as a bridge to help other pathogens to invade and proliferate inside the amniotic tissues. Co-migration properties between *F. nucleatum* and *P. gingivalis* have been demonstrated before in epithelial and endothelial cell-culture-based systems (Saito *et al.*, 2008). Studies evaluating co-aggregation properties have suggested that *F. nucleatum* might be a primary colonizer of host tissues and enhance attachment of *P. gingivalis* to the host cells in the oral cavity, which may explain co-occurrence (Metzger *et al.*, 2009). In contrast, Fardini *et al.* (2010) observed, in an animal model, that *F. nucleatum* but not *P. gingivalis* or any other member of the red-complex, was able to invade the murine placenta, although both species were present in the plaque sample that was inoculated directly into the bloodstream.

Porphyromonas gingivalis has been previously identified at high prevalence (30.8%) in the amniotic fluid of woman with a diagnosis of threatened preterm labour (León *et al.*, 2007); these bacteria being identified by colony morphology and biochemical tests. Another study identified *P. gingivalis* antigens in placental tissues using immunocytochemistry in women with chorioamnionitis (Katz *et al.*, 2009). It is therefore plausible that *P. gingivalis* can translocate to the amniotic tissues on its own.

In this study, *P. gingivalis* was identified by sequence comparison and it was also quantified. The relatively low total bacterial load in the *P. gingivalis*-positive samples (1.47E+03 and 1.50E+03 cells ml^{-1}) means that a high percentage was determined. Similarly to our results, Barak *et al.* (2007) reported an average level of 3.61E+02 cells cm^{-3} in homogenized placental tissue of women with pre-eclampsia and preterm birth; which supports our findings. However, the relative levels could be overestimated because of the difficulty in calculating precisely the levels of general bacteria in the NGA. The number of operons per bacterial genome in the samples is known to vary from 1 to 10; therefore we calculate that the relative levels of the bacteria could be overestimated by up to a factor of 10.

Barak *et al.* (2007) also identified *T. forsythia* (2.31E+02) at significantly high levels in placental samples compared with their control women with no pre-eclampsia (5.10E+01 and 1.70E+01 for *P. gingivalis* and *T. forsythia*, respectively). Both *A. actinomycetemcomitans* (1.88E+01), and *F. nucleatum*

(3.30E+01) were also observed in their study but only in the cases group. Similarly, *A. actinomycetemcomitans* was identified at very low levels (max: 4.65E+01 cells ml^{-1}) in this study. These low levels did not allow identification by sequencing even when performing a nested PCR (two successive PCR runs using universal primers followed by specific primers) therefore this study cannot demonstrate the presence of *A. actinomycetemcomitans* in the samples. Furthermore, in contrast to our study, Barak *et al.* (2007) identified *T. forsythia* at high levels, and *F. nucleatum* was identified at low levels in the placental samples. One reason for the differences may be the samples analysed; also, the fact that the gene copy number was not considered, therefore numbers may well be miscalculated (Rajendhran & Gunasekaran, 2011). No other study has identified periodontal bacteria in an amniotic environment using a quantitative approach.

The results presented demonstrate that the most common bacteria associated with periodontal disease (*A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia*) are not normally present in the vagina. It was suggested that oral bacteria might have reached the women's genitourinary tract during oro-genital sex practices (Saini *et al.*, 2010). However, in this study, oro-genital contact during pregnancy did not show any influence as regards the presence of periodontal bacteria in the vagina. Even though most of the women whose infants presented with *F. nucleatum* admitted having oro-genital contact, *F. nucleatum* in the vagina did not show an association with oro-genital contact ($P = 0.633$). In the case of *P. gingivalis* observed in the neonates, the same pathogen was not observed in the woman's oral cavity or vagina. In this particular case, the participant confirmed performing oro-genital contact which may explain the presence of the bacteria in the neonate. Nonetheless, other potential routes of transmission, such as the partners' oral periodontal bacteria, were not investigated in this study.

Levels of periodontal bacteria in the pregnant woman's oral cavity have been studied before (Persson *et al.*, 2008; Ryu *et al.*, 2010; Urbán *et al.*, 2010). Interestingly, in this study, *T. forsythia* was not associated with periodontal disease, as demonstrated previously (Mineoka *et al.*, 2008); instead, it was observed in 100% of the women and at relatively high percentage (median = 1.22E+04 cells ml^{-1}; 5.7%) compared with the other common periodontal bacteria (*A. actinomycetemcomitans* and *P. gingivalis*). In contrast, these other bacteria were observed at high levels in a few samples and usually in association with the presence of pockets. This is the first study reporting such high levels of *T. forsythia* in pregnant women. This could be explained by an increase in gingival inflammation in pregnant women, as demonstrated previously (Adriaens *et al.*, 2009). Our study also confirmed that *F. nucleatum* is highly prevalent in the oral cavity (Moore & Moore, 1994); nevertheless, it is also present in the vagina, although at lower prevalence and at low levels and percentages. Therefore, a woman's genitourinary tract may also represent a reservoir of the pathogen.

It can be inferred from our data that women who smoke during pregnancy may be at a higher risk of an intrauterine fusobacterial infection because an association between smoking and higher levels of *F. nucleatum* in the oral cavity was observed in this study. Presence of periodontal pockets may represent another risk factor as women with pockets present higher levels of *F. nucleatum* in NGA. However, other parameters such as plaque levels or bleeding on probing should be investigated along with translocation given that periodontitis was not found to be a condition required for potential transmission of bacteria. Interestingly, in all four cases that contained high levels of *F. nucleatum* (>100 cells ml^{-1}) in the NGA, the mothers presented with at least one pocket of 4–6 mm depth; three of the cases were emergency caesarean deliveries. The reason for the emergency caesarean section was unfortunately not always clarified and included cases of hypotension, hyperglycaemia, previous endometriosis, previous miscarriage and hypoxia. The opposite was also observed, infants whose mothers did not have pockets, did not present with *F. nucleatum*. However, except for one woman, all performed oro-genital contact during pregnancy; and some cases were shown to contain *F. nucleatum* in the vaginal sample as well; which supports the vagina being a potential origin. It could be observed that samples were usually only positive for the end-point PCR when levels of *F. nucleatum* were higher than 100 cells ml^{-1}. Few exceptions were observed specifically in those cases when the bacterium was present in a low relative amount, which may indicate a possible reason why previous studies do not detect the bacteria in vaginal samples.

To confirm the precise source of the bacteria present in the NGA; further analysis of the species/subspecies was performed by comparison of the aligned sequences. The primers used were designed to amplify any component of the genus *Fusobacterium*. In this study, those of most clinical significance were identified in the samples (*F. nucleatum*, *F. periodonticum*, *F. necrophorum* and *F. naviforme*). *Fusobacterium periodonticum* was limited to the oral cavity; even though it has been reported to not be restricted to the oral niche (Strauss *et al.*, 2008). *Fusobacterium necrophorum* was observed in the NGA and also in the vagina (of a different women), but not in the oral cavity. In accordance with Bennett & Eley (1993), they observed that *F. necrophorum* is more common in the lower part of the body and genitals, whereas *F. nucleatum* is more common in the oropharynx. Similar to previous suggestions (Hill, 1993), the range of subspecies observed in the infants resemble more closely those observed in the oral cavity of the mothers rather than their vaginas. Previous evidence confirmed that the subsp. *animalis* causing a stillbirth originated from the oral cavity. *Fusobacterium nucleatum* subsp. *animalis* has also been reported as an uncommon oral strain but was significantly more prevalent in necrotizing ulcerative gingivitis in a Chinese population (Gmür *et al.*, 2006), as was the women's ethnicity in this report.

Finally, the comparisons performed between samples of individual cases showed that the species observed in the NGA matched more closely with those in the oral cavity than with those in the vagina. It remains to be determined using a greater number of samples whether the vagina may also represent a reservoir for fusobacterial species able to translocate to the neonates. Usually, the presence of oral bacteria in the amniotic environment (amniotic fluid, placenta) has been published in case reports; which indicate that it is relatively rare that translocation of periodontal bacteria to the amniotic environment can cause an adverse pregnancy outcome or perhaps other factors, such as gene–environmental or gene–gene factors, or mechanisms may be involved (Romero *et al.*, 2006).

ACKNOWLEDGEMENTS

We would like to thank the UNESCO–L'OREAL partnership for supporting this study through the International Fellowship awarded to CG. 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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