MOLECULAR DETECTION OF BACTERIA FROM A POSSIBLE MATERNAL ORAL ORIGIN IN NEONATAL GASTRIC ASPIRATES OBTAINED FROM COMPLICATED PREGNANCIES

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ABSTRACT

It has been suggested that periodontal disease, a disease that affects the supporting tissues of the teeth, represents a risk factor for adverse pregnancy outcomes. Certain oral pathogens possess a demonstrated ability to translocate and invade the amniotic tissues. Once in the amniotic environment, these opportunistic colonisers could then initiate or contribute to a perinatal infection, and in this way be involved in the complications. **The overall aim of this study was to determine the presence, and confirm the origin, of suspected maternal oral microbiota in neonatal gastric aspirates (swallowed amniotic fluid) collected due to complications during pregnancy and/or evidence of neonatal sepsis.**

Non-cultural PCR-based methods directed to the ribosomal encoding genes (rDNA) were applied to analyse neonatal and maternal samples. The use of universal and species-specific primers that target the bacterial 16S rRNA gene allowed identification and quantification of broad-range and specific bacteria to the species level. Sequence comparative analysis of a more variable fragment, the intergenic spacer region located between the 16S and the 23S rDNA, was finally used to compare strains obtained from the neonates and their counterparts in the respective mother's oral and vaginal samples.

Data analysis allowed identification of a range of potential confounding factors for presence of bacteria in the infants, such as vaginal delivery and prolonged rupture of membranes. However, bacteria with a possible oral origin were mostly identified in the neonatal samples in low prevalence and not associated with any particular variable. Quantitative analysis of potential periodontal pathogens demonstrated the presence of *Porphyromonas gingivalis* (1%) and *Fusobacterium nucleatum* (16%) in the neonates. *F. nucleatum* was detected at relatively high levels (mean=4.41E+02 cells/ml); representing up to 50% of the total bacterial load, which strongly supports its possible role in pregnancy complications. Also, *F. nucleatum* subspecies analysis and comparisons at the strain level suggest the oral cavity as the most likely origin of this infectious agent. This study supports the need for further studies.

Declaration

I hereby certify that the work embodied in this thesis is the results of my own investigation, except where otherwise stated.

LIST OF PUBLICATIONS/PRESENTATIONS

Publications

Gonzales-Marin C, Spratt DA, Kempley S, Millar MR, Simmonds M, Allaker RP. Identification of bacteria in neonates at risk of infection delivered by caesarean and vaginal birth. [Manuscript submitted to the American Journal of Obstetrics and Gynaecology]. January 2011.

Gonzales-Marin C, Spratt DA, Kempley S, Millar MR, Simmonds M, Allaker RP. Levels of periodontal pathogens in neonatal gastric aspirates and possible maternal sites of origin. [Manuscript submitted to the journal Molecular Oral Microbiology]. February 2011.

Gonzales-Marin C, Spratt DA, Millar MR, Allaker RP. The 16S-23S rDNA intergenic spacer region to determine the precise origin of *Fusobacterium nucleatum* in neonates. [Manuscript submitted to the journal Molecular Oral Microbiology]. March 2011.

Presentations

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Prize awarded: Postgraduate funding award.
Tittle: Molecular techniques to study microbiology of gastric aspirates.
Meeting details: Division of Infection Journal Club, Barts and The London NHS Trust, London. 5th February 2010.

Title: Quantitative-PCR analysis of *Fusobacterium nucleatum* associated with adverse pregnancy outcomes.
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Dedication

This thesis is dedicated to my family;
my dad, mom, and sisters
for all their support and encouragement
during my career.

Special dedication to Patito,
Alexito and Camila
"Teach them well and
let them lead the way"

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LIST OF ABBREVIATIONS

AF	Amniotic fluid
APO	Adverse pregnancy outcome
ATCC	American Type Culture Collection
BA	Blood agar
BHI	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
BV	Bacterial vaginosis
CFU	Colony-forming units
CI	Confidence interval
Cp	Crossing point
DGGE	Denaturing gradient gel electrophoresis
DSM/DSMZ	German Collection of Microorganisms and Cell Cultures (for its acronym in german)
dNTP	Deoxyribonucleotide triphosphate
GBS	Group B streptococci
HOMD	Human Oral Microbiome Database
iATB	Intrapartum antibiotics
IL	Interleukin
ISR	Intergenic spacer region
IUGR	Intrauterine growth restriction
LBW	Low birthweight
LVS	Low vaginal swab
MIAC	Microbial invasion of the amniotic cavity
M_T	Melting temperature
NAM	N-acetylmuramic acid
NCTC	The National Collection of Type Cultures

NGA	Neonatal gastric aspirates
NICU	Neonatal Intensive Care Unit
OD/OD600	Optical density/ OD at a wavelength of 600 nm
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Periodontal disease
PGE_2	Prostaglandin E_2
PPROM	Preterm prelabour rupture of membranes
pROM	Prolonged rupture of membranes
PROM	Preterm rupture of membranes
PTB	Preterm birth
PT/LBW	Preterm and low birthweight
PTL	Preterm labour
q-PCR	Quantitative PCR
RDP	Ribosomal Database Project
rrn/ rRNA	Ribosomal RNA
*rrn*DB	The Ribosomal RNA Operon Copy Number Database
RT	Room temperature
SEND	Standarised Electronic Neonatal Database
SGA	Small for gestational age
T_A	Annealing temperature
TAE	Tris-acetate-EDTA
TNF-α	Tumour necrosis factor alpha
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 OVERVIEW

The oral cavity harbours one of the most complex microbial communities in the human body. Few of these microorganisms present the essential virulence factors that provide them with the ability to cause disease within and beyond the limits of the oral cavity. Periodontal disease (PD) is an inflammatory-based disease of the supporting structures of the teeth, usually as a result of bacterial infection. It refers to a broad group of pathological alterations of the periodontal tissues in which the periodontal vasculature proliferates and dilates, providing a greater surface area that facilitates the entry of microorganisms into the systemic circulation. Based upon these principles, PD (mainly periodontitis) has been previously associated with pregnancy complications in a number of case-control and cohort studies. However, the results from these studies are mixed and therefore controversial. The biological mechanisms of these associations can be explained by translocation of bacteria and/or their products and by the action of maternally produced inflammatory mediators. Nevertheless, to date, none of these theories have provided a clear evidence linking oral infection and adverse pregnancy outcomes (APO).

Pregnancy complications may originate due to multiple causes and infection is one of the most important causes. Presence of bacteria in the amniotic cavity may lead to a diverse range of clinical manifestations such as preterm birth (PTB), spontaneous rupture of membranes, clinical chorioamnionitis, and other adverse perinatal outcomes. Likewise, a significant percentage of newborns develop infection during the first hours of life, representing a major cause of both neonatal death and subsequent disease. Even though the main source of intrauterine infection may originate from the maternal genito-urinary tract, many cases remain unexplained. Therefore, the oral infection theory may provide an alternative explanation.

Animal studies and clinical findings have provided substantial evidence to support a possible oral-uterus translocation. It has been demonstrated that oral bacteria are able to translocate through the bloodstream, cross the placental membranes, and specifically invade the amniotic tissues resulting in an APO. Also, a number of

bacterial species with a possible oral origin have been found in the amniotic cavity associated with cases of PTB, chorioamnionitis, preterm labour (PTL), stillbirth, neonatal death and previous complications. Nevertheless, none of these studies has evaluated the precise origin of the pathogens. Microbiological similarities between the oral cavity and the female genital tract may be misleading the current evidence as to the origin of the infection.

This PhD project focussed on the hypothesis of direct bacterial translocation to the intrauterine cavity. The overall aim of this study was to investigate, using molecular-based techniques, the possibility that bacteria present in the amniotic environment may have originated from the maternal oral cavity in cases of APO. The use of neonatal gastric aspirates (NGA) was introduced in this study. These clinical samples are relatively easily available, avoid the need of invasive procedures, are routinely taken at hospitals from newborns that present with an adverse perinatal outcome, and have been demonstrated as an alternative to AF to investigate perinatal infection. Finally, this work is also supported by the use of novel and precise methods to compare bacterial species from two possible donor sites (maternal oral cavity and vagina) and the recipient site (NGA) at the subspecies, and strain levels.

1.2 THE ORAL MICROBIOTA AND PERIODONTAL DISEASE

1.2.1 The oral microbiota in health and disease.

The oral cavity harbours one of the most complex microbial communities of the human body. This complexity is due to the large number of diverse surfaces, such as soft shedding tissues (buccal and vestibular mucosa, hard and soft palate, tongue, and floor of the mouth) and hard non-shedding surfaces (teeth) that allow a wide variety of complex biofilms to develop (Spratt and Pratten, 2003). Even biofilms associated with teeth are sub-divided, depending on their location; these include supragingival plaque (above the gingival margin often giving rise to caries), gingival margin plaque, subgingival plaque (below the gingival margin and associated with PD), and

approximal plaque (between teeth). The dorsum of the tongue, due to unique structural features, also allows a significant and characteristic microbiota to reside. Each surface provides a unique habitat for microbial colonization with variable gradients of nutrients, oxygen, redox potential, and pH. Moreover, the oral tissues are bathed in saliva, which provides physical cleansing by virtue of fluid flow and dilution effects, as well as host immune and non-immune defence factors that together have profound consequences for the microbial ecology of the oral cavity (Lamont et al., 2006).

Also, different intraoral surfaces and bacterial species have different receptors and adhesion molecules that dictate the composition of biofilms on different oral surfaces (Mager et al., 2003), e.g. *Actinomyces* species colonize hard tissues at far higher proportions than soft tissues, while *Prevotella melaninogenica*, *Veillonella parvula* and *Streptococcus mitis* are found in higher proportions on soft tissue surfaces. Numbers of colonizers may also vary; in supragingival plaque the numbers of bacteria can exceed 10^9/mg on a single tooth surface, while counts in subgingival sites range from about 10^3 in healthy shallow sulci to more than 10^8 in deep periodontal pockets. Although the ecologic relationship between the microbiota and its host are usually benign, occasionally a subset of bacterial species either is introduced, overgrows or exhibits new properties that may lead to disease and the destruction of the oral tissues (Lindhe et al., 2008).

With the advent of culture-independent molecular methods to characterize microbial communities, it has been estimated that more than 600 different species are capable of colonizing the mouth, and any individual may typically harbour 150 or more different species (Aas et al., 2005). With the understanding that essentially all the human oral surfaces are covered by complex microbial biofilms, infection is now recognized to be caused by a group of organisms rather than a single pathogen (Dewhirst et al., 2010). Several bacteria have been associated with a healthy oral cavity (Aas et al., 2005), while other bacteria have been implicated in oral diseases, such as caries (Chhour et al., 2005), periodontitis (Paster et al., 2001, Tanner et al., 2006), and root canal infections (Munson et al., 2002, Sassone et al., 2008).

There is no bacterial species found exclusively in health or disease and the recent evidence, based on the latest methods, is changing previous beliefs that PD is initiated due to colonization by an stablished group of gram-negative anaerobes in the subgingival biofilm e.g., in a study looking for bacterial communities associated with periodontal status changes, increases in levels of *Veillonella* spp., a gram-negative bacterium and the most common member of the subgingival bacterial community, were associated with periodontal health, while *Filifactor alocis*, a gram-positive anaerobe, was found at higher levels in subjects with disease (Kumar et al., 2006, Parahitiyawa et al., 2010).

In addition, certain oral species have been implicated in several systemic diseases such as infective endocarditis, central nervous system infections, pneumonia, osteomyelitis, septicaemia, cardiovascular disease, and preterm and low birthweight (PT/LBW). Through a number of mechanisms, it is likely that bacteria from the oral cavity may gain access into the bloodstream and, in this way, reach distant body sites. Oral species associated with systemic infections include gram-positive and gram-negative bacteria (Table 1.1).

Table 1.1 Bacteria previously associated with systemic infections.*

GRAM-POSITIVE	GRAM-NEGATIVE
Streptococcus constellatus	*Aggregatibacter actinomycetemcomitans*
Streptococcus intermedius	*Agreggatibacter aphrophilus*
Streptococcus oralis	*Caphnocytophaga gingivalis*
Abiotrophia defectiva	*Dialister pneumosintes*
Actinomyces odontolyticus	*Fusobacterium nucleatum*
Solobacterium moorei	*Haemophilus aphrophilus*
	Haemophilus paraphrophilus
	Kingella kingae
	Leptotrichia buccalis
	Leptotrichia trevisanii
	Neisseria meningiditis
	Porphyromonas gingivalis
	Selenomonas spp.

Source: Parahitiyawa *et al.* (2009). *The list is not exhaustive.

1.2.2 Periodontal disease.

Periodontal disease refers to a broad group of pathological alterations of the periodontal tissues (the soft and hard tissues that support the teeth) and represents one of the most widespread infections of humans. The common forms of the disease are known as gingivitis and periodontitis. Gingival disease is manifested as a reversible inflammation of the marginal periodontal tissues and is most frequently associated with dental plaque (plaque-induced gingivitis). The severity and duration of gingivitis can be modified by factors that modulate bacterium-host interactions, including systemic factors (e.g. endocrine changes associated with puberty, pregnancy, and diabetes), medications, and malnutrition.

Periodontitis is an inflammatory-based disease of the supporting structures of the teeth characterized by progressive destruction of the periodontal ligament and alveolar bone that may result in tooth loss (Lamont et al., 2006). The primary clinical features of periodontitis include clinical attachment loss, alveolar bone loss, periodontal pocketing and gingival inflammation (Figure 1.1). In addition, enlargement or recession of the gingivae, bleeding of the gingivae following application of pressure and increased mobility, drifting and/or tooth exfoliation may occur. The histopathological characteristics of periodontitis include periodontal pocketing, location of junctional epithelium apical to the cemento-enamel junction, loss of collagen fibers subjacent to the pocket epithelium, alveolar bone loss, numerous polymorphonuclear leukocytes in the junctional and pocket epithelium and a dense inflammatory cell infiltrate with plasma cells, lymphocytes and macrophages.

The aetiology of PD implicates a bacterial infection as the primary cause of the disease. Several bacterial species residing in a biofilm on the tooth surface have been closely associated with periodontitis. It appears that various complexes of putative periodontal pathogens can initiate and perpetuate the disease in a susceptible host (Flemmig, 1999). Commonly species associated with chronic periodontitis are listed in Table 1.2. *A. actinomycetemcomitans* seems to be the prime candidate in the

aetiology of aggressive periodontitis. Therapy for infections like PD may consist of a combination of mechanical wound debridement and localize application of an antimicrobial agent (Christersson et al., 1991).

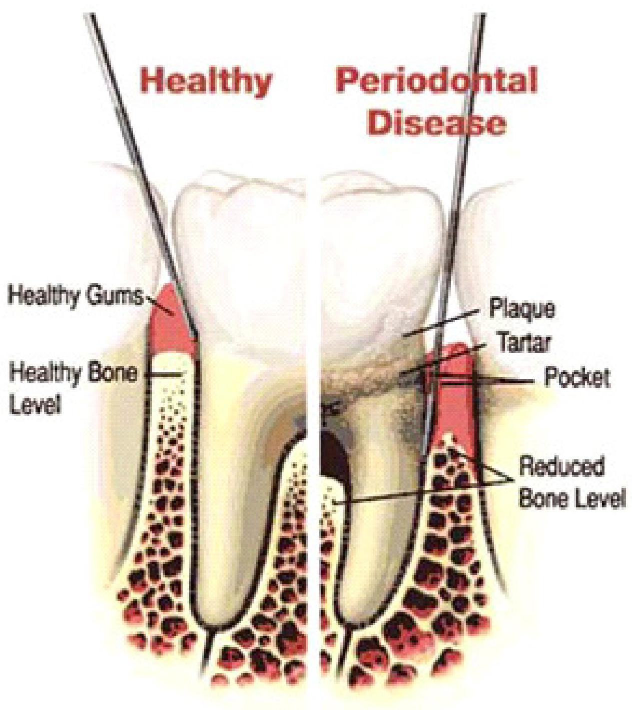


Figure 1.1 Diagrammatic representation of the periodontium in health and disease. Source: American Academy of Periodontology (http://www.perio.org/consumer/graphics/toothdiagram.gif)

Table 1.2 List of species commonly associated with chronic periodontitis.*

GRAM-POSITIVE	GRAM-NEGATIVE
Streptococcus intermedius	*Porphyromonas gingivalis*
Parvimonas micra (formerly *Peptostreptococcus micros*)	*Prevotella intermedia*
	Tannerella forsythia
Eubacterium spp.	*Fusobacterium nucleatum*
	Capnocytophaga spp.
	Eikenella corrodens
	Prevotella nigrescens
	Treponema denticola and other spirochetes

*The list is not exhaustive

1.2.3 The main microbial etiological agents of periodontal disease.

The World Workshop in Periodontology (1996) designated *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* as periodontal pathogens strongly associated with PD, disease progression, and unsuccessful therapy according to the criteria for defining pathogens of destructive PD, initially based on Koch's postulates but amended and extended in later years. These criteria include association, elimination, host response, virulence factors, animal studies, and risk assessment (Lindhe et al., 2008).

1.2.3.1 *Aggregatibacter actinomycetemcomitans.*

Aggregatibacter actinomycetemcomitans (formerly *Actinobacillus actinomycetemcomitans*) is a small, non-motile, gram-negative, saccharolytic, capnophilic, rounded rod that forms small, convex colonies with a "star-shaped" centre when grown on blood agar (BA) plates (Figure 1.2). The most convincing correlation between the presence of *A. actinomycetemcomitans* and PD is found in young patients diagnosed with an aggressive form of the disease (localized aggressive periodontitis). Aggressive forms of PD are rapidly progressive and characteristically present with significant periodontal attachment loss at an early age, with a tendency for familiar clustering of cases (Henderson et al., 2010).The majority of subjects with aggressive periodontitis present an elevated serum antibody response to this species. Its elimination or suppression results in successful therapy, while recurrent lesions harboured the species (Listgarten et al., 1981, Haffajee et al., 1984). Potentially damaging metabolites include leukotoxin and a cytolethal distending toxin (Saiki et al., 2001). It also induces disease in experimental animals and has been found to invade epithelial and vascular endothelial cells *in vitro* (Schenkein et al., 2000), and buccal epithelial cells *in vivo* (Rudney et al., 2001). The species has also been shown to induce apoptotic cell death (Kato et al., 2000).

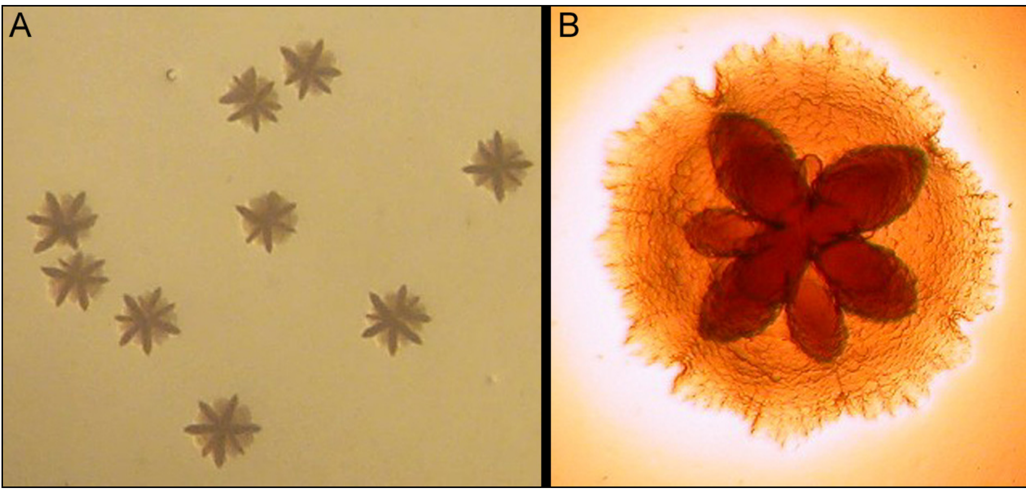


Figure 1.2 (A) Star-like morphologies of multiple colonies. (B) A single colony of *A. actinomycetemcomitans* demonstrating a 'crinkled-rough' surface as well as the characteristic star shape (photograph kindly provided by Dr Derren Ready, Eastman Dental Institute, UCL)

Aggregatibacter actinomycetemcomitans has been implicated in chronic forms of destructive PD, but its role is less clear. It has been isolated from chronic periodontitis lesions, but less frequently and in lower numbers than from lesions in aggressive periodontitis subjects (Rodenburg et al., 1990). Antibody data from treatment of *A. actinomycetemcomitans* infected patients with chronic or refractory periodontitis provided evidence of a possible etiologic role in chronic forms of PD, with decrease in probing pocket depth and gain in attachment levels (van Winkelhoff et al., 1992). There appears to be at least six serotypes of A. *actinomycetemcomitans* (a, b, c, d, e, and f), and its predominance varies from different populations and forms of disease.

It is suspected that *A. actinomycetemcomitans* initially colonizes the oral cavity by attachment to the surfaces of the oral epithelium through a specific protein adhesion, Aae (Fine et al., 2006), and then moves to the subgingival environment where it may attach to and invade the epithelial lining of the periodontal pocket and penetrate the underlying connective tissues (Rudney et al., 2001). Finally, *A. actinomycetemcomitans* has been cultured from atheromatous plaques and has been determined as causing or contributing to cause endocarditis (Paturel et al., 2004,

Kozarov et al., 2005). This pathogen is one of the most frequent oral species detected in cardiovascular disease specimens (Nakano et al., 2009, Wang et al., 2010).

1.2.3.2 *Porphyromonas gingivalis.*

Porphyromonas gingivalis is a gram-negative, anaerobic, non-motile, assaccharolytic rod that usually exhibits coccal to short-rod morphologies. *P. gingivalis* is a member of the "black-pigmented bacteria" characterised for their ability to form brown to black colonies on BA plates (Figure 1.3). *P. gingivalis* is considered the major etiological agent in the initiation and progression of chronic PD. This species is uncommon and present in low numbers in health or gingivitis but more frequently detected in destructive forms of disease (Haffajee and Socransky, 1994). In diseased subjects, there is a strong positive relationship with pocket depth and in sites exhibiting PD progression (Albandar et al., 1997, Kawada et al., 2004). *P. gingivalis* has been shown to be reduced in successfully treated sites but was commonly encountered in sites that exhibited recurrence of disease or persistence of deep periodontal pockets post-therapy (Kawada et al., 2004). It has been shown to induce elevated systemic and local immune responses in subjects with various forms of periodontitis (O'Brien-Simpson et al., 2000). *P. gingivalis* induces a chronic inflammatory response that, locally, results in inflammatory bone destruction which is manifested as PD, and systemically represents a contributory factor for several systemic diseases including diabetes, PTB, stroke, atherosclerosis and cardiovascular diseases (Hayashi et al., 2010).

A variety of virulence factors have been attributed to *P. gingivalis*, including those associated with its cell surface, bioactive metabolic products, and proteolytic enzymes. The bacterial cell surface serves as a dynamic interface between the bacterium and the external environment, and facilitates growth, nutrient acquisition, colonization, biofilm formation and evasion of host defence (Yoshimura et al., 2009). *P. gingivalis* has been shown to be able to invade human gingival epithelial cells *in vitro*, buccal epithelial cells *in vivo*, and endothelial cells (Rudney et al., 2001, Takahashi et al., 2006). Attachment to and invasion of epithelial cells appears to be

mediated by adhesins known as fimbriae (Weinberg et al., 1997). The most potent virulence factors of *P. gingivalis* are the gingipains, which consist of three cysteine proteases that bind to and cleave a wide range of host proteins. Gingipains participate in sequential phases of disease progress including adherence and colonization, nutrient acquisition, neutralization of host defence, manipulation of the inflammatory response, tissue destruction, invasion and dissemination to systemic sites (Guo et al., 2010). It has been shown that gingipains have inhibitory effects on host cell recognition by inactivation of Toll-like receptors that allow *P. gingivalis* to escape from the immune system (Kishimoto et al., 2006). Another important strategy used by this pathogen to evade the host immune response is based upon its ability to invade host cells, such as monocytes/macrophages or dendritic cells in inflamed oral mucosal lesions, to move from the initial colonization site, cross tissue barriers and disseminate in the circulation (blood or lymphatics) to other sites (Hayashi et al., 2010).

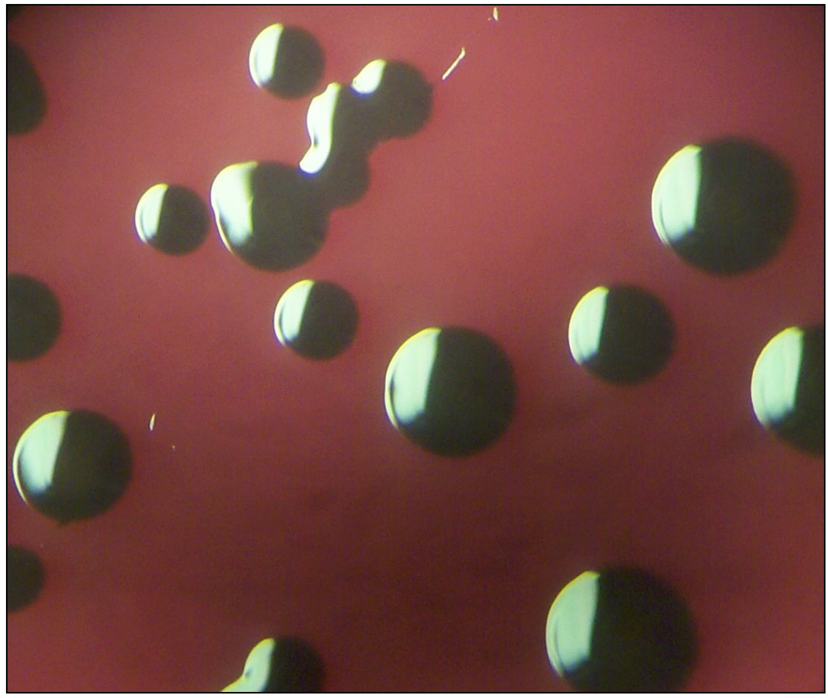


Figure 1.3 Black-pigmented colonies of *P. gingivalis* growing on agar supplemented with blood (photograph kindly provided by Dr Derren Ready, Eastman Dental Institute, UCL)

1.2.3.3 *Tannerella forsythia.*

Tannerella forsythia (formerly *T. forsythensis* and *Bacteroides forsythus*) is a gram-negative, anaerobic, spindle-shaped, highly pleomorphic rod. This pathogen has been classified into the group of red-complex bacteria together with *P. gingivalis* and *Treponema denticola*, which are also strongly implicated in the onset of periodontitis (Mineoka et al., 2008). *T. forsythia* is difficult to grow, often requiring 7-14 days for minute colonies to develop. The growth of the microorganism was shown to be enhanced by co-cultivation with *F. nucleatum*. The need for co-cultivation could be overcome by providing N-acetylmuramic acid (NAM) in the medium (Wyss, 1989). Inclusion of this factor markedly enhanced growth and the resulting cells were regularly shaped, short, gram-negative rods rather than the pleomorphic cells (Figure 1.4).

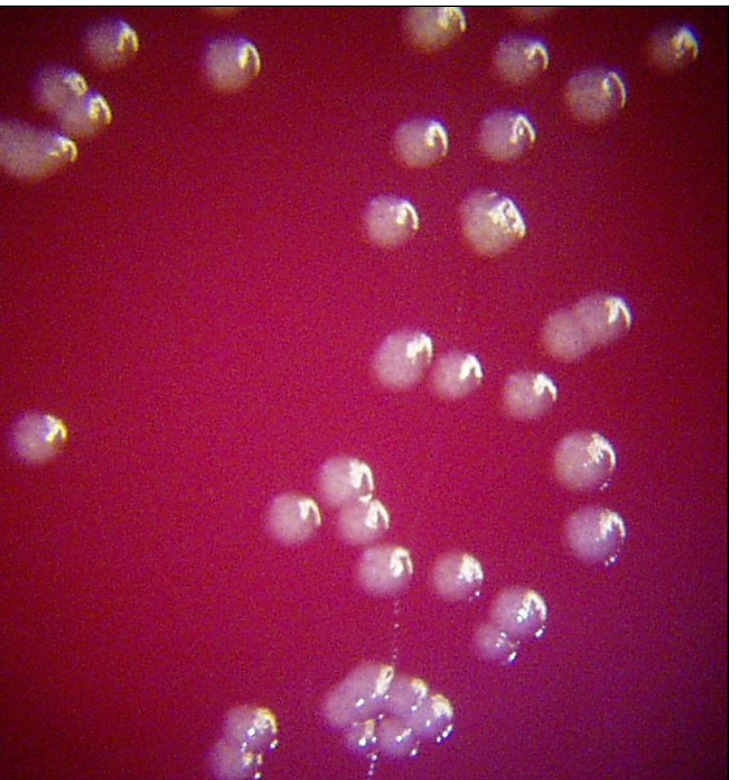


Figure 1.4 Small white colonies of *T. forsythia* growing on agar supplemented with blood and NAM (photograph kindly provided by Dr Derren Ready, Eastman Dental Institute, UCL)

Initially, this species was thought to be a relatively uncommon subgingival species. However, it has been demonstrated that the levels of *T. forsythia* are much higher in subgingival than supragingival plaque samples (Lai et al., 1987). This species was suggested to be the major species found at sites that converted from periodontal health to disease (Tanner et al., 1998). It was found in much higher counts, proportions, and prevalence in subjects with destructive PD, i.e. actively progressing periodontitis, and with greater risks for alveolar bone loss, attachment loss and tooth loss than in periodontally healthy subjects (Lai et al., 1987). *T. forsythia* has been shown to be decreased as regards frequency of detection and counts after successful periodontal therapy, including scaling and root planing, periodontal surgery, or systemically administered antibiotics (Haffajee et al., 2006). It was found at higher level at sites which showed breakdown after periodontal therapy than sites which remained stable or gained attachment (Shiloah et al., 1998). *T. forsythia* is also the most common species detected on or in epithelial cells recovered from periodontal pockets and is infrequently detected in epithelial cell samples from healthy subjects (Dibart et al., 1998).

In spite of overwhelming evidence implicating *T. forsythia* in PD pathogenesis, the virulence mechanisms of this bacterium remain unclear. This is partly due to the fastidious growth requirements for cultivation as well as the difficulty to perform genetic manipulations of this microorganism (Sharma, 2010). Animal studies have demonstrated the virulence potential of *T. forsythia*. It has been shown that pathogenic potential of *T. forsythia* is enhanced in the presence of other bacteria, such as *F. nucleatum* and *P. gingivalis,* e.g. synergistically enhancing abscess formation in rabbits and mice (Sharma, 2010). Despite its ability to interact in biofilms with pathogens that have been shown to contribute to systemic diseases, no previous studies have associated *T. forsythia* to non-oral infections.

1.2.3.4 *Fusobacterium nucleatum.*

Fusobacterium nucleatum is a gram-negative, non-spore forming, non-motile, spindle-shaped rod (Figure 1.5). The bacterium is anaerobic but grows in the

presence of up to 6% oxygen. This species is one of the most commonly occurring species in human gingival crevice (Moore and Moore, 1994). It increases in proportion as plaque forms and is highly prevalent in gingivitis in children and young adults. *F. nucleatum* is prevalent in subjects with periodontitis and periodontal abscesses and is reduced after successful periodontal treatment.

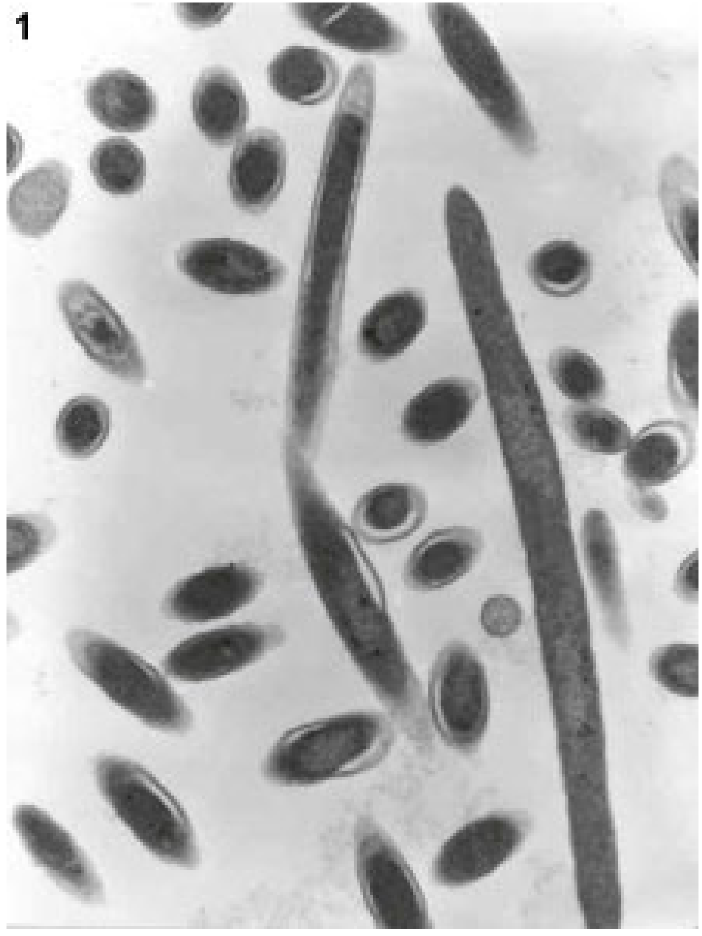


Figure 1.5 Transmission electron microscopy picture of *Fusobacterium nucleatum* showing spindle rod shape. Source: http://www.icb.usp.br/mariojac/

The pathogenic potential of *F. nucleatum* is based on its ability act as a bridge between early and late colonizers on the tooth surface and to form aggregates with other suspected pathogens in PD, increasing their pathogenicity in mixed infections (Jenkinson and Lamont, 2005). Its ability to obtain energy from fermentation of

simple carbohydrates or certain amino acids, as well as its aminopeptidase activity to metabolise small peptides when lacking fermentable carbohydrates, helps to explain its presence in both supra- and subgingival plaque and why it is often found together with organisms such as *P. gingivalis* which display powerful endopeptidase activities (Rogers, 1998). *F. nucleatum* is also capable of inhibiting the proliferation of human gingival fibroblasts, and may have the ability to penetrate the gingival epithelium (Bolstad et al., 1996).

The heterogeneity of *F. nucleatum* on the basis of physiological, biochemical and molecular variability demonstrate that distinct subgroups exist within the species (Dzink et al., 1990, Gharbia and Shah, 1992). Five subspecies of *F. nucleatum* have been proposed as *F. nucleatum* subsp. *polymorphum, F. nucleatum* subsp. *nucleatum, F. nucleatum* subsp. *vincentii, F. nucleatum* subsp. *animalis* and *F. nucleatum* subsp. *fusiforme.* It was also suggested that some subgroups may be associated with disease and others may be associated with health (Gharbia et al., 1990). In the oral cavity, it was determined that *F. nucleatum* subsp. *nucleatum* was more prevalent in diseased sites, whereas isolates from healthy sites were identified as *F. nucleatum* subsp. *polymorphum* and subsp. *fusiforme*. However, other studies have not detected difference between isolates from periodontally healthy or diseased individuals (Berres et al., 2003).

Of the microbial species associated with PD, *F. nucleatum* is the most common in clinical infections of other body sites. It has been isolated from several parts of the body and from infections including bacteraemia and liver abscesses (Crippin and Wang, 1992), intrauterine infections (Chaim and Mazor, 1992), bacterial vaginosis (BV) (Hillier et al., 1993), urinary tract infections (Ribot et al., 1981), infective endocarditis (Shammas et al., 1993), pericarditis (Truant et al., 1983), brain abscess (Kai et al., 2008), and lung infections (Bartlett, 1993).

1.3 SYSTEMIC TRANSLOCATION OF THE ORAL MICROBIOTA

The exact mechanisms certain species use to translocate into the systemic circulation are still far from clear. Translocation may be due to a combination of variables, such as mechanical procedures or certain virulence attributes linked to vascular invasion (Parahitiyawa et al., 2009).

1.3.1 Mechanical and triggering factors causing odontogenic bacteraemia.

A feature that is unique to the oral cavity is the ability of dental biofilms, if left undisturbed, to establish themselves on non-shedding tooth surfaces. These biofilms, particularly the subgingival plaque, are in close proximity to a highly vascularised milieu, the gingival tissues. In common inflammatory conditions such as gingivitis and periodontitis, oral bacteria titers are dramatically increased and the periodontal vasculature proliferates and dilates thereby providing a greater surface area that facilitates the entry of microorganisms into the bloodstream. Indeed, oral microbes residing in saliva, dental plaque, mucous membranes and gingival crevice may translocate to the systemic compartment through inoculation when performing personal oral hygiene measures, chewing, and periodontal and endodontic procedures i.e. periodontal probing, scaling, root canal instrumentation, tooth extraction, resulting in bacteraemia (Lamont et al., 2006, Parahitiyawa et al., 2009).

Parahitiyawa *et al*. (2009) proposed two possible routes of bacterial entry from the teeth into the systemic circulation (Figure 1.6): 1. entry via the route canal or from periapical lesions into the alveolar vessels; 2. entry from the periodontum, where bacteria in the gingival crevices translocate to the capillaries in the gingival connective tissues, possibly through the junctional epithelium. Commensal oral bacteria which enter into the blood or other systemic compartments may then become pathogenic due to microbial virulence factors which are able to cause disease in a non-native environment. The new environmental stressors may influence the pattern of expressed genes to ensure survival in a non-oral site (Lamont et al., 2006).

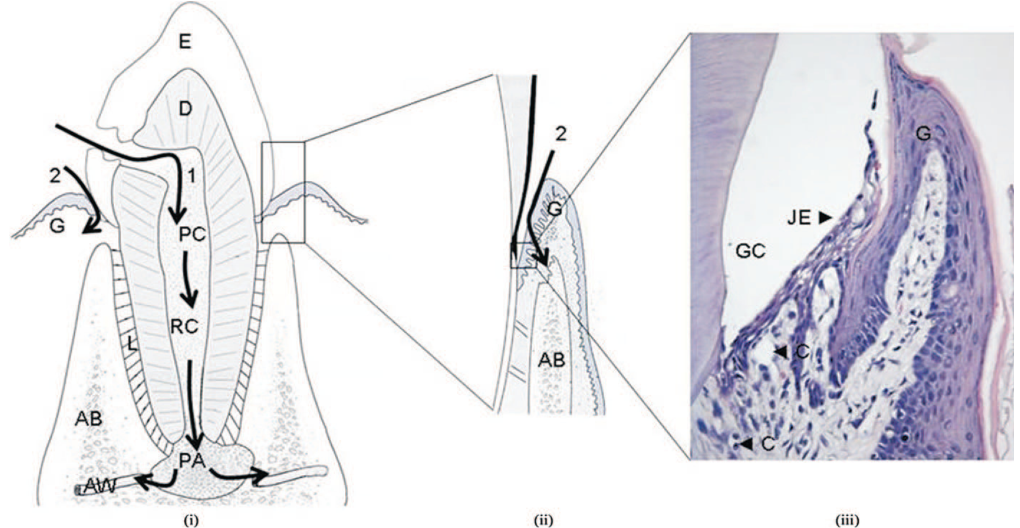


Figure 1.6 Possible routes of bacterial entry from the teeth into the systemic circulation. E, enamel; D, dentin; G, gingival; L, periodontal ligament; AB, alveolar bone; RC, root canal; PA, periapical lesion; AW, alveolar blood vessels; GC, gingival crevice; JE, junctional epithelium; C, capillaries. (i) Schematic representation of the tooth in its alveolo; (ii) Schematic representation of the gingivae and JE; (iii) Microscopical view of a hystologic specimen of the gingival tissues stained with hematoxylin and eosin. Source: Parahitiyawa *et al*. (2009).

1.3.2 Microbial virulence factors and systemic translocation.

Innate microbial factors may play an important role as only a few species are usually detected and able to persist for a prolonged time during bacteraemia, despite the diversity of bacteria residing in the periodontal biofilm. Also, it is generally accepted that odontogenic bacteraemia is transient in nature with the duration of bacteraemia after an extraction having been calculated as approximately 11 minutes in healthy children (Roberts et al., 2006) before being eliminated by the innate and adaptative defence mechanisms. However, a few bacterial species may be able to persist and survive host defences.

Species that are commonly found in the bloodstream have virulence attributes that could be linked to vascular invasion e.g. the endothelial adhesion of *Streptococcus* spp., degradation of intercellular matrices by *P. gingivalis*, and impedance of

phagocytic activity by *A. actinomycetemcomitans* and *F. nucleatum* (Parahitiyawa et al., 2009). The possible virulence properties linked to vascular invasion have been summarised previously for the most common oral species found in the bloodstream (Parahitiyawa et al., 2009) (Table 1.3).

1.4 ADVERSE PREGNANCY OUTCOMES

Adverse pregnancy outcomes denote those complications presented 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. Complications occur in 11% of pregnancies in the UK, and many of these are from unknown causes which represent a huge concern to both society and the healthcare system (Petrou, 2003).

1.4.1 Miscarriage and stillbirth.

Miscarriage or spontaneous abortion refers to the end of the pregnancy at any stage between the first day of the last menstrual period and the 24th week of pregnancy. It is the most common complication of pregnancy with around 15% of known pregnancies resulting in miscarriage. The rate in women under 35 years of age is 6%, rising to about 25% for women over 40 due to a higher risk of chromosomal abnormality. Miscarriages occurring before 13 weeks of pregnancy are referred to as ‘early miscarriages’ and those which occur after 13 weeks are ‘late miscarriages’. Miscarriages may be associated to diverse causes: genetic, hormonal, structural (cervical incompetence, abnormal shaped uterus and uterine septum), resulting from blood clotting problems or from an infection.

Stillbirth refers to the death of the fetus after the 24th week of pregnancy and before birth. The fetus may have died in the womb (intrauterine death) or during labour (intrapartum death). Stillbirths occur in 5.5 per 1000 of all births in England and Wales (Office for National Statistics, 2005). The rate of stillbirths is higher in

Table 1.3 Major bacterial species recovered from odontogenic bacteraemia and putative virulence factors thought to facilitate their entry and survival in blood.*

Organism	Factor, function, and/or characteristic	Reference
Streptococcus mutans	Ability to switch peripheral blood monocytes to dendritic cells which exhibit large numbers of adhesion molecules, such as ICAM-1, ICAM-2, and LFA-1, contributing to adhesion to the injured endothelium and to fibrinogen in blood clots	(Hahn et al., 2005)
Porphyromonas gingivalis	Cysteine proteinase (gingipain); degrades extracellular matrix proteins, such as laminin, fibronectin, and type IV collagen	(Andrian et al., 2004)
Abiotrophia defective	Strains associated with endocarditis in humans show a higher fibronectin-binding ability than that of related species	(Senn et al., 2006)
Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum	Inhibits binding of chemotactic peptides to neutrophils	(Van Dyke et al., 1982)
A. actinomycetemcomitans	EmaA (extracellular matrix protein adhesin A); forms antenna-like protrusions associated with the surface and mediates adhesion to collagen	(Ruiz et al., 2006)
Streptococcus intermedius	Hyaluronidase and chondroitin sulphate depolymerase break the ground substance in connective tissues, facilitating spread	(Shain et al., 1996)
Streptococcus anginosus	Binds to the endothelium, the basement membrane, and collagen	(Allen et al., 2002)
Streptococcus sanguis	Platelet aggregation-associated protein; enhances platelet accumulation	(Hahn et al., 2005)
Porphyromonas gingivalis	Porin-like activity of the outer membrane molecules (probably lipopolysaccharide) can depolarize neutrophils and immobilize polymorphonuclear leukocyte responses	(Novak and Cohen, 1991)
Viridans streptococci	Secreted factors could increase the production of interleukin-8, which is particularly important in lung pathology, such as acute respiratory distress syndrome	(Soto et al., 1998)

Source: Parahitiyawa *et al*. (2009). *List is not exhaustive

multiple pregnancies, women who smoke, women over 35 years of age or with a pre- existing medical condition, e.g. diabetes. Twelve percent of stillbirths are due to congenital malformations, 16% are caused by ante-partum haemorrhage (placenta previa), and 6% may be due to umbilical cord accidents or non-diagnosed ((Reddy et al., 2009)). Approximately 9% to 15% of stillbirths are caused by infections (Ahlenius et al., 1995). Infection is especially important as a cause of stillbirth occurring early in pregnancy (19% in less than 28 weeks) (Gibbs, 2002).

Infection may cause fetal death either indirectly due to a maternal infection leading to systemic illness or by direct infection of the placenta and later of the fetus (Goldenberg and Thompson, 2003). Recognized causes include syphilis, toxoplasmosis, parvovirus B-19, chorioamnionitis, and *Listeria monocytogenes*. Other organisms include *Ureaplasma urealyticum, Mycoplasma hominis, Chlamydia trachomatis*, HIV, and group B streptococci (GBS) (Gibbs, 2002). The patterns of infection vary between developed and developing countries and may originate due to a single microorganism or a variety of them. This is the case for ascending bacterial infections. Treatment of BV and sexually transmitted infections should prevent the occurrence of stillbirths or other pregnancy complications (miscarriage, PTB, preterm prelabour rupture of membranes [PPROM], and PTL), although the rates of stillbirths have not varied substantially in the last decade. In the UK, the exact cause of about 70% of stillbirth cases remains uncertain (Office for National Statistics, 2005).

1.4.2 Preterm birth.

Preterm birth is a major clinical problem associated with perinatal mortality, serious neonatal morbidity and moderate to severe childhood disability in developed countries (McCormick, 1985, Lumley, 2003). As defined by the World Health Organization (WHO), PTB is when the delivery occurs before 37 completed weeks of gestation. Based upon lower survival rates and increased risk of medical and developmental problems, PTB have been classified as mild preterm (32-36 weeks), very preterm (28-31 weeks) and extremely preterm (<28 weeks). Around 7% of all

births in the UK are preterm (Office for National Statistics, 2005). Improvements in neonatal intensive care during the last 20 years have increased the survival of the most immature newborns at 23 weeks (6-9% survival), 27–28 weeks (90% survival) 33 weeks (95% survival) (Ward and Beachy, 2003). Despite improvements in antenatal and neonatal care, the number of premature infants born each year has not significantly decreased since the 1960s.

The majority of PTB occur without any obvious cause or known risk factors. Several aetiologies and/or risk factors have been reported for PTB including prior PTB, maternal age ≤17 and >35 years, low socio-economic status, maternal low body mass index, parity, alcohol and tobacco use, drug abuse, inadequate prenatal care, medical conditions, uterine and cervical anomalies, infections, multiple gestations, and stress (Mavalankar et al., 1992, Horta et al., 1997, Petridou et al., 2001, Moutquin, 2003a, Moutquin, 2003b). Nevertheless, none of these risk factors can completely explain all PTB, thus suggesting a multifactorial origin. PTB usually occurs subsequently to a number of diverse clinical presentations, including spontaneous PTL, PTL complicated by additional factors (e.g. antepartum haemorrhage), PPROM, or an elective preterm delivery indicated on maternal or fetal grounds, e.g. due to pre-eclampsia (Lumley, 2003).

1.4.3 Preterm labour.

Labour is characterized by coordinated uterine contractions leading to cervical dilatation and finally the expulsion of the fetus (Williams et al., 2000). Spontaneous PTL precedes spontaneous PTB. Localized chronic inflammation as well as acute infections in an early stage of the pregnancy can cause production of autocrine/paracrine hormones and cytokines that then activate the myometrium causing PTL (Bryant-Greenwood and Millar, 2000).

1.4.4 Preterm prelabour rupture of membranes.

Release of hormones and cytokines can also act via an alternative pathway increasing matrix degradation of the fetal membranes and cervix leading to myometrial activation (labour) and the rupture of fetal membranes (Bryant-Greenwood and Millar, 2000). Rupture of the membranes before the onset of labour and at less than 37 weeks of gestation is defined as PPROM. Infection is usually regarded as the main cause of PPROM, although in some cases it is preceded by spontaneous PTL (a condition named preterm rupture of membranes [PROM]). Preterm rupture of membranes accounts for 25% of all PTB (7.1%–51.2%) (Moutquin, 2003a).

1.4.5 Prolonged rupture of membranes.

Prolonged rupture of membranes (pROM) is an event that occurs when the amniotic membranes, containing the fetus and the AF, ruptures prior to the start of labour and the amniotic cavity has been exposed to the external environment for more than 24 hours (Linder et al., 1995, Mandel et al., 2005). This term should be differentiated from PPROM. As described, PPROM occurs when rupture of membranes occurs before 37 weeks of gestation, resulting in the birth of a preterm infant. On the other hand, pROM is related to the time of exposure rather than the gestational age. The causes of pROM have not been clearly identified. Some risk factors include smoking, multiple pregnancies, polyhydramnios (excess AF), amniocentesis or cervical cerclage. Complications that may follow this condition include PTL and delivery, chorioamnionitis, endometritis, neonatal sepsis, and compression of the umbilical cord. When pROM occurs at term, labour usually follows or is induced to avoid complications as there is an increased risk of infection. However, earlier in the pregnancy, delay of labour is preferred to avoid higher risks of complications in a preterm neonate. Treatment and prognosis may vary and depend on the maturity of the fetus and the development of infection.

1.4.6 Low birthweight and small for gestational age.

Low birthweight (LBW) is defined by the WHO as weight at birth of less than 2.5 kg. Eight percent of births in the UK are LBW (WHO, 2004). Low birthweight is closely associated with fetal and neonatal mortality and morbidity, inhibited growth and cognitive development, and chronic diseases later in life (Doyle and Anderson, 2010). It is either the result of a short gestational period (preterm) or due to intrauterine growth restriction (IUGR). Low birthweight with a gestational age greater or equal to 37 weeks is defined as small for gestational age (SGA) (Williams et al., 2000). Birthweight is affected to a great extent by the maternal nutrition before and during pregnancy. Specific and non-specific infections also contribute to poor fetal growth (WHO, 2004).

1.4.7 Neonatal infection.

Neonatal infection is the colonisation of the neonate by an infectious agent. This is generally classified as early-onset and late-onset infection based on the initiation of bacterial colonisation (before or after delivery). Usually, the initial site of the infection is not always known since haematogenous dissemination to important organs occurs before diagnosis. Moreover, the clinical manifestations, whatever the source of infection, are frequently non-specific (tachycardia, tachypnea, abnormal temperature, leukocytosis) (Wynn et al., 2010). The multiplicity of associated risk factors and clinical manifestations makes it difficult to diagnose the infection. Despite advances in perinatal care, the prevalence of neonatal sepsis has not changed significantly over time. This represents a primary concern since, as indicated by the WHO data, 0.3% of pregnancies in England, Wales and Northern Ireland result in early neonatal death and 71.3% of these are classified as unexplained fetal death (WHO, 2008). The poor understanding of the events triggering these complications makes it difficult to prevent.

1.4.7.1 Early-onset neonatal infection.

Early-onset neonatal infection occurs during the first hours of life. It is also known as congenital or fetal infection. The colonisation initiates before birth, with bacteria originating either from the maternal genital tract or via the placenta, followed by infection of the membranes, AF, and the fetus (Martius et al., 1999). The mortality rate of neonates with congenital neonatal sepsis ranges from 25 to 90% and depends on the gestational age and the likelihood of survival (Romero et al., 2006).

1.4.7.2 Late-onset neonatal infection.

This occurs after the first 48 hours of life from microorganisms that may have been acquired from the mother and/or the immediate environment, e.g. hospital, breast/bottle feeding, mechanical ventilation. Organisms initially colonize superficial sites and the upper respiratory tract and infection may progress to cause widespread sepsis.

1.5 MICROBIAL INVASION OF THE AMNIOTIC CAVITY

The amniotic environment is normally sterile as less than 1% of women not in labour at term will present with bacteria in the AF. Detection of bacteria in the amniotic cavity is considered a pathological finding defined as microbial invasion of the amniotic cavity (MIAC) (Romero et al., 2006). Intra-amniotic infection is an important and frequent mechanism of obstetric complications. Women with MIAC are more likely to deliver preterm, have spontaneous rupture of membranes, develop clinical chorioamnionitis, and present with an adverse perinatal outcome (Romero et al., 2002). It has been associated with 25-40% of PTB (Goldenberg et al., 2000), which accounts for 75% of perinatal mortality and more than half of long-term morbidity cases (McCormick, 1985). Its prevalence varies according to the clinical manifestation and stage of the complication. The frequency of MIAC has been determined as 10-20% in women with PTL and intact membranes (Hill, 1998), 38%

in women with PTL and intact membranes who deliver preterm (Lettieri et al., 1993), 32.4% at admission in women with PPROM and 75% at the time of onset of labour (Romero et al., 2006). Also, fetal bacteraemia has been detected in 30% of cases where PPROM and positive AF cultures were presented (Carroll et al., 1996).

1.5.1 Pathways of intrauterine infection.

Possible sources of microorganisms related to MIAC have been proposed as originating from: 1). the maternal genital tract via an ascending route; 2). via the haematogenous route (transplacental infection), with an infection originating from a distant part of the body, such as the oral cavity; 3). retrograde seeding from the peritoneal cavity through the fallopian tubes; 4). accidental introduction at the time of invasive procedures, such as amniocentesis, percutaneous fetal blood sampling, chorionic villous sampling or shunting (Romero et al., 2002).

1.5.1.1 The ascending route.

Historically, the most common pathway of intrauterine infection is the ascending route. The pathogens responsible for intrauterine infection generally reflect the predominant vaginal microbiota of pregnant women. Bacterial vaginosis, characterised by a decrease in the normal levels of *Lactobacillus* spp. and an increase in other organisms such as *Gardnerella vaginalis*, *M. hominis, Prevotella* spp., *Bacteroides* spp., *Mobiluncus* spp., has been associated with numerous adverse outcomes including miscarriage, PTL, PTB, IUGR, and postpartum endometritis (Kimberlin and Andrews, 1998). In addition, *Streptococcus agalactiae* (also called GBS) from a vaginal origin is the most frequent cause of early-onset neonatal infection (Ohlsson and Shah, 2009). Some clinical trials have shown that antibiotic treatment of BV in women at high risk of preterm delivery results in a reduction of PTB. Furthermore, bacteria identified in cases of congenital infections are similar to those found in the lower genital tract (Stoll et al., 1996). However, pregnancy complications do not develop in all cases where women have been diagnosed with BV or were GBS-positive, and not every APO is associated with a genitourinary tract

infection with many cases remaining unexplained.

1.5.1.2 The transplacental route.

Secondary pathways for MIAC have also been proposed. Systemic maternal infections. e.g. pneumonia, pyelonephritis, malaria, typhoid fever have been associated with PTL and PTB. However, many of these conditions are rare in developed countries. Thus, the risk attributable to systemic maternal infection for prematurity is considered to be low (Romero et al., 2002). Nonetheless, recent evidence suggesting an association between PD or oral microorganisms with APO, has necessitated a re-examination of this possible route (Offenbacher et al., 1996, Jeffcoat et al., 2001, Offenbacher et al., 2001). Studies evaluating the simultaneous role of PD and BV as a risk factor for APO have shown that PD is significantly associated with APO, BV showed only a borderline significance, and the presence of both conditions appeared to increase the risks (Oittinen et al., 2005). These studies suggest that BV and PD are interrelated and increase the risk for APO through an additive effect. This could be explained particularly due to the microbiological similarities and common pathophysiology between the oral cavity and the female genital tract.

1.5.2 Stages of intra-amniotic infection.

The route of the infection has been found to be similar for the ascending and haematogenous route (Romero and Mazor, 1988, Han et al., 2004, Liu et al., 2007). Romero and Mazor (1988) described four stages of infection associated with the ascending route (Figure 1.7). Stage I consists of a change in the vaginal/cervical microbiota or the presence of pathologic organisms in the cervix. In stage II the microorganisms gain access to the intrauterine cavity and reside in the decidua. A localized inflammatory reaction leads to deciduitis. Microorganisms may then reside in the chorion and amnion. The infection may invade the fetal vessels (choriovasculitis) or proceed through the amnion (amnionitis) into the amniotic cavity, leading to MIAC (Stage III). Rupture of the membranes is not a prerequisite

for intra-amniotic infection, as microorganisms are capable of crossing intact membranes. Once in the amniotic cavity, the bacteria may gain access to the fetus through various ports of entry (Stage IV). Aspiration of the infected fluid by the fetus may lead to congenital pneumonia. Otitis, conjunctivitis and omphalitis may occur by direct spreading of microorganisms from infected AF. Seeding from any of these sites to the fetal circulation may result in fetal bacteraemia and sepsis.

Han *et al*. (2004) demonstrated in pregnant mice that inoculation of *F. nucleatum*, directly into the bloodstream, resulted in an infection with a similar pattern to that observed in the ascending route, i.e., bacteria first colonised the decidua, followed by spread to the fetal membranes, AF and fetus.

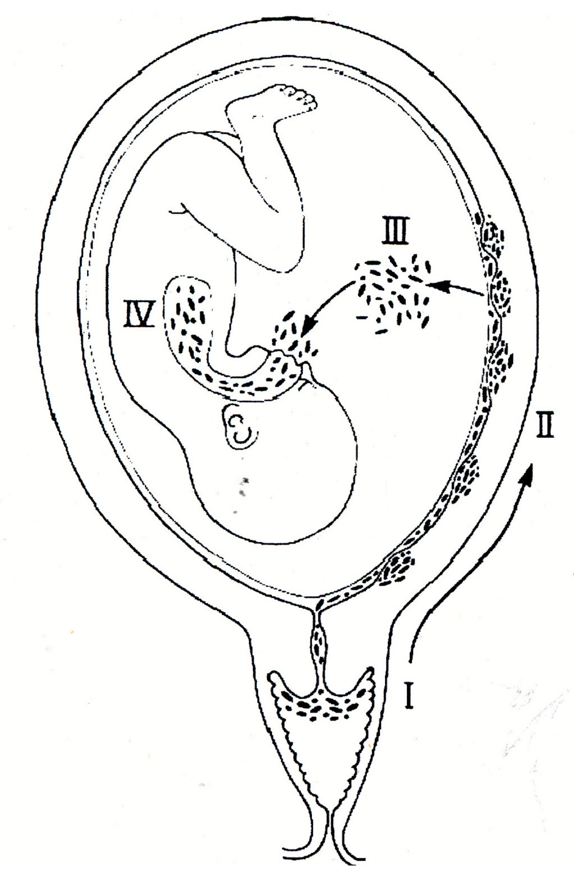


Figure 1.7 The stages of ascending infection. Source: Romero and Mazor (1988). Stage I, change in vaginal/cervical microbiota; Stage II, Invasion of the decidua, the chorion, and the amnion; Stage III, microbial invasion of the amniotic cavity; Stave IV, Invasion of the fetus.

1.5.3 Bacteria associated with microbial invasion of the amniotic cavity.

1.5.3.1 Study of body fluids and superficial samples to investigate intra-amniotic infection.

Isolation of microorganisms from body fluids such as blood and cerebrospinal, synovial, pleural, pericardial and peritoneal fluids remains the most valid method for the diagnosis of systemic bacterial infection in neonates. Surface culture obtained from skin or mucosal sites (axilla, external ear canal, umbilical stump, and aspirates from nasopharynx, endotracheal and gastric origin) are also used to evaluate suspect sepsis. Taking samples from surface body sites is based on the concept that colonization precedes infection; therefore, identification of potential pathogens in the surface sites may be predictive of those that later cause invasive disease (Evans et al., 1988). Samples obtained from surface body sites, such as NGA, can be analysed to identify those bacteria associated with fetal colonization and the cause of early-onset neonatal infections. On the other hand, body fluids analysis will identify those infectious agents that have already initiated a serious blood-borne bacterial infection and sepsis in the infants.

The gold standard for the investigation of MIAC has been through analysis of AF. Extraction of AF usually requires the need for an invasive amniocentesis procedure. Amniocentesis is performed by inserting a needle through the mother's abdominal walls into the amniotic sac and is normally used to investigate chromosomal abnormalities and, less commonly, fetal infections. Obtaining AF presents the risk of accidentally introducing bacteria into the amniotic cavity (Romero and Mazor, 1988). Also, there might be a very small risk of miscarriage or fetal death (Evans and Wapner, 2005).

Neonatal gastric aspirates are a relatively easily available sample which avoids the need for invasive procedures. This sample is routinely taken at hospitals (Health Protection Agency, 2004). In theory, this sample should contain swallowed AF to which the fetus has been exposed to *in utero*. NGA is acquired through aspiration

which involves inserting a sterile nasogastric tube into the stomach of the newborn during the first hours post-delivery and before feeding. Gastric aspirates contain bacteria from the AF and transient microorganisms derived from the birth canal. With the advent of molecular techniques, these samples have been suggested as an alternative to AF to investigate early-onset neonatal infections since positive bacterial 16S rRNA detection correlates 100% with the presence of chorioamnionitis in preterm infants (<33 weeks) and/or presentation of PROM (Miralles et al., 2005). Investigations that have evaluated the use of NGA as a tool to predict neonatal sepsis have been of mixed success (Leibovich et al., 1987, Evans et al., 1988, Puri et al., 1995). These might be due to technique limitations and the limited knowledge as regards the associated pathogen(s) and their pathogenicity.

1.5.3.2 Microbiology of the amniotic fluid.

Using the culture approach, the most common microbial isolates from the amniotic cavity in women with PTL and intact membranes have been *U. urealyticum*, *Fusobacterium* spp. and *M. hominis* (Gonçalves et al., 2002). Other microorganisms that have been found in AF include *S. agalactiae*, *Peptostreptococcus* spp., *Staphylococcus aureus*, *G. vaginalis*, and *Bacteroides* spp. Occasionally, *Lactobacillus* spp., *Escherichia coli*, *Enterococcus faecalis*, *Neisseria gonorrhoea* and *Peptostreptococcus* spp. have been encountered. *Haemophilus influenzae*, *Capnocytophaga* spp., *Stomatococcus* spp. and *Clostridium* spp. were rarely identified in AF.

Recently, with the introduction of molecular techniques, it has been shown that a higher frequency of microorganisms can be detected in the AF, particularly in women with evidence of intra-amniotic inflammation. Also, a broader range of species have been identified when investigating the microbiota associated with APO. Specific primers annealing to the 16S ribosomal RNA gene have been used for the identification of *U. urealyticum*, *M. hominis*, *G. vaginalis*, *E. coli*, *Fusobacterium* spp., *Peptostreptococcus anaerobius*, *Bacteroides fragilis*, *C. trachomatis*, *H. influenza*, *N. gonorroea*, and *Streptococcus* spp. Using a broad-range PCR technique,

an association between those cases of PTL and intact membranes, and a positive detection of bacteria was determined (Markenson et al., 1997, Oyarzún et al., 1998).

Moreover, an association between intra-amniotic infections, as determined by culture-independent methods, and clinical events including early-onset neonatal sepsis, clinical and histological chorioamnionitis, and funisitis have recently been demonstrated (Kotecha et al., 2004, Miralles et al., 2005, Han et al., 2009). Han *et al*. (2009) showed that up to 67% (14/21) of the species detected in AF from cases of PTB were uncultivable and difficult-to-cultivate bacteria, including *F. nucleatum*, *Leptotrichia* (*Sneathia*) spp., *Bergeyella* sp., *Peptostreptococcus* sp., *Bacteroides* spp., and species of the order *Clostridiales*. Miralles *et al*. (2005) reported a similar range of species and also identified *G. vaginalis* and *Streptococcus pneumoniae/milleri* in AF. DiGullio *et al*. (2010a, 2010b, 2010c) also described the microbiology of AF associated with APO. They identified *Ureaplasma* sp. (21%), *S. pneumoniae/mitis/oralis* (3%), and *F. nucleatum*, *Sneathia sanguinegens*, *S. agalactiae*, and *M. hominis* (1%) as the most prevalent bacteria in the samples, and also identified several less prevalent species including *G. vaginalis* and species of the genera *Prevotella*, *Bacteroides*, *Lactobacillus*, *Peptostreptococcus*, *Peptoniphilus*, *Staphylococcus* and *Haemophilus*.

1.5.3.3 Microbiology of neonatal gastric aspirates.

In terms of the microorganisms present, NGA should contain bacteria to which the fetus has been exposed to while in the uterus (swallowed AF), and transient microorganisms acquired during and immediately after birth. Any bacteria colonising the amniotic tissues before birth (early-onset neonatal infection) should be present in the samples. Hence, this relatively accessible sample is particularly useful to determine the potential pathogens associated with adverse perinatal outcomes.

Previous studies have characterized NGA using both traditional and molecular methods. Using the culture approach, bacteria from 229 (21%) of 1084 NGA samples were isolated with only one species per sample in 85% of samples being

identified, and more than one potential pathogen in 15% of samples (Evans et al., 1988). Microorganisms isolated by conventional culture techniques from these samples have to date been limited to a few species, including *E. coli*, *H. influenzae*, *S. aureus, L. monocygenes, S. pneumoniae*, GBS, and species of the genus *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Enterococcus*, *Pseudomonas*, and *Corynebacteria* (Evans et al., 1988, Thompson et al., 1992, Borderon et al., 1994, Hammoud et al., 2003).

Molecular studies have also been performed to describe the microbiology of NGA. *U. urealyticum,* and *M. hominis* has also been detected in these samples with species-specific primers (Egawa et al., 2007). Jones *et al*. (2010) recently demonstrated, using conventional and quantitative molecular methods, that culture analysis of NGA is only positive when bacterial DNA exceeds 4.50E+05 colony-forming units (CFU)/ml. They also showed that the bacterial load in these samples ranged from 3.00E+03 to 1.74E+08 CFU/ml (average= 3.10E+05). In their study, they identified many potential pathogens including *U. urealyticum*, *G. vaginalis*, *S. sanguinegens/ammionii*, *Lactobacillus* spp., *Veillonella* spp., *H. influenza* and *S. agalactiae*. Nevertheless, the techniques used were based upon direct sequencing and did not allow a detailed description of the microbiology of NGA.

Oue *et al*. (2009) identified 23 species in 42 gastric fluids positive for bacteria from a total of 103 specimens (41%) using nested PCR and clone analysis. The species of bacteria found in their study were as follows: *U. urealyticum/parvum, M. hominis, E. coli*, *S. agalactiae*, *S. mitis*, *Streptococcus salivarius*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus epidermidis*, *Bacteroides* spp., *Lactobacillus crispatus*, *Peptoniphilus* spp., *Sphingomonas* spp., *F. nucleatum*, *Prevotella bivia*, *Propionibacterium acnes*, and *Corynebacterium* spp. Recently, Payne *et al*. (2010) described the bacteria present in NGA using a combination of denaturing gradient gel electrophoresis (DGGE) and species-specific PCR for *M. hominis* and *Ureaplasma spp.* in preterm neonates. They demonstrated that the combined use of techniques identifies a higher diversity of species. Detection of *M. hominis* was increased from detection in one sample by DGGE to 11 (20%) using

species-specific PCR; similarly, *Ureaplasma* spp. identified in only two samples was then detected in 38% of the gastric fluids.

1.6 THE ASSOCIATION BETWEEN ORAL BACTERIA AND ADVERSE PREGNANCY OUTCOMES

1.6.1 Epidemiological and interventional studies.

Based upon the underlying biological plausibility that oral/periodontal infection may contribute to an APO by translocation of bacteria and/or their products and by the action of maternally produced inflammatory mediators; it has previously been suggested that a possible association between PD and PT/LBW exist. Offenbacher *et al*. (1996) first demonstrated in a case-control study that women with PD were 7.5 times more likely to present with a PT/LBW. Several other case-control and cohort studies have been performed since to investigate PD as a possible risk factor for APO, determining an association in some studies (Dasanayake, 1998, Jeffcoat et al., 2001, López et al., 2002a, Mokeem et al., 2004, Goepfert et al., 2004, Offenbacher et al., 2006a, Agueda et al., 2008), and a lack of association in others (Mitchell-Lewis et al., 2001, Davenport et al., 2002, Moore et al., 2004, Bassani et al., 2007, Vettore et al., 2008).

Similarly, few interventional studies have evaluated treatment of PD to reduce the incidence of PT/LBW. Some clinical trials report a significant effect of PD treatment on APO (López et al., 2002b, Sadatmansouri et al., 2006, Offenbacher et al., 2006b), while others did not find the same association (Michalowicz et al., 2006). Reasons for the inconsistencies include a genetic-environmental interaction effect (Romero et al., 2006, Pretorius et al., 2007), the population of study, variability among studies in definition of PD (Andriankaja et al., 2006, Manau et al., 2008) and APO, as well as inadequate control for confounding factors and possible effect modifiers for APO that make it difficult to draw meaningful conclusions (Wimmer and Pihlstrom, 2008). Wimmer and Pihlstrom (2008) provided an extensive critical review of the

literature for studies investigating PD as a possible risk factor for APO, recommending that adequate large prospective cohort studies followed by multicentre randomized-controlled trials are needed to clarify the potential role of PD in APO, and of periodontal therapy in preventing APO. Interestingly, in a recent randomized clinical trial, no significant difference was observed between the control and the periodontal treatment groups; however, a strong significant association was demonstrated between a successful periodontal treatment (characterised by the resolution of gingival inflammation and by the lack of progression of attachment loss or periodontal probing pocket depth) and term birth (Jeffcoat et al., 2010).

Recently, the hypothesis that destructive PD may not be strictly necessary for bacterial translocation to occur has been raised (Han et al., 2006, Han et al., 2010). It is likely that the sole presentation of gingival inflammation, as frequently observed in the common condition known as pregnancy gingivitis, in association with changes in the maternal immune system may suffice to allow opportunistic pathogens, which possess the required virulence factors, to translocate and invade the amniotic cavity.

1.6.2 Possible biological mechanisms.

1.6.2.1 Increased inflammatory mediators in periodontal disease.

There is a possible common cellular and biochemical pathway that mediates the pathogenesis of PD and the normal physiological mediators of parturition. It is established that the physiological pathway that initiates parturition at term is mediated by inflammatory events (Norman et al., 2007); therefore, distant infections such as PD may well activate the host inflammatory response and the parturition cascade. Such early activation of the normal parturition process will thus produce a pregnancy complication as a result, manifested as one of many different clinical conditions including PTL, spontaneous rupture of membrane, PTB, or early-onset neonatal infection (Romero et al., 2006).

The infected periodontum is considered to act as a reservoir for both microbial products (lipopolysaccharides, endotoxins) and inflammatory mediators such as prostaglandin E_2 (PGE_2) and tumour necrosis factor alpha (TNF-α) which have been shown to increase in PD. Prostaglandin E_2 normally rises throughout pregnancy until a critical threshold is reached to induce labour, cervical dilatation and delivery. Pregnant women with elevated AF levels of PGE_2, interleukin (IL)-6 and IL-8 and with periodontitis are at higher risk for PTB (Dörtbudak et al., 2005). Animal experiments with pregnant hamsters challenged with *P. gingivalis* showed a significant association between increasing levels of PGE_2 and TNF-α, and fetal growth retardation (Collins et al., 1994). Therefore, it is plausible that PD may represent a risk factor for APO via an indirect mechanism.

However, it remains unclear whether distant infections, such as PD, that result in elevated levels of circulatory inflammatory mediators, can influence parturition, as stated previously (Klebanoff and Searle, 2006), or if a more direct mechanism involving entry of the infectious agents into the amniotic cavity followed by a local elevation of mediators occurs regardless of the origin of the pathogen.

1.6.2.2 Bacterial translocation.

Translocation of bacteria represents another possible mechanism associated with the development of an APO. The mechanisms and virulence factors used by oral pathogens to translocate systemically have been explained previously (Section 1.2.3). Evidence of their translocation ability is described in following sections.

1.6.3 Evidence of microbial translocation.

A number of bacterial species with a possible oral origin have been found in the amniotic cavity in individuals with pregnancy complications. These microbiological findings have provided substantial evidence to suggest a possible oral-uterus translocation. *Capnocytophaga sputigena* was the first reported oral species recognized in the uterus from a preterm case with clinical chorioamnionitis and intact

membranes (Edwards et al., 1995, Douvier et al., 1999). *F. nucleatum*, a common oral species, is the most frequently detected oral species from AF cultures among women with PTL and intact membranes (Cahill et al., 2005, Hill, 1998).

Previous studies (Bearfield et al., 2002) have determined the presence of bacteria, such as *Streptococcus spp.* and *F. nucleatum*, in the amniotic cavity of healthy women attending for elective Caesarean section. A significant association was found between detection of these bacterial species and complications in previous pregnancies, including miscarriage, intrauterine death, neonatal death, preterm delivery and PROM. *Eikenella corrodens,* part of the normal flora in the human oral cavity, has been found to cause chorioamnionitis, PTB, and neonatal death (Andrés et al., 2002, Kostadinov and Pinar, 2005, Garnier et al., 2009). *P. gingivalis* has been detected in subgingival samples and the respective AF in women with a diagnosis of threatened PTL (León et al., 2007).

Recently, the first unequivocal evidence that bacteria from the oral cavity may be linked with PTB has been obtained. *Bergeyella* spp., a reported uncultivable bacterium from the oral cavity, was detected in the uterus from a patient with clinical chorioamnionitis and genetic typing matched it exactly with the oral strain (Han et al., 2006). Moreover, uncultivable and difficult-to-cultivate species have been identified in AF associated with evidence of intra-amniotic inflammation, including oral species such as *Bergeyella* spp. and *F. nucleatum* (Han et al., 2009). Evidence that oral bacteria have been associated with fetal death has recently been published in a case report in which *F. nucleatum* was the cause of a 39th week stillbirth (Han et al., 2010). These findings strongly indicate that bacteria from the mouth may be involved in APO.

1.6.4 Supporting evidence in animal experiments.

Animal models have also been developed to understand the possible mechanisms behind the findings in humans. Studies with *P. gingivalis* (Lin et al., 2003a, Lin et al., 2003b, Boggess et al., 2005), *F. nucleatum* (Han et al., 2004, Liu et al., 2007) and

Campylobacter rectus (Yeo et al., 2005, Arce et al., 2010) confirmed translocation and transplacental passage of oral bacteria to placental and fetal tissues after inoculation into the maternal bloodstream at a site distant from the uterus. Studies with *F. nucleatum* demonstrated that this bacterium is able to translocate via the haematogenous route, specifically invade the amniotic tissues, and cause PTB, fetal grown restriction, stillbirths and miscarriage.

Recently, Fardini *et al*. (2010) demonstrated, in a mouse model, that placental colonization with oral bacteria might be species-specific, since only a small proportion of the complex oral microbiota was found to be able to invade the murine placenta after bloodstream inoculation with samples of saliva and subgingival plaque. Interestingly, most of the oral bacteria able to cross the placental barriers are commensals in the oral cavity and show a relatively low virulence. However, once inside the uterus, they may stimulate the synthesis and release of proinflammatory cytokines, neutrophil infiltration and activation, and prostaglandin and metalloproteinase synthesis release, leading to cervical ripening, membrane weakening and rupture, uterine contractions and PTB. Some of these were observed to be enriched in the placenta, e.g. *Granulicatella adiacens*, *Neisseria* spp., *S. mitis*, *Veillonella* spp., *F. nucleatum*, and *Aggregatibacter segnis*. Also, a similar pattern has been reported to be associated with APO in humans (Romero et al., 1986, Barak et al., 2007, DiGiulio et al., 2008, Han et al., 2009, Han et al., 2010).

1.7 MICROBIAL IDENTIFICATION TECHNIQUES

1.7.1 Traditional microbial identification in clinical samples.

Traditional methods for microbial identification require the isolation of pure cultures followed by a diverse and complex range of phenotypical and biochemical tests (Amann et al., 1995). Culture techniques are considered the gold standard in microbiology and remain to be an important diagnosis method to detect bacterial species in hospitals. However, these techniques present important limitations not

only because of the large number of bacteria not able to be cultured, but also due to the difficulty to detect those fastidious strains that require very specific environmental or nutritional requirements to grow. One major limitation of the phenotypical and biochemical tests is related to unreliability due to characteristics that do not fit into the patterns of any known genus and species (Woo et al., 2008). Even cultivable species may be difficult to recover when they are present in low numbers. Also, anaerobic pathogens could become non-viable after sampling, during transport and analysis, thereby underestimating their presence in the samples. All these limitations together with the need for other requirements, such as experienced personnel, time and relatively high cost, have led to the need for non-cultural diagnostic methods, particularly nucleic acid-based detection methods.

1.7.2 Molecular identification techniques to study complex microbial populations.

Recently, improved methods based upon PCR and comparative DNA sequencing have made it possible to identify even extremely fastidious and uncultivable pathogenic bacteria in mixed species communities without the need for culture (Amann et al., 1995, Patel, 2001). Studies comparing traditional versus molecular techniques to define the bacterial content within a particular specimen have demonstrated the advantage of the molecular approach in detecting a broader range of species (Kroes et al., 1999). Molecular methods based on the analysis of the conserved small subunit 16S ribosomal RNA (rRNA) molecule have become very important in characterising complex microbial populations. The 16S rRNA gene has been proposed as the primarily tool for the identification and classification of bacteria (Rajendhran and Gunasekaran, 2010).

To adequately study microbes in complex microbial communities, genomic DNA is extracted directly from the mixed sample without the need for cultivation. Gene libraries derived from amplification products of a defined fragment can be then sequenced. Bacterial taxa are identified based on the 16S rRNA sequence homology with existing sequences in the databases. A cloning and sequencing approach is

dependent upon the number of clones that can be processed and sequenced. This may be a very expensive and labour-intensive technique which limits the number of samples that can be evaluated. In addition to cloning and sequencing, DNA fingerprinting techniques based on the 16S rRNA gene have been proposed as this may be a more affordable option. Nevertheless, to date cloning and sequencing have proven to be accurate and reliable tools and are still broadly used techniques for exploring microbial diversity from complex communities.

Currently developing technologies such as next-generation, high-throughput DNA sequencing offers a great leap forward in the ability to explore complex microbial ecosystems such as the oral microbiota by sequencing simultaneously thousands of isolates without amplification and cloning biases (Pozhitkov et al., 2011). The first paradigm shift in next-generation sequencing, to overcome the limitations of dideoxy chain termination sequencing, has been to create molecular clones *in vitro* by physically separating single template molecules and to replicate these in a physically isolated clonal population. The second shift has been to devise new sequencing chemistries enabling template sequence to be read as a series of discrete events in real time. These technologies are already being applied to the study of microbial community complexity (Nossa et al., 2010, Lazarevic et al., 2009). However, with such revolutionary advances in technology come new challenges in methodologies and informatics. Next-generation sequencing can produce terabytes of data that needs to be stored and analyzed. The biggest challenge will be this overwhelming production of data, requiring not only storage but also interpretation. In the future, next-generation sequencing will allow not only to catalogue the numbers and types of bacteria present but also to understand correlations with the function and fitness of a defined ecosystem. This will ultimately contribute to a more comprehensive understanding of human health, disease susceptibilities, and the pathophysiology of infectious and immune-mediated diseases (Snyder et al., 2009).

1.7.2.1 PCR and the16S ribosomal RNA gene.

Polymerase chain reaction is a primer-mediated enzymatic amplification of specific target sequence from genomic DNA (Saiki et al., 1985a). With this technique, a specific section of DNA can be copied multibillion-fold, allowing detailed molecular studies to be performed on as little as a single original cell. Briefly, PCR method was developed in 1985 (Saiki et al.). It relies on a thermal cycling consisting of cycles of repeated heating and cooling of the reaction producing DNA melting and enzymatic replication of the DNA. A high temperature (94 - 98 ^{o}C) is necessary first to physically separate the two strands in a DNA double helix; as PCR progresses, the heat-stable DNA polymerase (Taq) enzymatically ensembles at a lower temperature a new complementary chain of nucleotides by using a single-stranded DNA as a template. The fragment of DNA to be amplified is identified by a pair of specific oligonucleotides (primers) that designate the outer limits of the amplicon (Figure 1.8).

The 16S rRNA gene (Figure 1.9) has been selected as a candidate molecule for bacterial identification for several important reasons: 1. it is present in all bacteria and the RNA performs an important structural role as well as a crucial part in protein synthesis (as part of the ribosome); 2. it contains both highly conserved nucleotide sequences and hypervariable regions, thereby allowing detection of a broad range of bacteria as well as specific bacterial species; 3. the 16S rRNA gene is of sufficient size (ca. 1500 bases) to be easily sequenced and still contains sufficient information for identification and phylogenetic analysis (Clarridge, 2004). Generally, the comparison of 16S rRNA gene sequences allows differentiation between microorganisms at the genus level across all major phyla of bacteria. In addition, it often allows classification of bacteria to the species level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relates to several species that have the same or very similar sequences (Clarridge, 2004). The nucleotide sequence obtained by 16S rRNA-PCR can be compared to online databases which provide an increasing number of aligned 16S rRNA sequences and allows identification based upon similarity with the nearest neighbour.

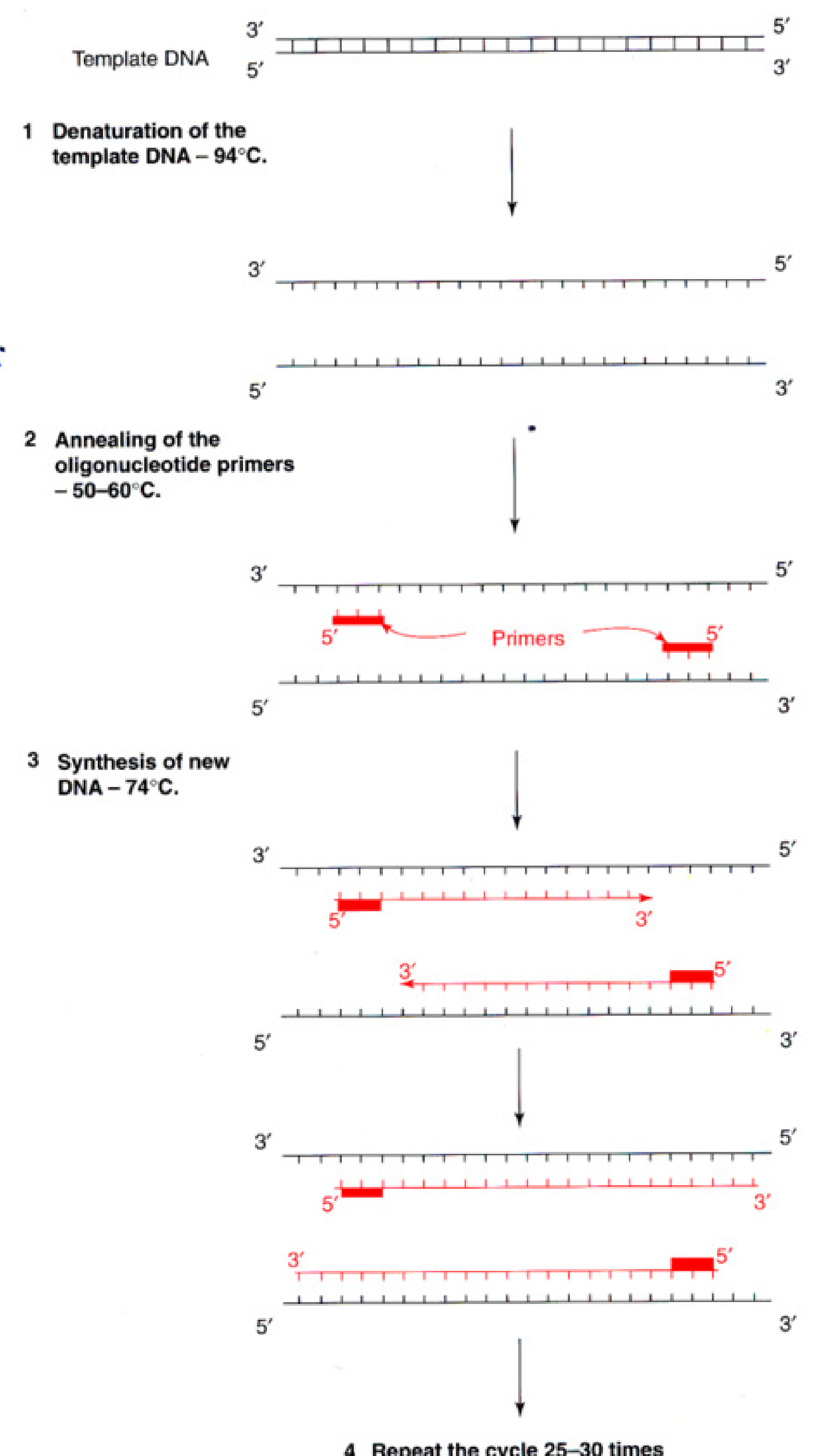


Figure 1.8 Schematic representation of a PCR amplification sequence. Source: Gene cloning and DNA analysis: an introduction (Brown et al., 2006).

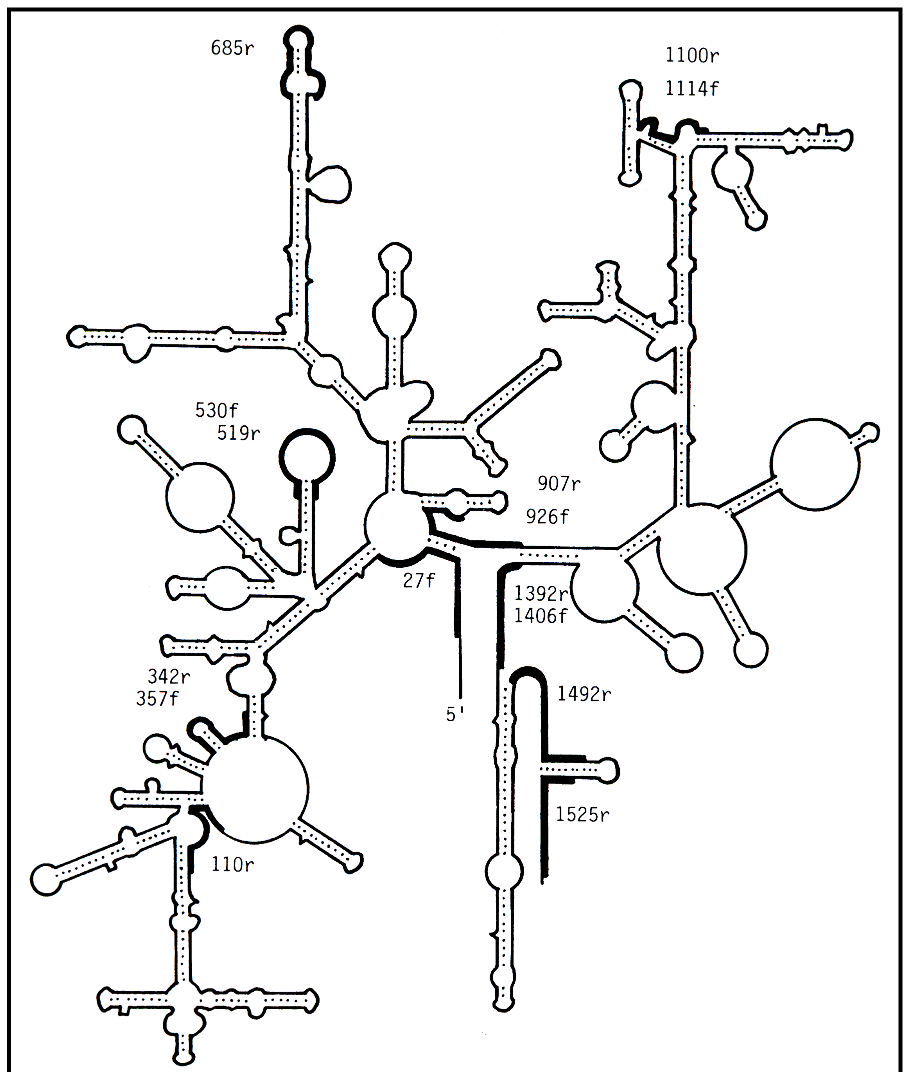


Figure 1.9 The 16S rRNA structure indicating (in black) the areas with the highest conserved regions which are the most common targets for the analysis of all (universal) bacteria. Source: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt and Goodfellow, 1991).

A 97% similarity level has been proposed for bacterial identification using 16S rDNA sequences (Patel, 2001). However, other studies has suggested that a >0.5% difference may be indicative of a new species (Drancourt et al., 2000). It is not possible to determine a universal cut-off for bacterial genus and species delineation as different bacterial species are likely to evolve at different rates and the cut-offs for

species and genus identification should vary depending on the bacterial genus under investigation. Janda and Abbott (2007) suggested that a minimum of >99%, and ideally >99.5%, sequence similarity be used as the criteria for species identification. They also proposed that for matches with distance scores <0.5% to the next closest species, other properties, e.g. phenotype, should be considered in final species identification. However, using these criteria, it is difficult to determine the species identity with sequences that have very similar distances to the closest and next closest matches, especially those within 0.5–1%.

1.7.2.2 Clone analysis and sequencing.

Direct sequencing is not accurate when applied to mixed samples. Therefore, procedures to isolate individual species from complex communities are necessary. Microbial diversity assessment is generally based on:

1. PCR amplification of 16S rRNA genes from genomic DNA.
2. Construction of 16S rRNA gene clone libraries.
3. Sequencing of randomly selected clones.
4. Comparative sequence analysis.

In gene cloning, a fragment of DNA is inserted into a circular DNA molecule called a vector to produce a recombinant DNA molecule (Figure 1.10). The vector transports the gene into a host cell, usually *E. coli*. Within the host cell the vector multiplies producing numerous identical copies of its genetic information including the inserted gene that it carries. When the host cell divides, the DNA recombinant is passed to the progeny. After a large number of cell divisions, each cell of a colony (or clone) will contain many copies of the gene of interest. Randomly selected colonies are picked and amplified by PCR for identification by sequencing (Brown et al., 2006).

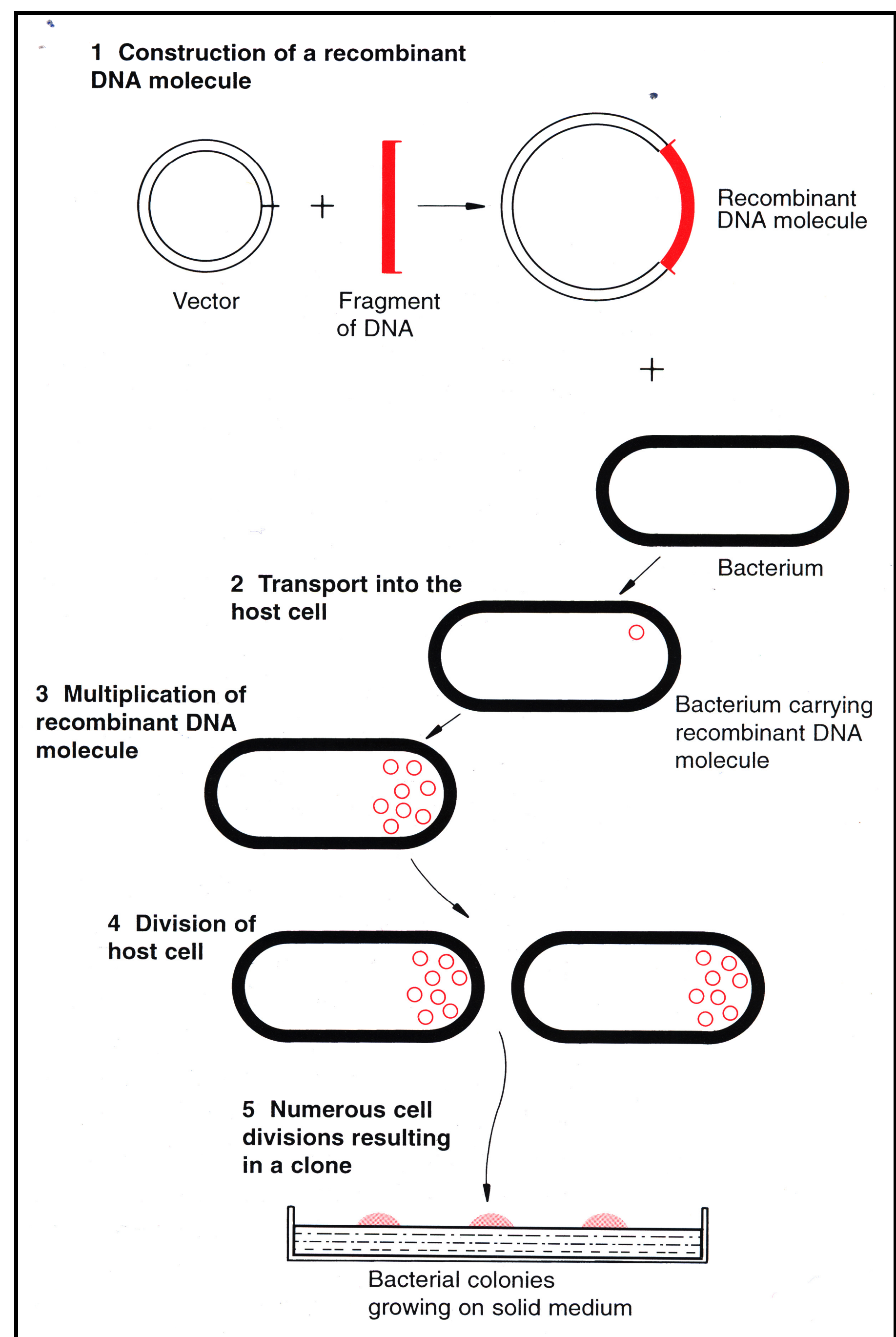


Figure 1.10 Schematic representation of clone analysis. Source: Gene cloning and DNA analysis: an introduction (Brown et al., 2006).

1.7.2.3 DNA fingerprinting techniques.

In addition to sequence analysis, DNA fingerprinting analysis based on 16S rRNA genes such as DGGE or temperature gradient gel electrophoresis, single-stranded conformation polymorphism, amplified ribosomal DNA restriction analysis and terminal restriction fragment polymorphism analysis are widely used to differentiate microorganisms (Bouchet et al., 2008).

Denaturing gradient gel electrophoresis has rapidly become a valuable tool in the study of complex microbial populations. It consists of the electrophoretic separation in a polyacrylamide gel of PCR-amplified 16S rDNA fragments of the same length based upon their sequence difference (Muyzer et al., 1993). The polyacrylamide gel contains a linear gradient of DNA denaturants (urea and formamide), and electrophoresis is run at a constant temperature and voltage. When the DNA domain with the lowest melting temperature (M_T) reaches its M_T, a transition from helicoidal to a partially melted molecule causes it to cease migrating. Different sequences will stop migrating at different positions in the gel based upon their respective G+C content and M_T (Figure 1.11). To increase specificity, the addition of a 30- to 50-nucleotide GC-clamp to the 5'-end of the DNA fragment is added during DGGE-PCR to act as a high melting domain, preventing the two DNA strands from complete separation; the GC-clamp will be co-amplified and thus introduced into the amplified DNA fragment.

The bands can then be observed with UV light after staining with ethidium bromide or an alternative dye (Muyzer and Smalla, 1998). The number of bands corresponds to the number of predominant members in the microbial community. However, bands at the same position in the gel do not necessarily belong to the same sequence. They merely have the same melting behaviour. Individual bands can be excised, eluted, re-amplified, and sequenced to determine identity. Several samples run in the same gel allow a visual comparison between profiles. Limitations of this technique may be related to the separation of only small fragments of the gene, therefore limiting the sequence information for phylogenetic inferences. Also, co-migration of the

fragments, particularly, those samples with very low variability, may represent a problem. Li *et al.* (2007) determined that cloning of each band produced an average number of species detected of 2.7. This would indicate that the richness of the samples is probably much higher than suspected. Gafan and Spratt (2005) proposed the use of a more narrow gradient of denaturants to provide higher resolution in order to resolve the limitations of co-migration.

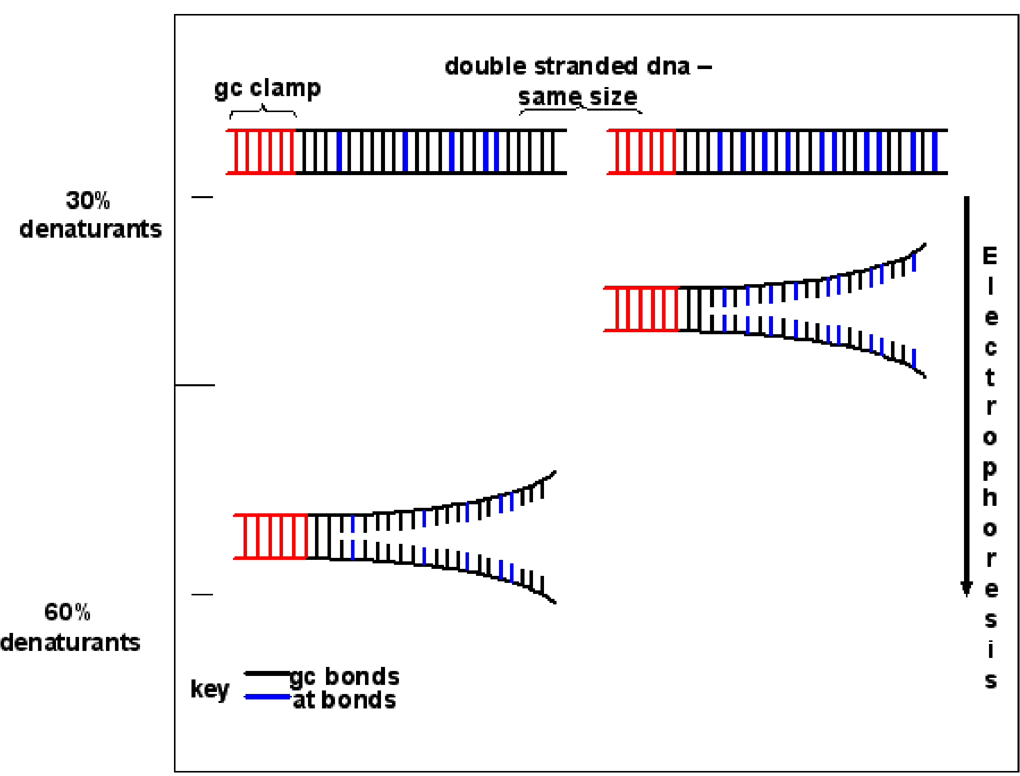


Figure 1.11 Schematic representation of DGGE demonstrating the difference in electrophoretic mobility of two DNA fragments of equal size as a result of differences in their base-pair sequence.

1.7.2.4 Quantitative PCR.

Quantitative PCR (q-PCR) or real-time q-PCR is being used in a growing number of research applications, including pathogen detection. It was introduced to overcome the main limitation of the conventional (end-point) PCR in which the data is

collected after the amplification reaction has been completed, giving only a final qualitative result (Wang et al., 1989). Therefore, end-point PCR cannot calculate or differentiate between variable initial levels of template in the samples being analysed. With q-PCR, the number of DNA molecules of a particular sequence in a complex sample can be determined with high accuracy and sensitivity. A fluorescent reporter molecule is used to monitor the progress of the amplification reaction. This molecule could be either: 1). a sequence-specific probe comprised of an oligonucleotide labelled with a fluorescent dye plus a quencher; or, 2). a non-specific DNA binding dye (such as SYBR Green I) that fluoresces when bound to double-stranded DNA. In each amplification cycle, the intensity of the fluorescence increases proportional to the increase in amplicon concentration. As PCR amplification is an exponential process, the extent of amplification (N) is given by the equation $N = N_0(1 + E)^n$, where N_0 is the initial amount of material, E is the efficiency, and n is the cycle number. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample (Kubista et al., 2006).

Figure 1.12 represents the phases of amplification occurring during a q-PCR reaction and the response curves generated when plotting the cycle number against the fluorescence produced during each cycle. During the initial cycles the signal is weak and cannot be distinguished from the background. As the amount of product accumulates a signal develops and increases exponentially. Thereafter the signal levels saturate due to the reaction running out of some critical component e.g. primers, deoxyribonucleotide triphosphate (dNTP), polymerase in which case the exponential amplification switches to linear amplification. An end-point PCR experiment provides information on the resulting product at the plateau phase; hence, it only distinguishes a positive from a negative sample. In q-PCR, the response curves in the exponential growth phase of the reaction are separated based upon the initial amounts of template molecules. The number of cycles at which the fluorescence of a sample rises above the background fluorescence is called the crossing point (Cp) of the sample. This Cp value can be directly correlated to the

starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the earlier the Cp value for that sample.

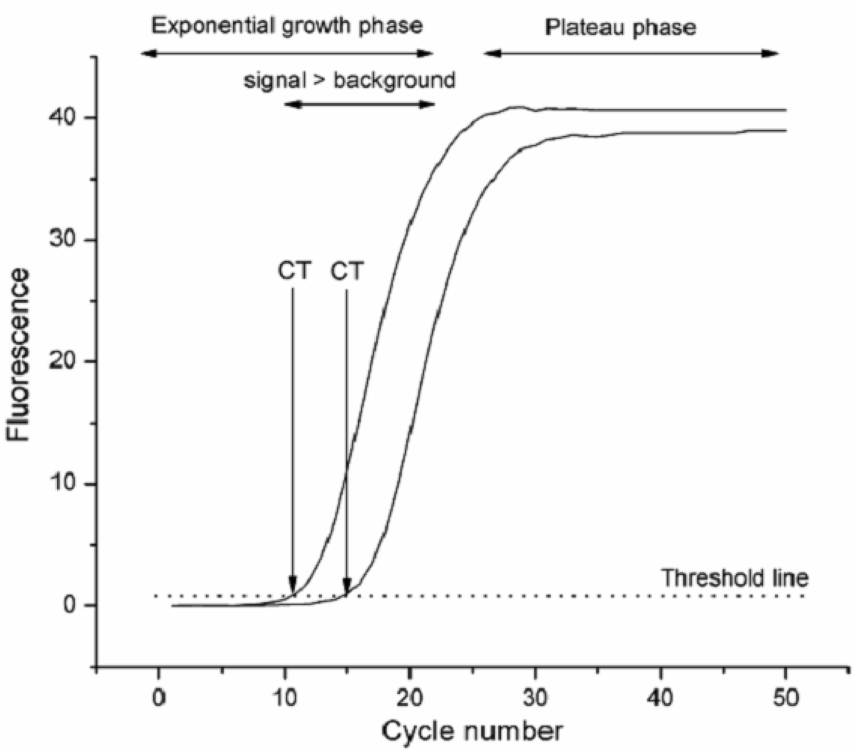


Figure 1.12 Real-time PCR response curves. A threshold level is set sufficiently above background and the number of cycles required to reach threshold, CT are registered. CT=crossing point (Cp). Source: Kubista *et al*. (2006)

There are two methods of PCR quantification: absolute and relative quantification. Absolute quantification allows measurement of the exact level of template in the samples. It uses a standard curve prepared from a dilution series of template of known concentration (Figure 1.13A). The standard curve is generated by plotting the logarithm of the initial copy number against the Cp generated for each dilution (Figure 1.13B). The plot of these points should be a linear regression line. Comparing the Cp values of the unknown samples to the ones produced by the standards allows the quantification of initial copy numbers. For this, a standard of known concentration should be included in every experiment.

A linear standard curve also implies that the efficiency of amplification is consistent at varying template concentrations. The slope of the standard curve describes the kinetics of the PCR amplification, indicating how quickly the amount of the target molecule can be expected to increase with the amplification cycles. The efficiency can be calculated from the slope using the formula $E=10^{-1/slope}$. Ideally, the slope should be near -3.3 (slope=-3.3→ E=2 or double the amount of target with each cycle).

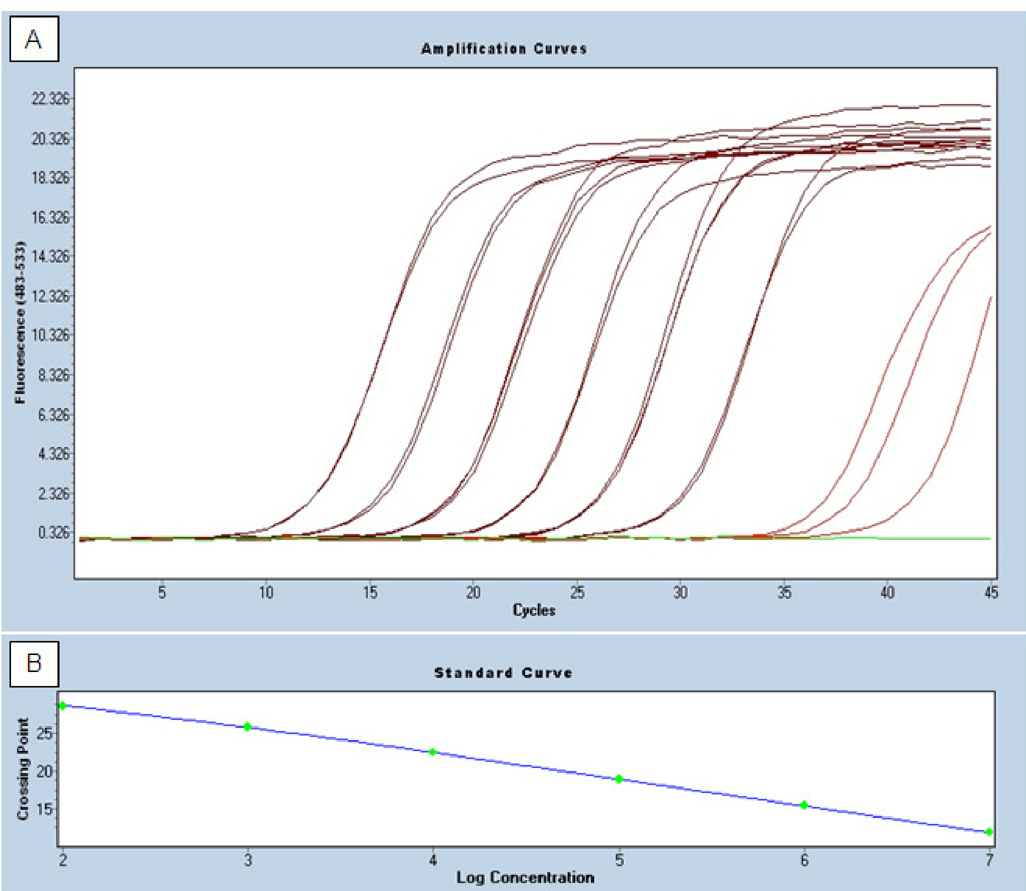


Figure 1.13 Standard curves for q-PCR. A. Amplification curves of the standard dilution series plotting fluorescense vs. number of cycles. B. Logarithmic linear representation of standard curve plotting initial copy vs. Cp.

The relative quantification is performed by measuring the relative concentration of the gene of interest in unknown samples compared to a calibrator, or control sample. Using this approach, differences in Cp value between an unknown sample and

calibrator are expressed as fold-changes (i.e., up- or down-regulated) relative to the calibrator sample. In addition to comparing the expression of the target gene in a control versus experimental sample, a normalizing reference gene or target, typically a gene whose expression is constant in both the control (calibrator) and experimental samples, can be added.

1.7.3 The 16S-23S rDNA Intergenic Spacer Region.

Typically, each ribosomal operon consists of the three genes encoding the structural rRNA molecules, 16S, 23S, and 5S. Among bacterial species, the average lengths of these genes are 1 522 bp, 2 971 bp, and 120 bp, respectively (Figure 1.14). The three rRNA genes are highly conserved within a bacterial species due to their fundamental role in protein synthesis. On the other hand, the 16S-23S rDNA intergenic spacer region (ISR) of the ribosomal operon is under minimal selective pressure during evolution and therefore contains more sequence variation than that of the 16S and 23S rRNA coding regions. It has been found that bacteria contain multiple copies (between one and 15) of the ribosomal operon in each genome (Lee et al., 2009). Moreover, the size of the ISR varies considerably for different species, and even among different operons within a single cell. It has been determined that the length of the 16S-23S rDNA ISR in bacteria varies between 164 bases in *Bacillus subtilis* (Green et al., 1985) and 1529 bases in *Bartonella elizabethae* (Roux and Raoult, 1995). The variation in length of an amplified ISR is mainly due to the presence of various numbers and types of transfer RNA (tRNA) genes (Morgan et al., 1977). It has been suggested that the length and sequence polymorphisms in the ISR, which are detectable by ribotyping techniques, can be used to distinguish different bacterial species (Bouchet et al., 2008). However, while in some species the ISR length is variable within and between isolates, in others the ISR lengths are conserved and do not allow discrimination (Christensen et al., 1999). Thus, the most accurate techniques rely on sequencing. Sequencing of the amplified ISR has been suggested previously for species identification and subtyping (Whiley et al., 1995).

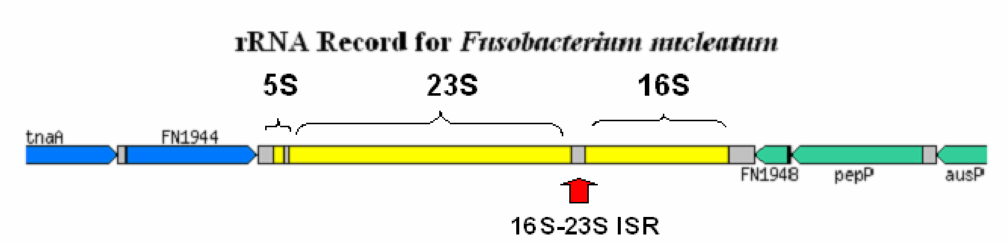


Figure 1.14 Ribosomal RNA map of *F. nucleatum* ATCC 25586- Operon 1. Source: Oral Pathogen Sequence Database, http://www.oralgen.lanl.gov

1.8 AIMS OF THE STUDY

TO INVESTIGATE THE MATERNAL ORAL CAVITY AS A POSSIBLE SOURCE OF PATHOGENS ASSOCIATED WITH COMPLICATED PREGNANCIES

- To determine the microbiology of neonatal gastric aspirates obtained from complicated pregnancies using a combination of molecular techniques (clone analysis and DGGE) based upon the 16S rRNA gene.

- To identify bacteria with a possible oral origin in samples of gastric aspirates.

- To specifically detect and quantify periodontal pathogens from a possible oral origin in samples of gastric aspirates using a quantitative PCR approach.

- To determine if any demographical or clinical variable might be independently associated with the presence of bacteria (including potential oral bacteria) in neonatal samples using a multivariable analysis approach.

- To compare, at the subspecies and strain level, suspected oral bacteria observed in the neonatal samples with their respective counterparts in maternal oral and vaginal samples using the 16S rRNA gene and the 16S-23S intergenic region to evaluate them as the possible origin of these bacteria.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design.

The following project is a laboratory-based analysis of clinical samples. Figure 2.1 represents a simplified scheme of the protocol followed during the study. Ethical approval was obtained for this project from the Outer North East London Research Ethics Committee (Possible Association between the Oral Microflora and Adverse Pregnancy and/or Neonatal Outcomes, REC Ref. No. 08/H0701/61) (ANNEX 1), and was later amended to cover all the stages of the project.

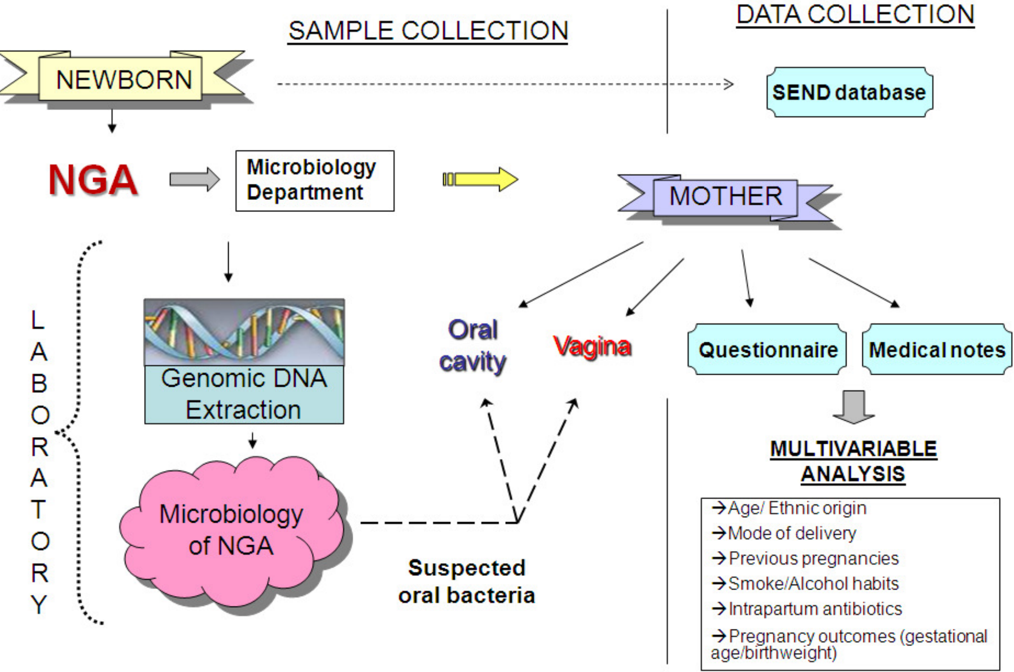


Figure 2.1 Schematic representation of the study design followed during this PhD project. NGA, neonatal gastric aspirates; SEND, Standarised Electronic Neonatal Database.

The samples of NGA were identified and collected for this study at the Microbiology Department, Royal London Hospital, Barts and The London NHS Trust. These samples are routinely collected as part of the normal clinical care of the newborns for investigation of infection and were not collected specifically for this study.

Demographical and clinical data was obtained from the Standarised Electronic Neonatal Database (SEND) and from the maternity notes. Data included were maternal age, ethnic origin, gravidity (number of times a woman has been pregnant), parity (number of times a woman has given birth to a fetus at $\geq$ 24 weeks of gestation), mode of delivery, onset of labour, date and time of birth, complications during this and previous pregnancies (i.e. miscarriages, PTB, LBW, pROM), gestational age, birthweight, administration of intrapartum antibiotics (iATB), and smoking during pregnancy. Other information was also obtained from the Microbiology Department database such as date, time and place of collection of the sample, as well as results regarding gram staining and culture.

Maternal samples from the oral cavity and the vagina were also collected for this study. Those mothers whose neonates had to provide NGA sample in the hospital were invited to participate in the study. Samples from neonatal and maternal origin obtained during the recruitment period were then analysed for the purposes of this study using the molecular techniques as explained in this and following chapters.

2.2 Samples of neonatal gastric aspirates.

Gastric aspirates are routinely taken at the Maternity and Neonatal Units, Royal London Hospital, Whitechapel under the 'Standard operating procedure for the investigation of gastric aspirates and infection screen swabs from neonates' Reference no. BSOP 23i4.1 (Health Protection Agency, 2004). These fluids are obtained from newborns that present with a clinical manifestation of neonatal sepsis (respiratory distress, unstable temperature and cardiovascular depression) or are at risk of developing an infection (congenital abnormalities, LBW, PTB, pROM, maternal fever, protracted and difficult births, respiratory distress syndrome, toxaemia of pregnancy, invasive procedure and devices, mother with previous infant affected by GBS sepsis). The samples are acquired through aspiration by inserting a tube in the newborn stomach at less than 4 hours post delivery and before feeding. These specimens are not normally taken from neonates that are not considered to be at risk of infection; therefore, healthy infants do not provide samples of NGA.

The samples of NGA were collected for this study on the following day, after being taken at the hospital and analysed at the Microbiology Department. The remaining volumes of those samples that contained at least 200 µl of non-diluted sample were obtained. Following collection, the samples were immediately aliquoted in 200 µl fractions. Two of the samples were immediately subjected to DNA extraction. The remainder of the sample was stored at -80°C.

2.3 Sample size.

A total of 240 samples of NGA were collected during this project. The minimum number of NGA samples to be included in this study was calculated from preliminary data with the analysis of 50 samples of NGA. As observed, the transmission rate for the most common pathogens was likely to be at least 52%. With 180 cases, it was expected to generate confidence intervals of about ±7%. Therefore, there was greater than 80% power to observe a difference between the presence and absence of bacteria for differences of 50 and 16%.

2.4 Participants approach.

Women attending the Royal London Hospital, who gave birth to a child with a suspected infection and were required to provide a NGA sample as part of the hospital's routine care, were invited to participate in the study. Initial ethical approval was amended to identify and approach these women either at the hospital or by post (ANNEX 2). The participants were requested to provide a sample of their oral cavity and a lower vaginal swab (LVS), and, they were also asked to sign a consent form (ANNEX 3) and complete a validated questionnaire (Davenport et al., 2002) (ANNEX 4). The samples were obtained either at the labour ward, at the Neonatal Intensive Care Unit (NICU), or at The Dental Hospital, Barts and The London NHS Trust. The questionnaire provided included questions related to smoking and drinking habits, illness and medication during pregnancy, dental attendance, diet, antenatal care, education, paternal information, and sexual relations and oro-genital contact practice during pregnancy.

2.5 Samples from the maternal oral cavity and vagina.

The oral samples consisted of a pool of saliva, supra- and subgingival plaque, and tongue surface scraping to ensure a representative sample of the diverse oral microbiota. A full-mouth periodontal examination (ANNEX 5) at six sites per tooth was performed in order to determine the periodontal status of the mothers and to identify those sites with deep pockets from which the subgingival samples were obtained. After completing the periodontal chart, a sample of saliva was obtained by asking the women to hold, and pass around the mouth, a sterile oral swab (Salimetrics Europe Ltd, Suffolk, UK) for 1-2 minutes. The Salimetric Oral Swab was then placed into a 7 ml bijou for transportation. A supragingival plaque sample was obtained from the gingival margins of 3-4 teeth using a sterile toothpick. This was then placed into a 0.5 ml tube containing 200 µl of phosphate buffered saline (PBS). After that, subgingival plaque was obtained from those sites with observed pockets (>4 mm) (if present) and/or from healthy sulci at the molar level. A validated paper point technique was used (Loomer, 2004, Jervøe-Storm et al., 2007) to collect subgingival plaque. Briefly, a paper point ISO#40 (SybronEndo, Kerr International, CA, USA) was inserted deeply into the pocket and held for 10 seconds. This was performed with five to 10 absorbent points and pooled into one 0.5 ml tube containing 200 µl of PBS. Finally, a tongue scraping sample was obtained with a nylon bristle brush (Cytosoft ™ Cytology Brush, CardinalHealth, Dublin) by making a few strokes on the tongue dorsum and placing it into a 1.5 ml tube containing 500 µl of PBS.

Lower vaginal swabs were asked to be self-collected by the mothers. This avoided requirement for medical staff or obstetricians to collect the samples. Previous studies support the use of self-collected low vaginal samples; it has been demonstrated that a substantial agreement exists between the vaginal microbiota when comparing physician-obtained speculum examination and self-obtained vaginal swabs (Strauss et al., 2005). Self-collected swabs have been shown to be well accepted by most women and the quality of the swabs to be satisfactory (Bresson et al., 2006). A card with instructions on how to obtain self-collected vaginal swabs was provided to the

women (ANNEX 6); this was modified from Rose *et al.* (2007). Once collected, the swabs were immediately placed in a 1.5 ml tube containing 600 µl of PBS.

All the samples were finally transported to the laboratory. The saliva sample was immediately extracted from the oral swabs by placing it in a Swab storage tube (Salimetrics Europe Ltd, Suffolk, UK); this was followed by centrifugation at 13,000 rpm for 1 minute as per manufacturer's instructions. A pooled sample of the oral cavity was composed of the following: 300 µl of saliva, 150 µl of supragingival plaque, 150 µl of subgingival plaque, and 200 µl of the tongue scraping placed in the same tube. This was then divided into 200 µl aliquots. The sample of vagina was aliquoted in a 300 µl volume per tube. The samples were then stored at +4°C and, soon after, subjected to DNA extraction.

2.6 Growth of bacterial strains.

Reference strains, including recognised pathogens associated with PD, were grown in this study with two main purposes: for construction of standards for q-PCR (Table 2.1), and for the analysis of the ISR of fusobacterial species (Table 2.2). All the bacterial strains were anaerobic or facultative anaerobic bacteria. The 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 solid media supplemented with 5% defibrinated horse blood (TCS Biosciences Ltd, Buckingham, UK) at a temperature of 37ºC. Unless specified, the bacteria were grown on BA (Oxoid, Basingstoke, UK) media (Table 2.1). Bacteria obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) were supplied lyophilized and were reconstituted according to the manufacturer's instructions.

Table 2.1 Oral strains of bacteria grown in this study for constructions of standards for q-PCR analyses.

	BACTERIUM	STRAIN TYPE	GROWTH CONDITIONS	SOURCE	REFERENCE
1	*F. nucleatum*	NCTC 10562	Grown anaerobically on BA for 3-5 days.	EDI	MI
3	*P. gingivalis*	DSM 20709	Grown anaerobically on BA for 3-5 days.	EDI	MI
4	*T. forsythia*	ATCC 43037	Grown anaerobically on FAA** for 2 weeks.	CID	(Lee et al., 2006)
5	*Granulicatella elegans*	DSM 11693	Grown anaerobically on BA* for 24 hours.	DSMZ	(Roggenkamp et al., 1998)

BA, blood agar; FAA, Fastidious Anaerobic Agar; * media supplemented with 0.01% L-cysteine hydrochloride + 5 µg/ml haemin (Sigma, Poole, UK)+ 1µg/ml menadione (Sigma); ** media supplemented with 0.001% (w/v) filter-sterilized N-acetyl muramic acid (Sigma), 5 µg/ml haemin, and 1µg/ml menadione. MI, manufacturer's instructions.

Table 2.2 Subspecies of *F. nucleatum* grown in this study for analysis of the ISR.

	BACTERIA*	**STRAIN TYPE**	**SOURCE**
1	*F. nucleatum* subsp. *polymorphum*	NCTC 10562	EDI
2	*F. nucleatum* subsp. *nucleatum*	ATCC 25586	CID
3	*F. nucleatum* subsp. *vincentii*	DSM 19507	DSMZ
4	*F. nucleatum* subsp. *fusiforme*	DSM 19508	DSMZ
5	*F. nucleatum* subsp. *animalis*	DSM 19679	DSMZ

*All the strains were grown anaerobically on BA for 3 to 5 days.
EDI, Eastman Dental Institute; CDI, Centre for Infectious Disease, Barts and The London, QMUL; DSMZ, German Collection of Microorganisms and Cell Cultures.

2.7 Counts of bacteria and storage of stocks.

Each strain was grown on multiple agar plates as specified above. Freshly collected colonies were transferred into sterile tubes containing 1.5 ml Brain Heart Infusion (BHI) broth + 10% sterile glycerol. The mixture was vortexed and the number of bacteria in the neat sample was quantified by a standard plate count method and spectrometric (turbidimetric) analysis. Briefly, the samples were serially diluted in BHI broth. Dilutions from 10^{-5} to 10^{-9} were plated in duplicate using two different amounts (10 and 100 µl) and those plates containing between 30 to 300 CFU were used to determine the number of bacteria in a given dilution (Figures 2.2 and 2.3).

The concentration of bacteria in the neat suspension was calculated as CFU/ml using the following equation:

Number of colonies (CFU) / dilution x amount plated = Number of bacteria/ml

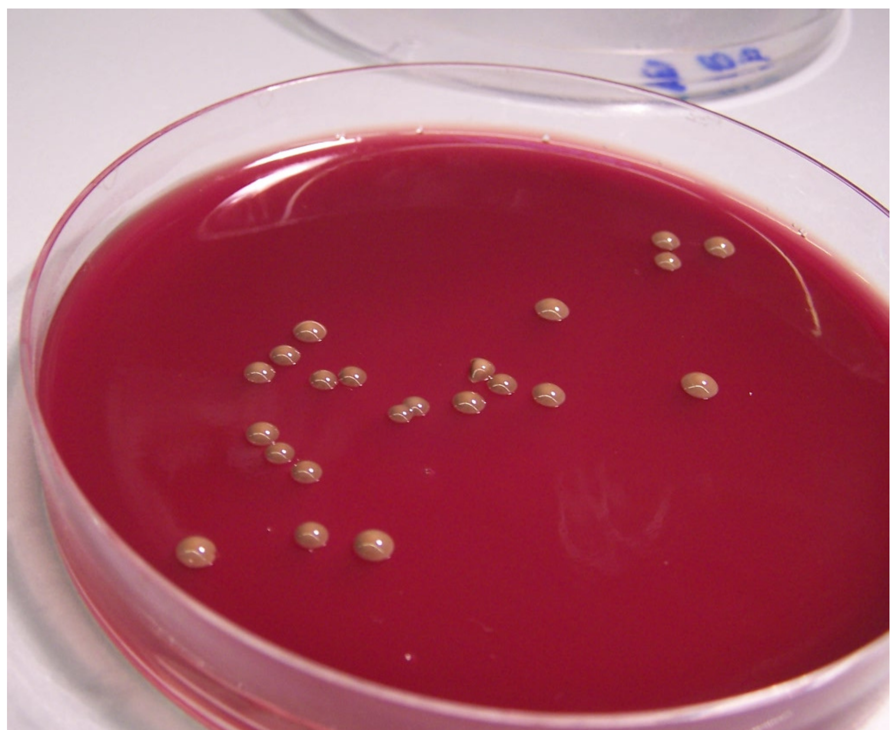


Figure 2.2 Photograph of *P. gingivalis* pure culture grown in BA medium after 8 days incubation in an anaerobic cabinet (dilution 10^{-7} ,10 µl).

The turbidity of the samples was calculated measuring the optical density (OD) at a wavelength of 600 nm (OD600). The OD600 of the neat sample, as well as the OD600 for the dilutions 1:2, 1:4, 1:8, 1:16 and 1:32, were determined for spectrometric analysis. The number of bacteria in the neat and dilutions were plotted against absorbance using Microsoft Excel® software. The trend line and slope equation was used to determine the OD600 for the closest 10^N dilution. Figure 2.4 represents an example of the technique.

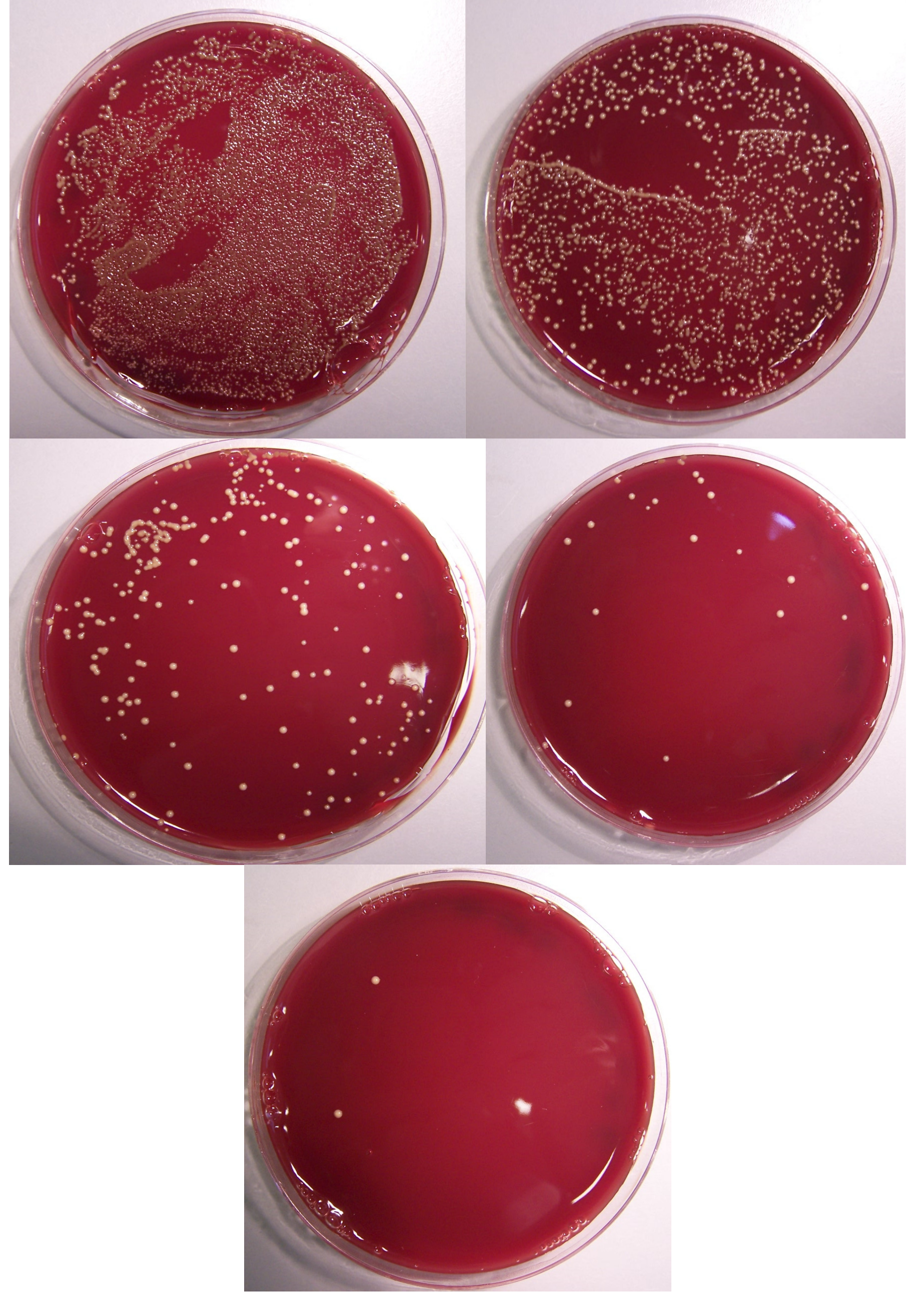
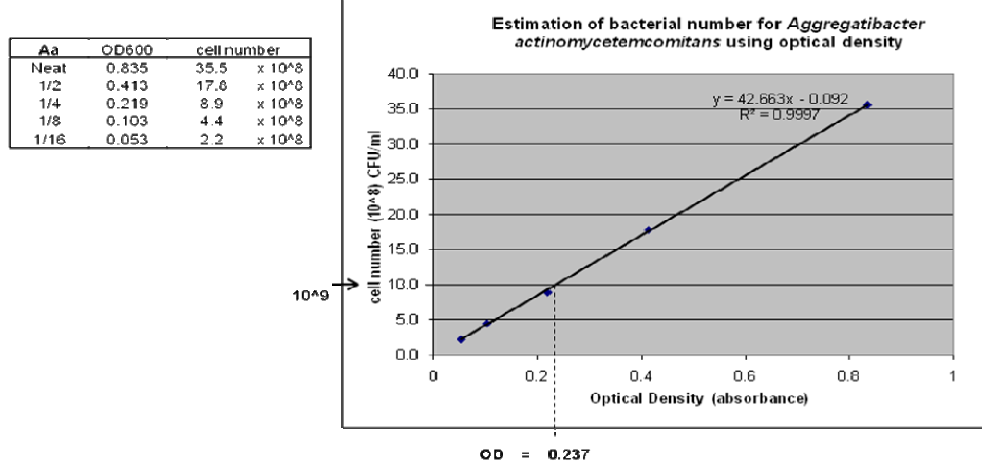


Figure 2.3 Serial dilutions of an unknown concentration of *P. gingivalis* (100 µl) on BA plates after 5 days incubation in an anaerobic cabinet. Dilutions are shown from the highest concentration (10^{-5}) in the top left plate, to the lowest concentration (10^{-9}) in the bottom plate.



Aa	OD600	cell number	
Neat	0.835	35.5	x 10^8
1/2	0.413	17.8	x 10^8
1/4	0.219	8.9	x 10^8
1/8	0.103	4.4	x 10^8
1/16	0.053	2.2	x 10^8

Figure 2.4 Estimation of bacterial number as determined by the corresponding OD600 (example represents data for *A. actinomycetemcomitans*).

2.8 Genomic DNA extractions.

Genomic DNA from the clinical samples and reference strains was extracted by using the ArchivePure DNA Purification Kit for Yeast and Gram -/+ (Flowgen Bioscience, Nottingham, UK). Due to the high viscosity, samples of NGA received pre-treatment with Dithiothreitol (Mucolyse, Pro-Lab Diagnostics, Cheshire, UK), a sputum liquefying agent, before DNA isolation. An aliquot of 200 µl of the sample was diluted with 200 µl of PBS solution and 10 µl of 5% mucolyse was added. The mixture was vortexed for 30 seconds and incubated at 37°C for 1 hour or until easily pipetted. No other sample (oral cavity, vaginal swab, or laboratory-cultured bacteria) required pre-treatment before DNA extraction. The DNA extraction protocol used in this study was optimised and is detailed below.

The samples were pelleted by centrifugation at 13 000 x g in a bench top microcentrifuge for 20 minutes. The pellet was resuspended in 300 µl of Cell Suspension Solution and 1.5 µl of lytic enzyme was added. The samples tubes were inverted 25 times and incubated for 30 minutes at 37°C with occasional inverting. After incubation the cells were pelleted by centrifugation for 10 minutes and the

supernatant discarded. This was followed by resuspension of the pellets in 550 µl of Cell Lysis Solution and incubation at 80°C for 5 minutes in order to complete cell lysis. For a maximum yield of DNA, 1.5 µl of Proteinase K solution (20mg/ml, Flowgen Bioscience, Nottingham, UK) was added, mixed by inverting 25 times and incubated at 55°C overnight. After incubation, the cell lysate was treated with 3 µl of RNase solution, inverted 25 times and further incubation at 37°C for 15 minutes. Afterwards, the samples were cooled to room temperature (RT) and 100 µl of Protein Precipitation Solution was added to the lysate. Thorough mixing was then ensured by vortexing at high speed for 20 seconds. Following this, the protein was pelleted by centrifugation for 10 minutes and the supernatant was poured into a tube containing 300 µl of 100% isopropanol. The addition of 0.5 µl Glycogen Solution (20mg/ml, Bioline London, UK) for expected low DNA yields was included. This was mixed by inverting 50 times, centrifuged for 20 minutes and the supernatant discarded. The DNA was then washed by adding 300 µl of 70% ethanol and inverting several times. The samples were then centrifuged for 10 minutes and the supernatant discarded. The DNA pellet was air dried for 10 minutes and then re-suspended in 20 µl of DNA Hydration Solution and left at RT overnight. Finally, the samples were stored at 4°C until the amplification step was performed or kept at -20°C for long term storage.

2.9 16S rRNA gene PCR.

In general, bacterial 16S rRNA genes were amplified from genomic DNA following a protocol previously described by Gafan et al. (2004). Briefly, a final reaction volume of 50 µl was prepared containing 1 X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of each primer, 0.15 U of BioTaq DNA polymerase (Bioline, London, UK), and either 5 µl of the extracted genomic DNA from clinical samples or 2 µl of the extracted DNA from the reference strain. The PCR conditions used were: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, and elongation at 72°C for 1.5 minutes, and a final 5-minute extension step at 72°C.

Primers used were either universal for conserved sequences of the gene (Table 2.3) or species-specific to amplify certain genera or species (Table 2.4). A modification of the commonly used 27F forward primer was used to analyse samples of NGA. This formulation was suggested by Frank *et al*. (2008) for the analysis of human vaginal samples to avoid problems with inefficient primer binding commonly observed for certain important species. The primer 27F-YM+3, which contained four parts of 27F-YM (Table 2.3), plus one part of each primer specific for *Bifidobacteriaceae* 27F-Bif 5'-AGGGTTCGATTCTGGCTCAG-'3, *Borrelia* 27F-Bor 5'-AGAGTTTGATCCTGGCTTAG-'3, and *Chlamydiales* 27F-Chl 5'-AGAATTTGATCTTGGTTCAG-'3, together with the reverse primer 1492R (Table 2.3) amplified most of the 16S rRNA gene, approximately 1500 bp length.

The presence of the DNA fragment of interest was confirmed by electrophoresing 4 µl of the PCR product mixed with 1 µl of loading buffer (New England Biolabs, Hitchin, UK) in an 1 – 2 % agarose gel using a size marker (HyperLadder I, Bioline, London, UK) to confirm the correct fragment size (Sambrook et al., 1989). DNA was stained with ethidium bromide (10 mg/ml) and visualized under UV light to confirm amplification. Few modifications due to variations in fragment size or primers used were performed accordingly. Techniques that imply a great variation from this protocol, such as touchdown-PCR and q-PCR, are explained in later sections.

2.10 Cloning of bacterial 16S rRNA genes.

Amplicons generated by end-point PCR and Taq DNA polymerase were cloned into *E. coli* by using the TOPO TA Cloning® kit (Invitrogen, Paisley, UK). Fresh PCR product (0.5 to 4 µl, depending on DNA concentration) was mixed gently with 1 µl salt solution, 1 µl of the TOPO vector plasmid and sterile water (final volume 6 µl). Incubation of the mixture at RT (22-23ºC) for 30 minutes allowed for ligation of PCR product to the plasmid. A 2 µl volume of the reaction mixtures was then added to individual vials of One Shot® chemically competent TOP10 *E. coli* (provided with the kit), mixed gently and then incubated on ice for 30 minutes. After incubation the cells were heat-shocked for 30 seconds in a 42ºC water bath.

Table 2.3 Universal primers used in this study for amplification and sequencing of fragments within the 16S rRNA gene.

PRIMER NAME	Primer direction	PRIMER SEQUENCE	position in *E. coli*	REFERENCE
27F-YM[a]	forward	5'-AGAGTTTGATYMTGGCTCAG-3'	8-27	
1492R	reverse	5'-TACGGYTACCTTGTTACGACTT-3'	1506-1492	(Lane, 1991, Weisburg et al., 1991)
357F[b]	forward	5'-CTCCTACGGGAGGCAGCAG-3'	341-357	
357R	reverse	5'-CTGCTGCCTCCCGTAGGAG-3'	357-341	
518R	reverse	5'-ATTACCGCGCTGGTGG-3'	534-518	(Muyzer et al., 1993)
C11R	reverse	5'-ACGTCATCCCCACCTTCCTC-3'	1246-1227	(Loy et al., 2008)

[a] 27F-YM+3 (Frank et al., 2008) was used for analysis of NGA (p.88) [b] 357F-GC was used for DGGE analysis (p. 90). Modifications are explained in the text.

Table 2.4 Species-specific primers used in this study for amplification of a fragment within the 16S rRNA gene.

TARGET BACTERIUM	PRIMERS	PRIMER SEQUENCE	position in *E. coli*	REFERENCE
F. nucleatum	Fn1F	5'-GACAGAGCTTTGCGTCC-3'	212-228	(Nagano et al., 2007)
	Fn2R	5'-TGGGCGCTGAGGTTCGAC-3'	821-804	
A. actinomycetemcomitans	AaF*	5'-ATTGGGGTTTAGCCCTGGTG-3'	889 - 911	(Tran and Rudney, 1999)
P. gingivalis	Pg2F	5'-GCGTATGCAACTTGCCTTAC-3'	89-108	(Wahlfors et al., 1995)
	Pg2R	5'-GTTTCAACGGCAGGCTGAAC-3'	612-593	
T. forsythia	Tf2F*	5'-GCGTATGTAACCTGCCCGCA-3'	120-140	(Sakamoto et al., 2001)
G. elegans	GeF*	5'-AGAAGGAAAAGAGGCTTCGG-3'	196 - 215	(Roggenkamp et al., 1998)

* The following primers were complemented with a universal reverse primer for specific bacterial amplification (see Table 2.5).

This was followed by the addition of 250 µl S.O.C. medium (Invitrogen) at RT. The cells were then shaken horizontally at 37ºC for 1 hour and then 50 and 100 µl volumes were aliquoted onto pre-warmed Luria-Bertani agar supplemented with 50 µg/ml kanamycin and finally incubated at 37ºC overnight. Colonies were then selected based upon the presence of an insert with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The presence of the insert was confirmed by PCR amplification of the white colonies using the same protocols as performed in the first amplification reaction.

2.11 Denaturing gradient gel electrophoresis.

2.11.1 DGGE profiling - Touchdown PCR.

DGGE analysis was performed to identify the profile of bacteria present in samples of NGA. The V2-V3 region of the 16S rRNA gene was amplified using the PCR primers 357F-GC, which consisted of the 357F primer and a 40 bp GC clamp starting from the 5' end, and the 518R (Table 2.3). The amplification reaction mixture contained 1 X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of each primer, 2.5 U of BioTaq DNA polymerase (Bioline, London, UK), and 5 µl of the genomic DNA. The cycling parameters for a touchdown PCR were as follows: after pre-incubation at 94°C for 5 minutes, 34 cycles were performed at 94°C for 1 minute, annealing temperature (T_A) for 1 minute and 72°C for 1 minute. The T_A decreased step-wise by 1°C every 2 cycles from 65°C in the first cycle to 56°C in the 20th cycle. The T_A for the last 14 cycles was 55°C. Cycling was followed by 5 minutes incubation at 72°C. The expected product length (~235 bp) was confirmed by electrophoresis in a 2.6% agarose gel stained with ethidium bromide and run together with a size marker (HyperLadder V, Bioline, London, UK).

2.11.2 Casting parallel gels.

Parallel gels containing 10% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) were cast using a DCode system (BioRad laboratories Inc., Hertfordshire, UK) (Figure 2.5). The gels contained a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 60% at the bottom (with 100% denaturants corresponding to 7 M urea and 40% [v/v] deionised formamide). PCR products (15 µl) and loading buffer (6 µl) were loaded onto the gels and run at 58 V for 17 hours at a constant temperature of 60°C in 7 litres of 1 X TAE buffer (40 mM Tris base-acetic acid buffer and 1mM ethylenediaminetetraacetic acid pH 8.0) (Figure 2.6). Gels were stained for 20 minutes in 1 X TAE containing 0.0001% SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes, Paisley, UK) and photographed under UV light.

2.11.3 DGGE band excision.

Band excision was carried out using a sterile scalpel on a Syngene GVM20 transilluminator (Synoptics Ltd, Cambridge, UK). DNA fragments were then eluted into 20 µl of molecular grade water at 4ºC for 24 hours (Ampe et al., 1999). One microliter of the eluted DNA from each DGGE band was reamplified by using the same conditions described for the DGGE-PCR. To evaluate the success of this procedure and to verify whether single DGGE bands had been excised, 3 µl of the PCR reaction was electrophoresed through an identical DGGE gel.

2.12 Purification of PCR products.

All PCR products were purified before sequencing using the QIAquick PCR Purification kit (QIAGEN, Crawley, UK) as indicated by the manufacturer.

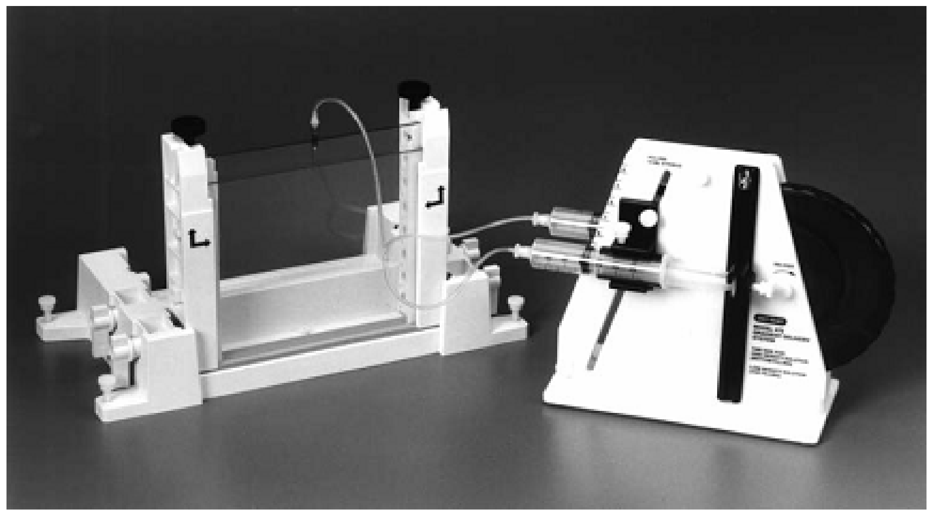


Figure 2.5 Casting stand and gradient delivery system for casting a parallel gradient gel for DGGE analysis. Source: Instruction Manual, DCode Universal Mutation Detection System, Bio-Rad Laboratories.

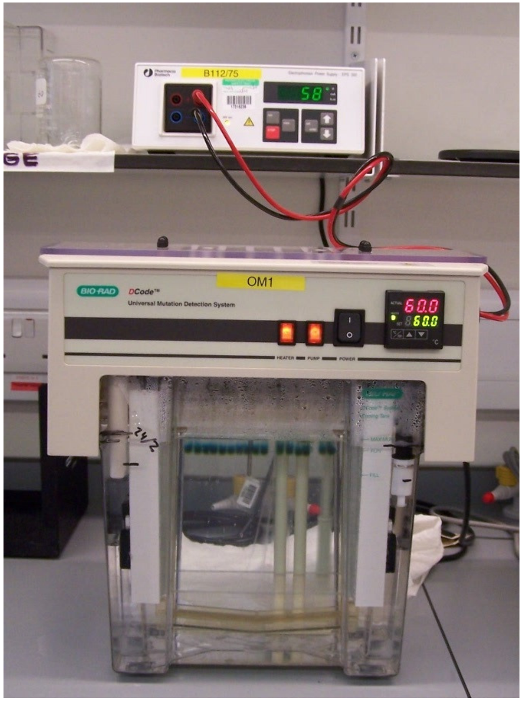


Figure 2.6 DGGE-PCR products running in a gel containing a linear gradient of denaturants increasing from 30% at the top to 60% at the bottom. Electrophoresis was performed at 58 V at a constant temperature of 60°C in TAE buffer for 17 hours.

2.13 Quantitative PCR analysis.

Quantitative PCR analyses were performed using the SYBR Green I method. Multiwell-96-plates were used, each well containing 4 µl of the 2 x LightCycler® FastStart DNA Mastermix (Roche Diagnostic, West Sussex, UK), 5 µM of each primer, 2 µl of the template and de-ionised H_2O to a final reaction volume of 10 µl. All multiwell plates were sealed, centrifuged at 3000 g for 5 s, and then amplified in a LightCycler LC480 instrument, with activation of polymerase (95°C for 5 min), followed by 45 cycles of 10 s at 95°C, 6 s at the T_A determined for each set of primers, and a predetermine extension time at 72°C.

The set of primers used for the q-PCR analyses and the respective T_A and extension times are detailed in Table 2.5. In general, the T_A was calculated as 3 to 5°C below the M_T of the primer with the lowest M_T (Sambrook et al., 1989). Extension time was based upon the expected fragment length and was calculated as 1 second for every 25 bp. The temperature transition rate was 4.4°C/s for all steps. Double-stranded PCR product was measured at 76°C for 1 s by detection of fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analysed and the absolute amounts were determined by the LightCycler® software v. 1.5. Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 30 s at 95°C, 30 s at 65°C and acquisition at 99°C. Temperature change rates were 4.4°C/s, except in the final step, which was 0.1°C/s. The melt peak generated represented the specific amplified product. Positive controls, comprising by a known dilution of the strain, were included in triplicate in all tests. Negative controls were also included, containing de-ionised H_2O instead of the DNA template and no H_2O/template. All samples were initially processed in a single reaction and, when possible, confirmed in triplicate when a positive result was found.

Table 2.5 Primers used in this study for bacteria quantification and conditions for each set of primers.

BACTERIAL TARGET	PRIMER SET*	Fragment length	Annealing Temperature	Extension Time
General bacteria	357F- 518R	200 bp	58°C	10 sec
F. nucleatum	Fn1F- Fn2R	609 bp	57°C	25 sec
A. actinomycetemcomitans	AaF- C11R	360 bp	61°C	15 sec
P. gingivalis	Pg2F- Pg2R	518 bp	59°C	21 sec
T. forsythia	Tf2F- 357R	240 bp	62°C	11 sec
G. elegans	GeF- 357R	160 bp	59°C	7 sec

* Primer sequences are detailed in Tables 2.3 and 2.4.

2.14 Primer set specificity test.

Each set of primers used to amplify a specific genus or species were tested in this study for specificity using end-point PCR. The specificity of the primers were determined through the application of the following: (i) using probeCheck tool, Department of Microbial Ecology, University of Vienna (Loy et al., 2008); (ii) against genomic DNA extracted from a pure culture of the specific species; (iii) against several oral bacterial strains (Table 2.6); (iv) against 10 common strains determined as prevalent in the samples of NGA in this study (see Section 3.5.1 in Chapter 3, Construction of a standard for DGGE analysis). De-ionised H_2O was included as a negative control in each experiment. Finally, the primers were also tested by sequencing the products as obtained from clinical samples in order to confirm its identity.

2.14.1 Oral bacterial strains to test primer specificity.

The bacteria used in this study to test primer specificity consisted of a list of common oral species (Table 2.6). Genomic DNA was extracted from each strain as previously detailed. The quality and concentration of the extracted DNA was then measured using a Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, East Sussex, UK), and stocks at approximately 50 ng/μl were prepared from each strain.

Table 2.6 Oral strains of bacteria used for primer specificity test.

	BACTERIUM	STRAIN TYPE	SOURCE
1	*A. actinomycetemcomitans*	HK998	EDI
2	*Actinomyces naeslundii*	DSM 17233	EDI
3	*Fusobacterium nucleatum*	NCTC 10562	EDI
5	*Lactobacillus casei*	ATCC 334	EDI
6	*Neisseria subflava*	DSM 17610	EDI
7	*Prevotella intermedia*	DSM 20706	EDI
8	*Porphyromonas gingivalis*	W50	EDI
9	*Pseudomonas aeuriginosa*	PAO1	EDI
10	*Streptococcus anginosus*	NCTC 10713	EDI
11	*Streptococcus gordonii*	NCTC 7865	EDI
12	*Streptococcus mitis*	NCTC 122	EDI
13	*Streptococcus mutans*	ATCC 700610	EDI
14	*Streptococcus salivarius*	NCTC 8618	EDI
15	*Streptococcus oralis*	NCTC 11427	EDI
16	*Streptococcus sanguinis*	NCTC 02863	EDI
17	*Staphylococcus aureus*	8325-4	EDI
18	*Tanerella forsythia*	ATCC 43037	CID
19	*Veillonella dispar*	NCTC11831	EDI

EDI, Eastman Dental Institute, UCL; CID, Centre for Infectious Disease, Barts and the London SMD, QMUL.

2.15 Specific amplification of the 16S-23S rDNA intergenic spacer region.

A forward primer within the 16S rRNA gene specific for the genus *Fusobacterium* (Fn1F, Table 2.3), and a universal primer within the 23S rRNA gene, either 456R or the 129R (Table 2.7), were used to amplify the complete ISR from any component of

the genus. PCR reaction conditions used to amplify this considerably longer fragment of DNA were a modification of the method previously described. A final reaction volume of 50 μl contained 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM of dNTPs, 0.2 μM of each primer, 0.5 U of BIO-X-ACT™ Long DNA Polymerase (Bioline, London UK), and either 2 μl of the extracted DNA from the reference strain, or 5 μl of the extracted genomic DNA obtained from the clinical samples. The PCR cycle was identical to the one previously described (Section 2.9) except for the extension time that was increased to 3.5 minutes.

2.16 Extended agarose gel analysis.

An extended agarose gel analysis was used to confirm amplification of the ISR, and in order to discriminate even small variations of the fragment sizes in the gel. The amplified products of ca. 2000 bp were resolved on a 0.8% agarose gel in TAE electrophoresis buffer at 65V using a size marker HyperLadder I (Bioline, London, UK). An extended electrophoresis time of 4 hours was determined in this study as the most efficient time to resolve the bands.

2.17 Sequencing.

All sequencing was carried out at the Genome Centre, Barts and The London using BigDye 3.1 chemistry with visualization on an ABI 3700 automated DNA sequencer. The primers used in the sequencing reactions varied according to the technique. The 357F primer (Table 2.3) was used for sequencing the fragments obtained from neonatal samples. The species-specific forward primers described in Table 2.4 were used to amplify fragments of specific bacterial species. A list of the primers used for sequencing of the ISR is detailed in Table 2.7.

Usually, sequencing produced an 800 bp sequence length (whereas DGGE analysis produced 150bp fragments). The quality of the sequences was checked using the Chromas software package (http://www.technelysium.com.au/chromas.html). Sequences of the ISR products amplified using both a forward and a reverse primer

were aligned and corrected using Multalign, a multiple sequence alignment tool (Corpet, 1988) and Chromas.

Table 2.7 Primers used in this study for the amplification and sequencing of fragments of the 16S-23S rDNA ISR.

PRIMER NAME*	PRIMER SEQUENCE	position in *E. coli*	REFERENCE
1114F	5'-GCAACGAGCGCAACCC-3'	1114-1130	(Stackebrandt and Goodfellow, 1991)
129R	5'-GGTTBCCCCATTCRG-3'	129-114	(Hunt et al., 2006)
456R	5'-CCTTTCCCTCACGGTACT-3'	456-438	(Hunt et al., 2006)

* Primer FnF1 was also used for the amplification of the ISR. Sequence details in Table 2.4.

2.18 Sequence comparative analysis.

Bacterial sequences were identified by comparison with libraries of sequences based upon ≥97% similarity with the closest relative (Patel, 2001). The on-line libraries used comprised thousands deposit bacterial 16S rRNA gene sequences from the Ribosomal Database Project (RDP) (Cole et al., 2007), the Human Oral Microbiome Database (HOMD) (Chen et al., 2010), and the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast).

2.19 Phylogenetic analysis.

Phylogenetic analysis was used to determined evolutionary relationships between strains of *F. nucleatum*. Multiple alignments of the sequence data were performed using the AlignX tool of the VectorNTI software (Advance 11.5 version; Invitrogen). Phylogenetic trees corresponding to the alignments were created using the ClustalW algorithm (pairwise alignments). Evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987) based upon the principle of

minimum evolution, which defines the topology of a tree by successively finding and joining pairs of neighbours. The method provides also the branch length determined from a matrix of distances between all pairs of sequence. These distances are related to the degree of divergence between the sequences which determined the alignments of the tree.

2.20 Statistical analyses and data presentation.

Data was summarized in tables organised by counts, mean, or median, depending on the type of data. In general, counts or prevalence were used to describe qualitative data. Mean was used for quantitative data that was normally distributed and median was used for quantitative data that did not present this characteristic.

All the graphs were performed using the IBM®SPSS® Statistical 19 software (IBM UK Ltd, Middlesex, UK). Clustered bar charts were used for qualitative data, usually when a significant statistical difference between two variables was observed. To illustrate quantitative data, i.e. the absolute and relative levels of bacteria, box-plots were constructed. Simple and clustered boxplots with interquartile range and outliers were used. The bottom and top of the box corresponded to the 25th and 75th percentiles, the band near the middle of the box represented the median (the 50th percentile). The results that substantially differed from the rest of the data, the outliers, were also included in the diagrams as small circles (mild outliers) or stars (extreme outliers). The addition of outliers in the box-plots was particularly useful to observe cases with high levels of bacteria and/or in high percentage. The data is presented on either logarithmic or linear scale, depending on the levels observed in the samples.

Statistical analyses were performed to compare prevalence, levels, and percentage of general and specific bacteria in NGA with important demographical and clinical data, as well as characteristics of sample collection. Presence of bacteria was treated as a binomial variable: present or absent. The 2-sided Fisher's exact test was used to compare detection of bacteria with the binomial covariates included in this study.

One-way ANOVA was applied to compare presence of bacteria with those variables with more than two categories, i.e. type of PTB and LBW. The independent t-tests were used when analysing parametric data to compare prevalence of bacteria with variables presented as quantitative data, i.e. gestational age and birthweight. Finally, the non-parametric Mann-Whitney U Test was used to compare quantitative levels of bacteria with the demographical/clinical variables. A p-value of <0.05 was considered to indicate statistical significance.

Finally, multivariate analyses were performed to control for the effect of those important variables in the occurrence of broad or specific bacteria in samples of NGA. The odds ratio (OR) and confidence intervals were reported. All statistical analyses were performed using the IBM® SPSS® Statistics Software, version 19.0.

CHAPTER 3
MICROBIOLOGY OF NEONATAL GASTRIC ASPIRATES

3.1 Introduction.

It is likely that the bacteria causing an infection in the fetus and/or neonate may have originated from a maternal site harbouring a complex community (e.g. the vagina, the gut, or the oral cavity), and that more than one single pathogen may have translocated from the contributor site to the amniotic environment. Few studies have fully characterized the bacterial diversity associated with APO. The lack of this information is due to the techniques traditionally used (i.e. culture or species-specific molecular analysis), which usually identify only one or a few pathogens (Evans et al., 1988). As a consequence, the use of routine techniques would not allow identification of the bacterial complexity possibly associated with the complications.

The overall aim of this project was to evaluate whether oral bacteria may have successfully translocated from the oral cavity to the amniotic environment. It is therefore important to have knowledge of the range of bacteria associated with cases of intrauterine infection, and which are possibly involved with APO. In addition, in order to identify the broader range of bacteria, including those present in low prevalence and low levels, improved and more sensitive protocols were necessary.

Another limitating factor to the understanding of the potential pathogens associated with APO is related to the suitability of the sample investigated. The analysis of AF has been considered the 'gold standard' for investigating bacteria associated with intra-amniotic infection. However, samples of AF are usually obtained from healthy mothers/infants at 16-20 weeks of gestation for genetic testing. Obtaining AF samples requires an invasive procedure, making it sometimes difficult to get the participant's consent and ethical approval. Moreover, contrary to the reasons for these studies, this procedure presents the risk of accidentally introducing bacteria into the amniotic cavity (Romero and Mazor, 1988). Therefore, these samples would not provide be of great predictive value when compared to samples obtained from disease-associated cases.

In this study, the analysis of NGA has been proposed. It has been suggested that "surface body samples", such as NGA, should contain the bacteria that reached and colonised the amniotic cavity before birth and therefore include those potential pathogens that possibly initiated an early-onset infections in neonates (Evans et al., 1988). Historically, investigations that have evaluated the use of NGA as a tool to predict neonatal sepsis were of mixed success (Leibovich et al., 1987, Evans et al., 1988, Puri et al., 1995). This may have been due to technique limitations (use of culture analysis) and the limited knowledge of the truly associated pathogen(s). As recently demonstrated, molecular detection of bacteria in NGA correlated with presence of chorioamnionitis and/or pROM (Miralles et al., 2005). Therefore, the study of NGA obtained from complicated pregnancies should provide a new insight into the investigation of the species of bacteria possibly associated with APO.

In this chapter, the array of microorganisms present in samples of NGA obtained from neonates that were born from complicated pregnancies was determined. A combination of clone analysis and DGGE was applied in order to increase the probability of identifying the less prevalent species, including the bacteria present at low prevalence/levels. Furthermore, a detailed analysis of possible oral bacteria in the samples was introduced.

3.2 Routine analysis of neonatal gastric aspirates as determined at the Microbiology Department, Barts and The London NHS Trust.

The neonatal samples analyzed in this study were obtained as a surplus to the NGA routinely obtained under the regulations of the UK's National Standard Methods. In accordance with these regulations, the samples were investigated using minimal microbiology standards, which consist of gram staining and basic microbial culture. These are the primary diagnostic methods used to date in most hospitals to determine the microorganisms present in a specific sample. Gram stain smears will not give a definite diagnosis; their purpose in medical settings is to allow rapid identification of bacteria to aid treatment and prognosis. Microbial culture allows growth and isolation of microorganisms using predetermined media under controlled laboratory

conditions. Culture particularly focuses on the range of bacteria that have been historically associated with neonatal sepsis (Table 3.1). All these procedures may take as long as 72 hours and are usually followed by further antibiotic susceptibility testing (Health Protection Agency, 2004).

Table 3.1 Summary of the routine culture procedures performed under the "Standard Operating Procedure for the investigation of gastric aspirates and infection screen swabs from neonates" (BSOP23). Source: Health Protection Agency (2004).

Clinical details/ conditions	**Standard media**	**Incubation**			**Cultures read**	**Target organism(s)***
		Temp °C	**Atmos**	**Time**		
Prolonged rupture of membranes **Possible sepsis**	Blood agar	35-37	5-10%	40-48 h	daily	Any of the following: β-haemolytic streptococci Coagulase-negative staphylococci *Enterococci* *Enterobacteriacea* *L. monocytogenes* *Pseudomonas* *S. aureus* Yeasts Pure growth of any other organisms
	CLED agar	35-37	air	16-24 h	≥ 16 h	

* Other organisms for consideration: *N. gonorrhoeae*, *C. trachomatis*, *Mycoplasma* species and *U. urealyticum*

The analyses routinely performed at the hospital determined that 25.8% (62/240) of the NGA samples included in this study were positive using the gram staining technique; similarly, 25% (60/240) were positive with the culture analyses. This information was not produced in this study but data was obtained from the Microbiology Department's database and is summarized in Tables 3.2 and 3.3.

Table 3.2 Number and percentage of samples of NGA positive with the gram staining technique as observed at the Microbiology Department, Royal London Hospital, Barts and The London NHS Trust. A total of 240 samples were analysed.

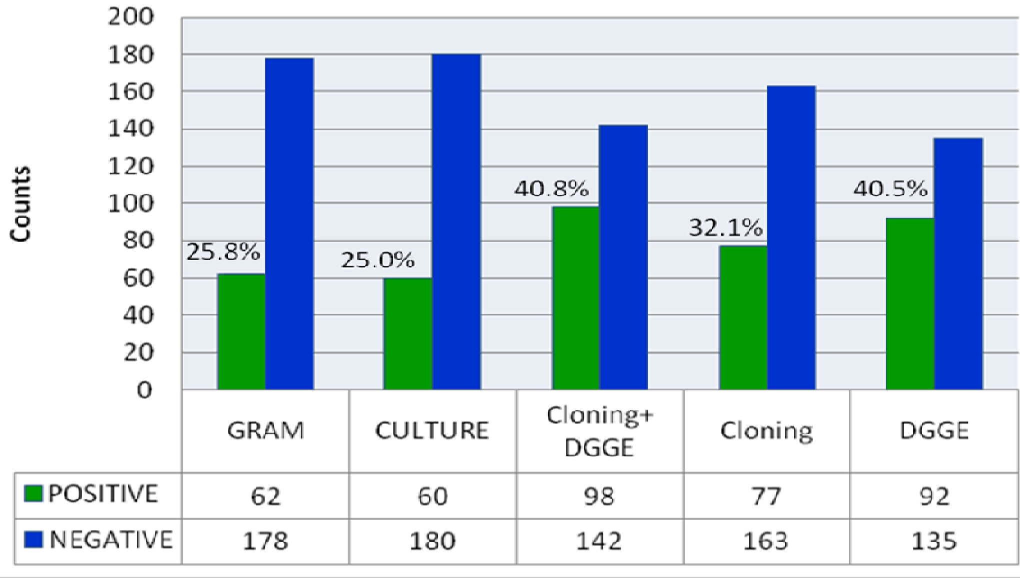
Gram result		#	%
G(+) cocci	(total)	43	17.9
	G(+) cocci pairs	5	2.1
	G(+) cocci chains	8	3.3
	G(+) cocci clusters	3	1.3
G(+) rods		15	6.3
G(-) cocci		3	1.3
G(-) rods		21	8.8
G(-) bacillus		2	0.8

Table 3.3 Number and percentage of samples of NGA positive with the culture technique as observed at the Microbiology Department, Royal London Hospital, Barts and The London NHS Trust. A total of 62 samples were positive by culture.

Culture result	#	%
α-haemolytic streptococcus (including ‘viridans’ streptococci)	7	11.7
group B streptococcus (GBS)	9	15.0
Streptococcus spp. (*S. bovis*)	2	3.3
coagulase-negative staphylococcus	7	11.7
Staphylococcus aureus	1	1.7
Corynebacterium spp.	2	3.3
Enterococcus spp.	2	3.3
E. coli	4	6.7
Klebsiella pneumoniae	1	1.7
coliform group bacteria	5	8.3
‘skin flora’	11	18.3
‘mouth flora’	3	5.0
Bacillus spp.	1	1.7
Gardnerella vaginalis	1	1.7
‘anaerobes’	4	6.7
Candida albicans	4	6.7
yeast	1	1.7

3.3 Molecular detection of bacteria in neonatal gastric aspirates.

Samples of NGA were analysed in this study using clone analysis and DGGE approaches. Detection of bacteria in the samples was defined as clone analysis-PCR and/or DGGE-PCR positive as confirmed using 1% and 2.6% agarose gel electrophoresis, respectively. With the clone analysis-PCR approach, 77 of the 240 samples analysed (32.1%) were positive. Due to limited amount of the samples, only 227 samples were available for the DGGE analysis of which 92 samples (40.5%) were positive. The percentage of positive samples with clone analysis when including only the 227 samples used for DGGE was 76 (33.5%). This excluded a smaller sample size as the reason for the higher detection rate with DGGE. Positive samples were then analysed in order to identify the bacterial species. Figure 3.1 illustrates a comparison of the same set of samples (N=240) analysed for the presence of bacteria using different detection techniques.



	GRAM	CULTURE	Cloning+ DGGE	Cloning	DGGE
POSITIVE	62	60	98	77	92
NEGATIVE	178	180	142	163	135

Figure 3.1 Clustered bar graph representing number of positive and negative samples for presence and absence of bacteria using different detection techniques. The percentage of positive samples out of the total evaluated are indicated on top of the positive bars.

3.4 Clone analysis.

The number of clones analysed per sample was initially 10 due to a low expected richness of the samples based upon previous studies (Evans et al., 1988). Nevertheless, it was observed in a preliminary analysis in 50 samples that some samples showed a richness of as many as seven different species in 10 clones analysed. Therefore, the following experiment was performed to determine the appropriate number of clones to be evaluated from each sample.

3.4.1 Richness of the samples and number of clones to evaluate.

Table 3.4 summarises the findings when evaluating as many as 47 clones from rich and less rich samples. The samples included in this experiment were randomly selected from different richness as observed in the initial 10 clones. It could be observed that in the 20 first clones selected, the number of taxa gradually increased for all the samples including the least rich, less rich, rich, and richest samples. After 20 clones, the number of taxa detected only increased significantly for the richest sample. Therefore, it was decided to analyse 20 clones of each sample and, if necessary, include the analysis of a further 10 clones depending on the richness.

Table 3.4 Number of taxa (richness) in NGA samples determined by cloning and sequencing.

Test Sample	Richness*	No. of clones evaluated				
		10	**20**	**30**	**40**	**47**
T1	**LEAST RICH**	1	1	2	3	3
T2		1	2	2	2	2
T3	**LESS RICH**	3	4	4	---	---
T4	**RICH**	5	6	6	8	---
T5	**RICHEST**	7	12	14	15	18

* Richness of the samples were determined from the analysis of the first 10 clones.

3.4.2 Taxa identified with clone analysis.

Due to technique-sensitive protocols and limited amounts of the samples, bacteria were identified in 33 (43%) of the 77 PCR-positive samples. A total of 932 clones were selected and sequenced for identification. Forty-two taxa were detected using this approach (Table 3.5), and the number of taxa per sample ranged between one and 11 with an average (mean±SD) of 2.4±2.1. To identify the species, the fragments of 16S rRNA gene were sequenced using the 357F primer and analysed by sequence comparison with both the RDP and the HOMD databases.

3.5 Denaturing Gradient Gel Electrophoresis.

3.5.1 Construction of a standard for DGGE analysis.

The most prevalent bacteria, identified by clone analysis in this study, were used to construct a standard for DGGE in order to facilitate the analysis of the gel bands. Purified PCR products from clones containing the inserts that were identified as originating from a range of taxa (*E. coli*, *G. vaginalis*, *L. crispatus*, *L. iners*, *S. agalactiae*, *Streptococcus pneumoniae/mitis/oralis/infantis*, *Sneathia sanguinegens/amnionii*, *U. urealyticum/parvum*, *V. montpellierensis*, and *V. parvula*) were subjected to a nested DGGE-PCR. The amplifications were performed separately for each species, and also in the same reaction to confirm that each species arrested at different levels in the gel (Figure 3.2). The identity of the bands was confirmed after band excision and sequencing. The DGGE standard was run together with the samples positive for the DGGE-PCR primers.

3.5.2 Taxa identified with DGGE.

Forty-one percent (92/227) of the samples available for DGGE-PCR were positive, detecting a total of 37 operational taxonomic units in the 59/92 positive samples (64%) that were successfully characterized with this technique. A number of bands (n=372) were excised from the polyacrylamide gels and identified by sequencing.

Table 3.5 Bacteria identified in NGA samples using a 16S rRNA gene PCR, cloning, and sequencing approach (in 33 of the 77 PCR-positive samples).

	SPECIES IDENTIFIED BY CLONE ANALYSIS	***No.***	***%^***
1	*Streptococcus pneumoniae/mitis/oralis/infantis* ¶*	16	48.5
2	*Escherichia coli ¶*	11	33.3
3	*Gardnerella vaginalis ¶*	8	24.2
4	*Ureaplasma urealyticum/parvum* ¶*	7	21.2
5	*Streptococcus sanguinis/gordonii* ¶*	6	18.2
6	*Lactobacillus crispatus ¶*	5	15.2
7	*Lactobacillus iners ¶*	5	15.2
8	*Streptococcus agalactiae ¶*	5	15.2
9	*Sneathia sanguinegens/amnionii* ¶*	5	15.2
10	*Veillonella montpellierensis (V. atypica/parvula)* ¶*	4	12.1
11	*Aerococcus christensenii ¶*	3	9.1
12	*Peptoniphilus* spp.¶	3	9.1
13	*Brevundimonas diminuta ¶*	3	9.1
14	*Staphylococcus epidermidis ¶*	2	6.1
15	*Streptococcus constellatus/intermedius/anginosus* ¶*	2	6.1
16	*Streptococcus salivarius/vestibularis* ¶*	2	6.1
17	*Peptostreptococcus anaerobius ¶*	2	6.1
18	*Hyphomicrobium zavarzinii*	2	6.1
19	*Sphingomonas* spp.¶	2	6.1
20	*Propionibacterium acnes ¶*	1	3.0
21	*Corynebacterium riegelii*	1	3.0
22	*Bifidobacterium* spp.	1	3.0
23	*Atopobium minutum*	1	3.0
24	*Bacteroides* spp.	1	3.0
25	*Prevotella* spp.¶	1	3.0
26	*Staphylococcus* spp.	1	3.0
27	*Granulicatella elegans¶*	1	3.0
28	*Enterococcus faecalis*	1	3.0
29	*Clostridium perfringens*	1	3.0
30	*Anaerococcus vaginalis*	1	3.0
31	*Faecalibacterium prausnitzii*	1	3.0
32	*Veillonella parvula/dispar* ¶*	1	3.0
33	*Fusobacterium necrophorum*	1	3.0
34	*Fusobacterium nucleatum ¶*	1	3.0
35	*Fusobacterium periodonticum*	1	3.0
36	*Sphingopyxis alaskensis ¶*	1	3.0
37	*Caldimonas taiwanensis*	1	3.0
38	*Haemophilus influenzae ¶*	1	3.0
39	*Moraxella osloensis ¶*	1	3.0
40	*Pseudomonas aeruginosa/otitidis* ¶*	1	3.0
41	*Pseudomonas fluorescens ¶*	2	6.1
42	*Silanimonas lenta*	1	3.0

^ Determined as percentage of the samples positive to the universal bacterial 16S rDNA primers used analysed with this technique (out of 33)
* Species considered in a same group due to difficulty in differentiating by 16S rRNA gene sequencing.

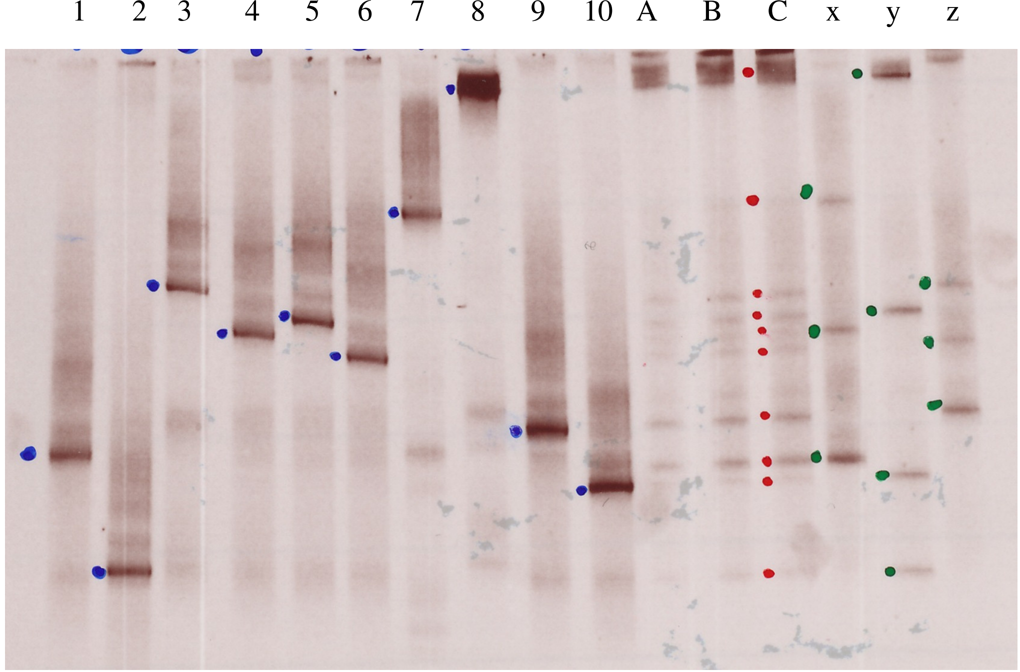


Figure 3.2 Negative image of a DGGE gel containing PCR products used for the construction of the standard for DGGE. Bacteria were run independently (1-10), in groups of 3-4 (x, y, z), and all together (A,B,C). Coloured dots indicate the levels at which each band arrested. Blue dots, single bacteria; green dots, groups of 3-4; red dots, all the bacteria. 1, *E. coli*; 2. *G. vaginalis*; 3, *L. crispatus*; 4, *L. iners*; 5, *S. agalactiae*; 6, *S. mitis* group; 7, *S. sanguinegens*; 8, *U. urealyticum/parvum*; 9, *V. montpellierensis*; 10, *V. parvula*; A-C contain all bacteria in different amounts; A, 10 µl; B, 15 µl; C, 20 µl. x, *S. sanguinegens*, *L. iners*, *E. coli*; y, *U. urealyticum/parvum*, *S. agalactiae*, *V. parvula*, *G. vaginalis*; z, *L. crispatus*, *S. mitis* group, *V. montpellierensis.*

Twenty bands were identified by comparison with the standard and/or a band sequenced from a nearby sample that arrested at the same level (Figure 3.3). This was kept to a minimum since different species were observed to provide more than one consecutive band in the gel. This varied between samples and might occur due to high levels of a specific species in the sample. Therefore, excision and sequencing of the bands was considered the more reliable technique. The number of taxa identified per sample using a DGGE approach ranged from one to seven with an average of 1.9±1.3. Table 3.6 summarizes bacteria species identified by DGGE in the samples of NGA.

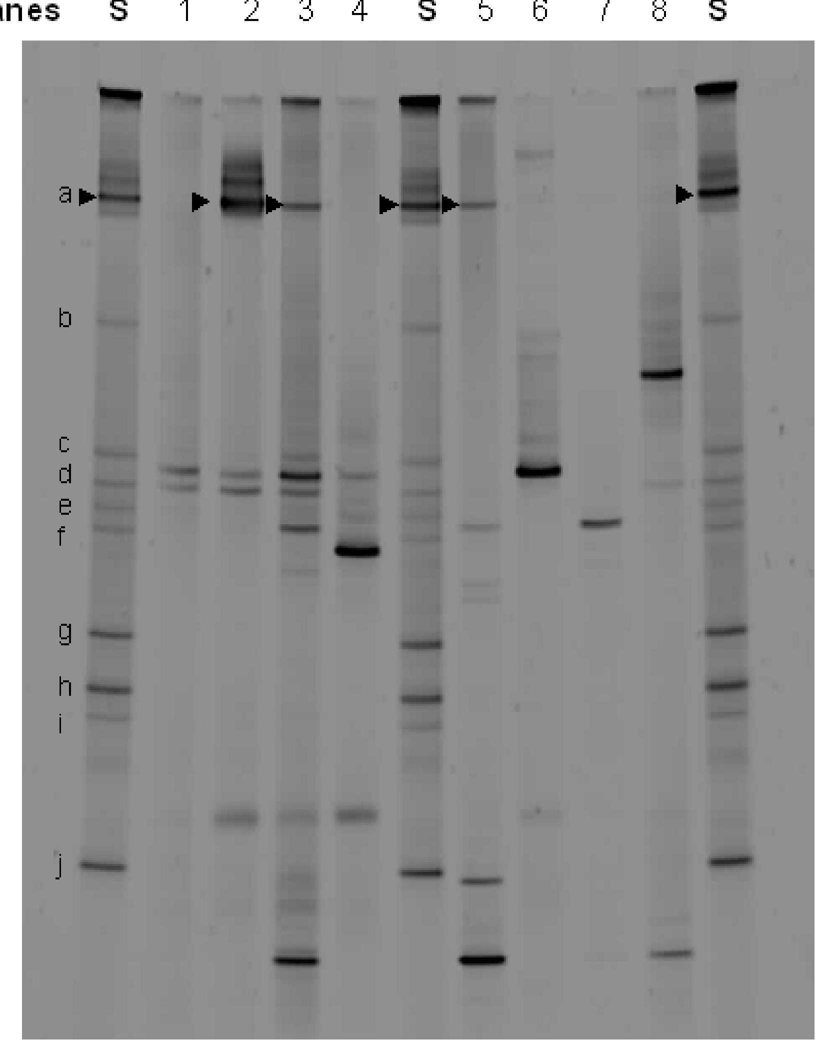


Figure 3.3 Negative image of a DGGE profile of ~235 bp 16S rDNA fragments extracted from samples of NGA. Samples (1-8) were run in separate lanes between standards (S) originating from the following taxa: a. *U. urealyticum/parvum**, b. *S. sanguinegens/amnionii**, c. *L. crispatus*, d. *S. agalactiae*, e. *L. iners*, f. *S. pneumoniae/mitis/oralis/infantis**, g. *V. montpellierensis*, h. *E. col*i, i. *V. parvula/dispar**, j. *G. vaginalis*. **Arrows** indicate an example of bands arresting at the same level of the polyacrylamide gel and identified as *U. urealyticum/parvum** as compared with standards and sequence of the PCR product. **Sample 2** shows an example of multiple consecutives bands for *U. urealyticum/parvum*. * Species considered in a same group due to difficulty to differentiate by 16S rRNA approach.

Table 3.6 Bacteria identified in NGA samples using a DGGE-PCR and band sequencing approach (in 59 positive samples).

	SPECIES IDENTIFIED BY DGGE ANALYSIS	***No.***	***%^***
1	*Streptococcus pneumoniae/mitis/oralis/infantis** ¶	20	33.9
2	*Ureaplasma urealyticum/parvum** ¶	16	27.1
3	*Lactobacillus iners* ¶	10	16.9
4	*Sneathia sanguinegens/amnionii** ¶	8	13.6
5	*Streptococcus agalactiae* ¶	8	13.6
6	*Lactobacillus crispatus* ¶	7	11.9
7	*Pseudomonas aeruginosa/otitidis** ¶	7	11.9
8	*Gardnerella vaginalis* ¶	6	10.2
9	*Staphylococcus epidermidis* ¶	5	8.5
10	*Escherichia coli* ¶	5	8.5
11	*Haemophilus influenzae* ¶	4	6.8
12	*Prevotella bivia*	3	5.1
13	*Streptococcus sinensis/parasanguis**	3	5.1
14	*Peptoniphilus* spp.¶	3	5.1
15	*Veillonella montpellierensis (V. atypica/parvula)** ¶	3	5.1
16	*Pseudomonas fluorescens* ¶	3	5.1
17	*Aerococcus christensenii* ¶	2	3.4
18	*Streptococcus constellatus/intermedius/anginosus** ¶	2	3.4
19	*Streptococcus salivarius/vestibularis** ¶	2	3.4
20	*Peptostreptococcus anaerobius* ¶	2	3.4
21	*Fusobacterium nucleatum* ¶	2	3.4
22	*Brevundimonas diminuta* ¶	2	3.4
23	*Propionibacterium acnes* ¶	1	1.7
24	*Prevotella* sp.¶	1	1.7
25	*Granulicatella elegans*¶	1	1.7
26	*Lactobacillus gasseri*	1	1.7
27	*Streptococcus sanguinis/gordonii** ¶	1	1.7
28	*Megasphaera* sp.	1	1.7
29	*Veillonella parvula/dispar** ¶	1	1.7
30	*Sphingopyxis alaskensis* ¶	1	1.7
31	*Sphingomonas* spp.¶	1	1.7
32	*Massilia* sp.	1	1.7
33	*Klebsiella pneumoniae*	1	1.7
34	*Proteus mirabilis*	1	1.7
35	*Acinetobacter baumannii*	1	1.7
36	*Moraxella osloensis* ¶	1	1.7
37	*Mycoplasma hominis*	1	1.7

^ Determined as percentage of the samples positive to the universal bacterial 16S rDNA primers used analysed with this technique (out of 59).

* Species considered in a same group due to difficulty to differentiate by 16S rDNA approach
¶ Species detected by 16S rRNA gene cloning and sequencing and DGGE.

3.6 The combined molecular approach to determine the microbiology of neonatal gastric aspirates.

When comparing the results obtained with the cloning and the DGGE analyses, it was observed that they differed greatly as regards the prevalence of bacteria described. Forty-two species were identified with cloning, while 37 bacteria could be identified with DGGE. However, bacteria observed only matched for 28 species (55%) out of the 51 observed with both techniques. This difference observed between the techniques was basically due to those less prevalent taxa. When the bacteria observed only once were not included, 30 and 29 species were identified for cloning and DGGE respectively matching in 90% (28 of 31) of cases. Clone analysis used in isolation identified most of the bacteria observed only once (n=22).

The results obtained from both techniques were combined in order to describe the microbiology of NGA. With the combined molecular approach 142 (59%) of the 240 samples were negative with the molecular techniques used. In the 98 (40.8%) samples positive, the total number of taxa detected was 51. The taxa identified in the NGA fell into six different phyla, namely Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria and Tenericutes. Table 3.7 summarizes the bacteria identified in the samples as organized by phylum, class, order, family, genus, and species.

Bacteria most prevalent (% out of 98 positive samples) were: *S. pneumoniae/mitis/oralis/infantis* (28%), *U. urealyticum/parvum* (18%), *Lactobacillus* spp. (18%), *E. coli* (14%), *Pseudomonas* spp. (12%), *S. agalactiae* (11%), *G. vaginalis* (10%), and *S. sanguinegens/amnionii* (9%).

Table 3.7 Taxonomic classification of the bacteria identified in NGA using clone analysis and DGGE in a combined molecular approach.

	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	*No.*	%^
1	**Actinobacteria**	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Propionibacterium*	***Propionibacterium acnes ¶***	**2**	**2.0**
2				Propionibacteriaceae	*Corynebacterium*	***Corynebacterium riegelii*** [a]	**1**	**1.0**
3			Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	***Bifidobacterium* sp.**[a]	**1**	**1.0**
4					*Gardnerella*	***Gardnerella vaginalis ¶***	**10**	**10.2**
5			Coriobacteriales	Coriobacteriaceae	*Atopobium*	***Atopobium minutum*** [a]	**1**	**1.0**
6	**Bacteroidetes**	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	***Bacteroides* sp.**[a]	**1**	**1.0**
7				Prevotellaceae	*Prevotella*	***Prevotella bivia*** [b]	**3**	**3.1**
8						***Prevotella* sp.¶**	**2**	**2.0**
9	**Firmicutes I**	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	***Staphylococcus epidermidis ¶***	**6**	**6.1**
10						***Staphylococcus* sp.**[a]	**1**	**1.0**
11			Lactobacillales	Aerococcaceae	*Aerococcus*	***Aerococcus christensenii ¶***	**4**	**4.1**
12				Carnobacteriaceae	*Granulicatella*	***Granulicatella elegans¶***	**2**	**2.0**
13				Enterococcaceae	*Enterococcus*	***Enterococcus faecalis*** [a]	**1**	**1.0**
14				Lactobacillaceae	*Lactobacillus*	***Lactobacillus crispatus ¶***	**8**	**8.2**
15						***Lactobacillus gasseri*** [b]	**1**	**1.0**
16						***Lactobacillus iners ¶***	**13**	**13.3**
17				Streptococcaceae	*Streptococcus*	***Streptococcus agalactiae ¶***	**11**	**11.2**
18						***Streptococcus constellatus/intermedius/anginosus* ¶***	**2**	**2.0**
19						***Streptococcus salivarius/vestibularis* ¶***	**4**	**4.1**
20						***Streptococcus sanguinis/gordonii* ¶***	**7**	**7.1**
21						***Streptococcus sinensis/parasanguis**** [b]	**3**	**3.1**
22						***Streptococcus pneumoniae/mitis/oralis/infantis* ¶***	**27**	**27.6**

^ Determined as percentage of the samples positive to the universal bacterial 16S rDNA primers used (out of 98); ¶ Species detected with both molecular techniques;
[a] Species identified with cloning analysis only; [b] Species identified with DGGE approach only

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Table 3.7 (cont.)

23				Clostridiaceae	*Clostridium*	***Clostridium perfringens*** [a]	**1**	**1.0**
24				Incertae Sedis XI	*Anaerococcus*	***Anaerococcus vaginalis*** [a]	**1**	**1.0**
25					*Peptoniphilus*	***Peptoniphilus* spp.¶**	**6**	**6.1**
26				Peptostreptococcaceae	*Peptostreptococcus*	***Peptostreptococcus anaerobius ¶***	**3**	**3.1**
27	**Firmicutes II**	Clostridia	Clostridiales	Ruminococcaceae	*Faecalibacterium*	***Faecalibacterium prausnitzii*** [a]	**1**	**1.0**
28					*Megasphaera*	***Megasphaera* sp.** [b]	**1**	**1.0**
29				Veillonellaceae	*Veillonella*	***Veillonella montpellierensis (V. atypica/parvula)* ¶***	**5**	**5.1**
30						***Veillonella parvula/dispar* ¶***	**2**	**2.0**
31						***Fusobacterium necrophorum*** [a]	**1**	**1.0**
32	**Fusobacteria**	Fusobacteria	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	***Fusobacterium nucleatum ¶***	**2**	**2.0**
33						***Fusobacterium periodonticum*** [a]	**1**	**1.0**
34				Leptotrichiaceae	*Sneathia*	***Sneathia sanguinegens/amnionii* ¶***	**9**	**9.2**
35			Caulobacterales	Caulobacteraceae	*Brevundimonas*	***Brevundimonas diminuta ¶***	**4**	**4.1**
36		Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	***Hyphomicrobium zavarzinii*** [a]	**2**	**2.0**
37			Sphingomonadales	Sphingomonadaceae	*Sphingopyxis*	***Sphingopyxis alaskensis ¶***	**2**	**2.0**
38					*Sphingomonas*	***Sphingomonas* spp.¶**	**3**	**3.1**
39		Betaproteobacteria	Burkholderiales	Comamonadaceae	*Caldimonas*	***Caldimonas taiwanensis*** [a]	**1**	**1.0**
40				Oxalobacteraceae	*Massilia*	***Massilia* sp.** [b]	**2**	**2.0**
41					*Escherichia/Shigella*	***Escherichia coli ¶***	**14**	**14.3**
42	**Proteobacteria**		Enterobacteriales	Enterobacteriaceae	*Klebsiella*	***Klebsiella pneumoniae*** [b]	**1**	**1.0**
43					*Proteus*	***Proteus mirabilis*** [b]	**1**	**1.0**
44			Pasteurellales	Pasteurellaceae	*Haemophilus*	***Haemophilus influenzae ¶***	**4**	**4.1**
45		Gammaproteobacteria		Moraxellaceae	*Acinetobacter*	***Acinetobacter baumannii*** [b]	**2**	**2.0**
46			Pseudomonadales		*Enhydrobacter*	***Moraxella osloensis ¶***	**2**	**2.0**
47				Pseudomonadaceae	*Pseudomonas*	***Pseudomonas aeruginosa/otitidis* ¶***	**8**	**8.2**
48						***Pseudomonas fluorescens ¶***	**5**	**5.1**
49			Xanthomonadales	Xanthomonadaceae	*Silanimonas*	***Silanimonas lenta*** [a]	**1**	**1.0**
50	**Tenericutes**	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	***Mycoplasma hominis*** [b]	**1**	**1.0**
51					*Ureaplasma*	***Ureaplasma urealyticum/parvum* ¶***	**18**	**18.4**

3.7 Bacteria from a possible oral origin.

Extensive lists of bacteria characterized from a diverse range of healthy and/or diseased sites in the oral cavity have been published in a number of studies. The species identified in samples of NGA were compared with those studies that have used non-specific molecular approaches to characterise oral bacteria (Dymock et al., 1996, Kroes et al., 1999, Paster et al., 2001, Munson et al., 2002, Paster et al., 2002, Hutter et al., 2003, Kazor et al., 2003, Aas et al., 2005, Kumar et al., 2005, Chhour et al., 2005, Paster et al., 2006, Tanner et al., 2006, de Lillo et al., 2006, Diaz et al., 2006, Sakamoto et al., 2006, Ledder et al., 2007, Siqueira et al., 2007, Riggio et al., 2007, Riggio et al., 2008, Faveri et al., 2008, Siqueira and Rôças, 2009, Kanasi et al., 2010). This was followed by an exhaustive search in Medline specific to the bacteria of interest to determine those species that are also resident in other non-oral sites.

In this study, 15 of the species detected in the samples of NGA have been selected as potential oral pathogens due to a demonstrated association in the previous literature with the oral cavity either in health or disease (Figure 3.4). The oral cavity can act as the portal of entry of many bacteria from the external environment, and is also in close proximity with the nasopharynx and laryngopharynx microbiota. As a consequence, many of the microorganisms normally present in these non-oral sites might have been inevitably detected in the oral cavity in this and other studies. Therefore, to avoid selection of possible transient species, the species were further investigated to determine their relevance to the oral cavity (Table 3.8). Species such as *A. baumannii* (Paster et al., 2001) and *P. bivia* (Dymock et al., 1996) were excluded as previous studies did not provide conclusive evidence of the association with their oral cavity. Other bacteria known to be prevalent in the oral cavity but were not identified at the species level in this study were also excluded (*Bacteroides* spp., *Bifidobacterium* spp., and *Megasphera* spp.).

Figure 3.4 Pie chart representing prevalence and percentage of the bacteria observed in samples of NGA using a combined molecular approach. Those species from possible oral origin are highlighted in red.

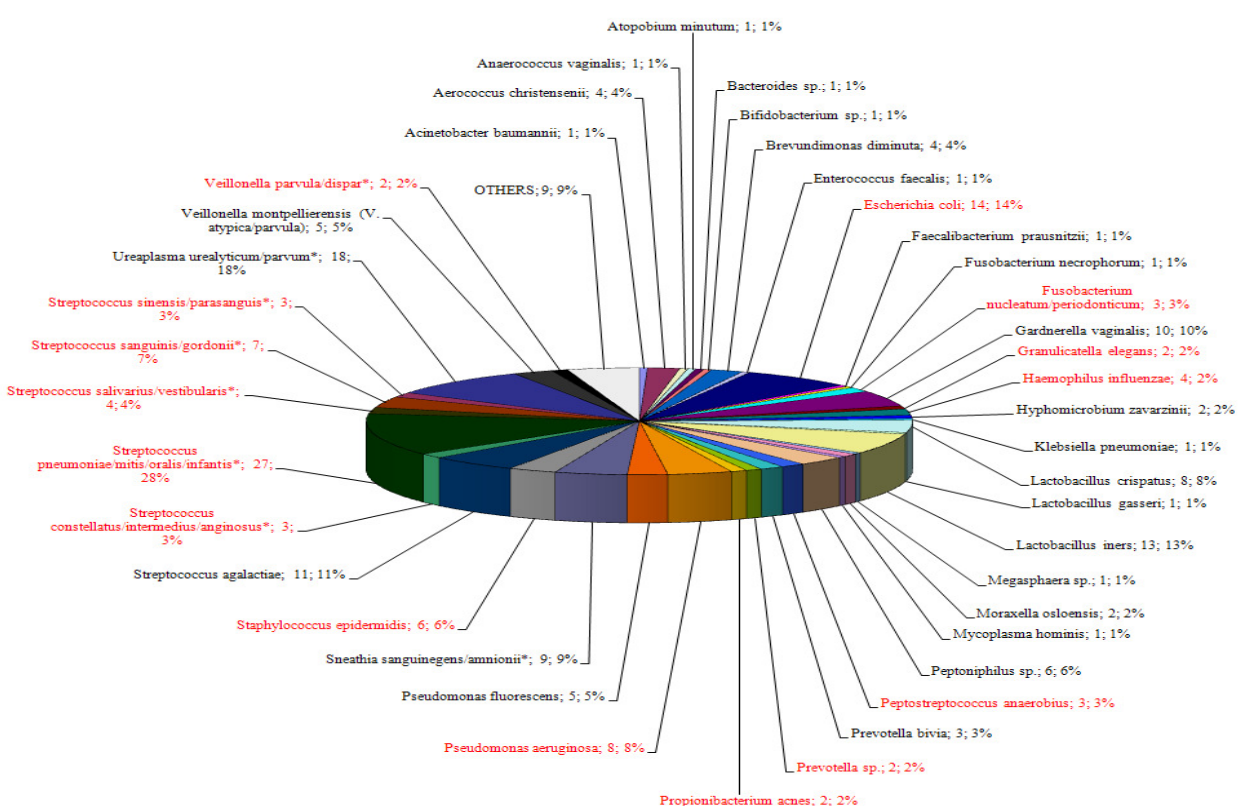

Table 3.8 Bacteria from a possible oral origin identified in NGA as previously determined using broad-range molecular techniques; their presence in non-oral sites, and associations with pregnancy complications.

	BACTERIA (prevalence in NGA)[#]	Presence in the oral cavity	REFERENCES^*	Presence in non-oral sites	REFERENCES^	Association with pregnancy complications	REFERENCES^
1	***Escherichia coli*** **(14%)**	periodontitis, dentoalveolar absess, halitosis	A, D, H	GUT, GIT, genito-urinary tract and relate infections	Wilson 2008	PTB, Chorioamnionitis, stillbirth, pROM, meningitis in neonates, early onset neonatal infection, PPROM	Bhola *et al*., 2008, Kuhn *et al*., 2010
2	***Fusobacterium nucleatum*** **(2%) /** ***periodonticum*** **(1%)**	health, periodontitis, caries, dentoalveolar absess, endodontic infection, halitosis	A, B, C, D, E, F, H, I, J, K, L, M, N, O, P, R	GUT, GIT, genito-urinary tract, pericarditis, IE, brain abscess, UTI, AF	Wilson 2008 Truant *et al*., 1983, Shammas *et al*., 1993, Kai *et al*., 2008, Ribot *et al*., 1981	PTB, stillbirth, preeclampsia, chrorioamnionitis, previous complications, pROM, AF, PROM	Bearfield *et al*., 2002, Barak *et al*., 2007, Hill 1993, Madianos *et al*., 2001, Han *et al*., 2010, Han *et al*., 2009, Cahill *et al*., 2005, Jalava *et al*., 1996
3	***Granulicatella elegans*** **(2%)**	health, periodontitis, caries, dentoalveolar absess, endodontic infection, halitosis	A, B, E, G, H, L	IE	Sato *et al*., 1999, Ohara-Nemoto *et al*., 2005, Casalta *et al*., 2002	n/a	n/a
4	***Haemophilus influenzae*** **(4%)**	health, periodontitis	A, E, L	respiratory tract, genito-urinary tract, nasal cavity, oropharinx, eyes, pneumonia	Okuda *et al*., 2005, Wilson 2008	maternal sepsis, PTB, PTL, chorioamnionitis, neonatal infections, PPROM	Warren *et al*., 2010, Gill *et al*., 1995, Takala *et al*., 1991, Jalava *et al*., 1996
5	***Peptostreptococcus anaerobius*** **(3%)**	periodontitis, dento alveolar absess, endodontic infection	A, D, E, F, K, O, S	GUT, GIT, genito-urinary tract, liver absess, BV	Wilson 2008, Truant *et al*., 1983, Shammas *et al*., 1993, Kai *et al*., 2008, Ribot *et al*., 1981, Pybus and Onderdonk, 1998	BV in pregnant women, endometriosis, AF	Hillier *et al*., 1993, Thadepalli *et al*., 1978
6	***Prevotella sp.*** **(2%)**	health, periodontitis, dentoalveolar absess, endodontic infection, caries	A, B, D, E, F, I, P	respiratory tract, GUT, GIT, vagina	Wilson 2008, Persson *et al*., 2009	intrauterine infection, BV, chorioamnionitis (P. bivia)	Gibbs *et al*., 2004, Hecht *et al*., 2008

Table 3.8 (cont.)

7	***Propionibacterium acnes* (2%)**	endodontic infection, Noma	F, R	GUT, GIT, genituurinary tract, skin, endocarditis, endophthalmitis, osteomyelitis, joint, CNS, cranial neurosurgery infections, implanted biomaterial contamination	Sasaki et al., 1980, Wilson 2008	placentitis and abortion in animals	Lyons et al., 2009
8	***Pseudomonas aeruginosa* (8%)**	periodontitis, periodontitis, endodontic infection, immunocompromised subjects	A	GUT, GIT, respiratory tract, dermatitis, soft tissue infection, bacteremia, bone and joint infections, pneumonia	Okuda et al., 2005, Wilson 2008	intraamniotic infection, neonatal death	Oláh 2004
9	***Staphylococcus epidermidis* (6%)**	endodontic infection, Noma	F, R	skin and mucous membranes, frequent hospital infections	Otto, 2009	neonatal infection	Nelson et al., 2009
10	***Streptococcus constellatus/intermedius/anginosus* (2%)**	health, periodontitis, ANUG, endodontic infection, absess	A, B, E, F, J, K, L, M, S, T	GUT, genito-urinary tract, respiratory tract, cardiovascular disease, abdominal infection, CNS	Wilson 2008	chorioamnionitis, BV in pregnant women	McDonald and Chambers, 2000, Rabe et al., 1988
11	***Streptococcus mitis/oralis* (27%)**	health, periodontitis, ANUG, endodontic infection, halitosis, caries	A, B, C, E, F, H, I, J, K, L, M, P, Q, R, T	GUT, genito-urinary tract, respiratory tract, IE	Wilson 2008, Aoyagi et al., 2005	breast milk, PTB, PPROM, neonatal meningitis	Martín et al., 2007, DiGiulio et al., 2008, Bignardi and Isaacs, 1989,
12	***Streptococcus salivarius /vestibularis* (4%)**	health, ANUG, halitosis	A, B, C, H, J, L	genito-urinary tract, GIT	Wilson 2008	uterine pathology in animals	McDougall, 2005
13	***Streptococcus sanguinis/gordonii/parasanguis* (3%)**	health, ANUG, periodontitis, halitosis, caries	A, B, C, E, G, H, I, J, K, L, M, P, Q	brain absess, IE	Kassis et al., 2010, Turner et al., 2009, Xiong et al., 2008	n/a	n/a
14	***Streptococcus sinensis* (3%)**	health	E, K	IE	Woo et al., 2002, Faibis et al., 2008	n/a	n/a
15	***Veillonella parvula/dispar* (2%)**	health, periodontitis, ANUG, endodontic infection, halitosis, caries	A, B, C, E, F, G, H, I, K, L, M, P, Q, T	GUT, GIT, genito-urinary tract, IE, meningitis, osteomielitis	Bhatti and Frank, 2000, Boo et al., 2005, Singh and Yu, 1992	PTB, PPROM	Romero et al., 1986

GUT, genitourinary tract; GIT, gastrointestinal tract; UTI, urinary tract infection; IE, infective endocarditis; CNS, central nervous system; ANUG, acute necrotizing ulcerative gingivitis

[#] Percentage of the samples positive to the universal bacterial 16S rDNA primers used (out of 98)

* Legend for the reference for oral cavity studies are detail below.

REFERENCES: A, Paster *et al* ., 2001; B, Aas *et al* .,2005, C, Kroes *et al* ., 1999; D, Dymock *et al* ., 1996; E, Kumar *et al* ., 2005; F, Munson *et al* ., 2002; G, Kanasi *et al* ., 2010; H, Riggio *et al* ., 2008; I, Ledder *et al* ., 2007; J, Tanner *et al* ., 2006; K, de Lillo *et al* ., 2006; L, Diaz *et al* ., 2006; M, Hutter *et al* ., 2003; N, Sakamoto *et al* ., 2006; O, Siqueira and Rôças, 2009; P, Chhour *et al* ., 2005; Q, Kazor *et al* ., 2003; R, Paster *et al* ., 2002; S, Riggio *et al* ., 2007; T, Faveri *et al* ., 2008.

Table 3.8 presents a description of those potential oral bacteria observed in NGA. The first column of this table summarizes those studies that have previously detected microorganisms as potential oral species using broad-range molecular techniques. The second column denotes the presence of these oral soecies in non-oral sites. Most of these pathogens are also normally found in non-oral sites such as the genitourinary tract, gastrointestinal tract, respiratory tract, and genito-urinary tract. Wilson (2008) provided an extensive overview of the distribution of microbial communities inhabitating humans that was used as a main reference source for this study. Also, a Medline search was performed to determine bacteria frequently found in other non-oral sites in disease. A few exceptions, namely *G. elegans*, *S. sanguinis/gordonii/parasanguis* and *Streptococcus sinensis*, have only been found within the oral cavity and not at any other body site. Interestingly, they have been associated previously with infection in other body sites, including infective endocarditis and brain abscesses.

Finally, a Medline search using "(*the specific bacteria name*)" AND "pregnancy" was performed to identified those microorganisms that have been previously associated with pregnancy complications. Again, most of the species have been associated previously with pregnancy complications, with the exception of *G. elegans*, *S. sanguinis/gordonii/parasanguis* and *S. sinensis*. Most of the studies that have identified *F. nucleatum* in association to an APO have also proposed the oral cavity as the source of the infection (Han et al., 2009, Han et al., 2010); other bacteria have been associated with the vagina or from diverse sources of contamination.

3.8 Discussion.

This chapter introduces a detailed description of the bacterial species contained in samples of NGA using molecular techniques. The specimens analysed were obtained from newborns that presented with an APO and/or were suspected to have an infection. Hence, the bacteria identified in the samples may be associated with the complications.

No healthy neonates could be sampled for NGA due to the invasiveness of the procedure. It could be anticipated that gastric fluids in newborn infants should be sterile since a healthy fetus is thought to develop in a bacteria-free environment and presence of bacteria in the amniotic cavity is considered a pathological finding (Romero et al., 2007). However, bacteria acquired during labour, at the time of delivery, or through other sources of contaminations may be present in these samples. Previous work have determined that presence of bacteria increased in labour from 3.5% to 13% when cervical dilatation increases from less than 4 cm to more than 4 cm (Seong et al., 2008). This has been explained by a suction-like effect during uterine contractions (Zervomanolakis et al., 2007). The microorganisms were more likely to originate from vaginal fluid aspirates since only few of these cases showed signs of chorioamnionitis and funisitis (umbilical cord infection).

Also, a recent study has demonstrated that the initial microbiota in healthy neonates, as determined in multiple body habitats (skin, oral mucosa, and nasopharyngeal aspirate), reflects the mother's vaginal bacteria (*Lactobacillus* spp., *Prevotella* spp., and *Sneathia* spp.) in the case of vagina delivery, and the skin microbiota (*Staphylococcus* spp., *Corynebacterium* spp., and *Propionibacterium* spp.) if delivered by Caesarean section (Dominguez-Bello et al., 2010). However, in the study mentioned, the samples were obtained within 5 minutes of delivery, from more superficial sites (in comparison to NGA), and before removing the vernix caseosa. The vernix is the waxy 'cheese-like' film that covers the fetus during the last trimester. Its function is to act as a barrier to protect the newborn from bacterial infection. It is in direct contact with the maternal vagina and skin at the time of delivery, therefore presence of certain levels of vaginal and skin bacteria is expected. No other study reporting first colonisers in healthy neonates has been reported. In this report, a high percentage of samples (59%; 142/240) were negative for detection of bacteria which supports the assumption that either no bacteria, or low levels of vaginal and skin contaminants should be present in the gastric fluids from healthy infants.

The laboratory methods used in this study consisted of a combination of two broad-coverage molecular techniques namely 16S rRNA gene sequencing (clone analysis) and DGGE profiling. A combination of methods to improve detection of microbial prevalence, diversity and abundance has been previously suggested (DiGiulio et al., 2008, Payne et al., 2010). In this study, the use of a combined approach was introduced in order to benefit from the inherent positive aspects of each method and to overcome their limitations. The initial PCR varied for each technique in the fragment size amplified, the set of primers, and the conditions used (Muyzer et al., 1993, Frank et al., 2008). This could explain the detection variations observed between both techniques i.e., 42 samples were positive with cloning-PCR, 38 samples were positive with DGGE-PCR, and only in 28 did these methods coincide. At first glance, the techniques show great variability; nonetheless, the results demonstrated that both techniques have shown to be efficient in detecting the most prevalent bacteria. The combined approach therefore showed great advantage also detecting less prevalent species. This feature is important for the aims of this study since potential oral pathogens may be present in low prevalence and perhaps at a low numbers.

Previous studies have investigated NGA using both traditional and molecular methods. Using the culture approach, microorganisms isolated have been limited to a few species, including *E. coli*, *H. influenzae*, *S. aureus, L. monocygenes, S. pneumoniae*, GBS, and species of the genus *Lactobacillus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Enterococcus*, *Pseudomonas*, and *Corynebacteria* (Evans et al., 1988, Thompson et al., 1992, Borderon et al., 1994, Hammoud et al., 2003). Recently, Jones *et al*. (2010) demonstrated, using a q-PCR approach, that culture analysis of NGA was only positive when bacterial numbers exceeded 4.50E+05 CFU/ml (mean: 3.10E+05; range: 3.00E+03- 1.74E+08). Therefore, routine analysis may underestimate the number of samples containing microbes. In this study 41% of the samples (98/240) were positive with the combined molecular approach compared to only 26% (60/240) culture-positive as determined at the Microbiology Department, Royal London Hospital. More importantly, culture-dependent methods may severely underestimate bacterial richness (Amann et al., 1995) usually reporting only one

single microorganism, while the results in this study describe up to 11 different species per sample (mean±SD: 2.2±2.2). This study supports the statement that routine culture analysis may represent a practical tool in the hospital to guide treatment in already established infections but is not useful to identify those unusual pathogens involved in fetal colonization and early-onset neonatal infections.

Other studies have given an insight into the microbiota associated with APO. In fact, as determined by 16S rRNA gene PCR techniques, an association between intra-amniotic infections and clinical events including early-onset neonatal sepsis, clinical and histological chorioamnionitis, and funisitis was demonstrated (Kotecha et al., 2004, Miralles et al., 2005, Han et al., 2009). In these studies, species detected in the AF of women who present with an APO, were observed as *Ureaplasma* spp., *M. hominis*, *G. vaginalis*, *F. nucleatum*, *S. sanguinegens, S. agalactiae*, *S. pneumoniae/mitis/oralis*, and species of the genus *Bergeyella, Prevotella*, *Bacteroides*, *Lactobacillus*, *Peptostreptococcus*, *Peptoniphilus*, *Staphylococcus* and *Haemophilus* (Miralles et al., 2005, Han et al., 2009, Digiulio et al., 2010a, Digiulio et al., 2010b, Digiulio et al., 2010c). In general, the microbiota this study has identified in the NGA resembles those bacteria described in the mentioned AF studies. This observation supports the ability of NGA to be used as an alternative to AF to investigate the infections associated with APO. In addition, results from the study published by Miralles *et al*. (2005) contribute to this assumption as NGA showed an exact correlation with chorioamnionitis and/or pROM.

Jones *et al*. (2010) identified many potential pathogens in NGA including *U. urealyticum*, *G. vaginalis*, *S. sanguinegens/ammionii*, *Lactobacillus* spp., *Veillonella* spp., *H. influenza* and *S. agalactiae*. However, the techniques they used were based upon direct sequencing and may not allow a detailed description of the microbiology of NGA. Also, Oue *et al*. (2009) identified 23 species that were also observed in this study. However, an additional 31 species were also identified using the combined molecular approach, including the more prevalent species *G. vaginalis*, *L. iners*, *S. sanguinegens/amnionii*, *S. sanguinis/gordonii*, *V. montpellierensis*, and *A. christensenii*, and some less prevalent taxa e.g. *G. elegans*, *S.*

constellatus/intermedius/anginosus, *P. anaerobius*, *H. influenzae*, *B. diminuta*, *M. micronuciformis*, *S. sinensis/parasanguis*, *P. bivia*, and *V. parvula*, most of which have not previously been detected in NGA.

Finally, bacteria from a possible oral origin have been found in samples of NGA in this study. *G. elegans, S. sanguinis/gordonii/parasanguis* and *S. sinensis* unlike the other possible oral species, have only been described as inhabitants of the oral cavity and not at any other body site, therefore it is quite possible that these bacteria may have originated from the oral cavity. These species require particular attention as they are known to become blood-borne and have been associated with other systemic disease such as infective endocarditis (Roggenkamp et al., 1998, Ohara-Nemoto et al., 2005, Faibis et al., 2008, Woo et al., 2008, Xiong et al., 2008, Kassis et al., 2010). The potential of these possible oral species to translocate via the bloodstream is supported by a number of studies (Soto et al., 1998, Allen et al., 2002, Turner et al., 2009). Interestingly, *G. elegans* was initially described as a member of the nutritionally variant streptococci (NVS) (Roggenkamp et al., 1998), which may explain shared virulence characteristics with the *Streptococcus* spp., the most commonly cause of endocarditis. These bacteria may have originated from the oral cavity, although it remains to determine whether they possess the ability to cross and invade the placental tissue.

Species such as *F. nucleatum/periodonticum, P. anaerobius, Prevotella* spp., *V. parvula,* and members of the *Streptococcus mitis*, *anginosus* and *salivarius* groups found in this study are recognised as common oral bacteria as shown to be present in most of the broad-range molecular studies (Table 3.8). However many of these species are also common to the genito-urinary and gastrointestinal tracts (Srinivasan et al., 2009). Although in low prevalence (2%), special attention should be given to *F. nucleatum*. This is, in fact, the most common species isolated from AF in cases of PTL and intact membranes (Hill, 1998).

Recently, the first evidence of *F. nucleatum* as the etiological cause of a stillbirth was reported (Han et al., 2010). Similarly to the findings reported in this study, Oue

et al. (2009) identified *F. nucleatum* in two (5%) of 42 bacteria-positive samples of NGA, whereas Han *et al.* (2009) detected the same species in 33.3% of AF positive samples. One reason for low detection may be due to the associated PCR amplification bias, which may preferentially amplify certain species (Kraytsberg and Khrapko, 2005). In this report, the use of a special set of primers including broad-range segments for normally vaginal strains may have contributed to the preferential amplification of other species over fusobacteria (Frank et al., 2008). With the use of species-specific primers, Bearfield *et al.* (2002) amplified *F. nucleatum* from 15% of AF samples and found a significant association with previous pregnancy complications. Further evidence regarding the potential ability of *F. nucleatum* to cross the placental tissues has been demonstrated in animal studies. Indeed, this microorganism has been observed to specifically invade the murine uterus without spreading systemically, resulting in preterm delivery, stillbirths and non-sustained live births (Han et al., 2004). Also, other work has been performed to evaluate the potential mechanisms used by *F. nucleatum* to colonise the placenta. It has been shown that the adhesin *fad*A may be involved in the attachment and invasion of placental tissue using *in vitro* and *in vivo* models (Han et al., 2005, Ikegami et al., 2009). Most of the studies usually imply *F. nucleatum* has an oral origin, although there is no conclusive evidence to support this. Other possible means whereby an oral pathogen might be transferred to the samples, need to be evaluated such as from medical staff during delivery and earlier colonization through oral-genital transmission from the partner followed by subsequent spread through the ascending route (Saini et al., 2010). Whether the bacteria have originated from the oral cavity or the vagina will be further analysed in the following chapters.

CHAPTER 4

ASSOCIATIONS BETWEEN PRESENCE OF BACTERIA IN NEONATAL GASTRIC ASPIRATES AND DEMOGRAPHICAL AND CLINICAL CHARACTERISTICS

4.1 Introduction.

Adverse pregnancy outcomes can occur in a range of clinical presentations and may be due to multiple causes. Intrauterine infection is a frequent and important mechanism for pregnancy complications. The causal link of infection leading to a diversity of clinical outcomes including fetal loss, preterm delivery, PROM, SGA infant, PTL, and neonatal morbidity and mortality has been well established (Gomez et al., 1995, Romero et al., 2002, Goldenberg and Thompson, 2003). The diagnosis of infection is not always performed since it could remain clinically silent for weeks or even months before it is manifested as one of many obstetric disorders (Gray et al., 1992). The predisposition to one of these presentations might be influenced by a gene-environment interaction and/or a gene-gene interaction involving maternal and fetal genotype (Romero et al., 2006). Also, several risk factors for the presentation of an APO have been suggested, including non-white ethnicity, low socio-economic status, low pre-pregnancy weight (41-45 Kg) or low BMI (body mass index), smoking and drinking habits, previous LBW or PTB, uterine and cervical anomalies, multiple pregnancies, maternal medical complications, gestational bleeding, urogenital infection and social isolation (Mavalankar et al., 1992). Other factors such as maternal age, parity, anxiety-stress during pregnancy, drug abuse, strenuous physical work-load, sexual activities, inadequate or no pre-natal care have also been proposed (Petridou et al., 2001, Moutquin, 2003a).

In this study, prevalence of bacteria in NGA was investigated and is detailed in Chapter 3. However, multiple aetiology and the complex nature of pregnancy complications should be carefully considered at the time of interpretation of the data together with other considerations. For example, the presence of bacteria could indicate colonisation rather than infection. The term ‘colonisation’ refers to the persistent presence of indigenous bacteria in a commensal relationship with the host without causing any harm. Alternatively, the successful multiplication of a pathogen within, and at the expense of, a host is defined as an infection (Finlay and Falkow, 1989). Infection does not always result in disease and, conversely, colonisation may sometimes lead to an opportunistic infectious disease (Asikainen and Chen, 1999).

Opportunistic species may proliferate as a result of the disease, but not necessarily be the cause of the disease. These opportunists may proliferate in numbers concomitantly with or after the true pathogens, thus making it difficult to determine the true pathogens involved with the complications.

Another limitation when using NGA as a sample for analysis was the number of potential transient microorganisms that could be present in the samples. These are: a) bacteria from the maternal vagina and skin acquired during delivery (Dominguez-Bello et al., 2010); b) possible bacterial contaminants from the hospital environment and personnel that may have reached the samples during delivery, collection and transportation of the sample. Finally, the routine administration of intrapartum antibiotic as a preventive strategy, which could have altered the microbiota in mothers and neonates, may have influenced the bacterial content in the samples.

Therefore, a diverse range of demographical, clinical and outcome characteristics of the mother and infant, as well as the time and place of sample collection, were analysed in this study using a bivariate analysis (the analysis of two variables simultaneously) followed by a multivariable analysis (the analysis of multiple variables simultaneously).

4.2 Demographical and clinical data.

Demographical and clinical characteristics were obtained from all the cases evaluated in this study and are detailed in Tables 4.1 and 4.2.

4.3 Comparisons between detection of bacteria and demographical/clinical data.

Detection of general bacteria with the combined molecular approach showed significant association with both vaginal delivery ($p = 0.001$) and pROM ($p<0.001$) using the bivariate Fisher's exact test (Table 4.3). Clustered bars representing the comparisons for these variables are shown in Figures 4.1 and 4.2. The p-value was placed to indicate differences between the groups.

Table 4.1 Demographical data.

DEMOGRAPHICAL DATA	TOTAL (%)
NEONATE GENDER[a]	
Female (n) (%)	104 (43.5)
Male (n) (%)	135 (56.5)
MATERNAL AGE[b]**;** mean (SD), range	28.6 (5.8), 17- 46
MATERNAL ETHNIC ORIGIN[a]	
Asian-Bangladeshi/Indian/Pakistani (n) (%)	117 (49.0)
Asian-other (n) (%)	16 (6.7)
Black African/Caribbean/other (n) (%)	30 (12.6)
White British/Irish/other (n) (%)	68 (28.5)
Other-mixed (n) (%)	8 (3.3)

[a] data only known for [a]239 cases, [b] 237 cases

Table 4.2 Clinical characteristics of the pregnancy, and place and time of collection of the samples of neonatal gastric aspirates.

CLINICAL CHARACTERISTICS	TOTAL (%)
Multiple delivery (n) (%)	19 (7.9)
Vaginal delivery[a] (n) (%)	117 (49.6)
Caesarean section[a] (n) (%)	119 (50.4)
Primiparous[b] (n) (%)	120 (54.8)
Parity (median) (range)	1 (0- 13)
Previous complications[c] (n) (%)	63 (56.3)
Gestational age (wk); mean, (SD)	34.9, (4.9)
Preterm birth (n) (%)	140 (58.3)
Birthweight (g)[d]; mean (SD)	2322.1 (962.9)
Low birthweight[d] (n) (%)	131 (54.8)
Time of ROM (h)[e] (median) (range)	3 (0- 2184)
Prolonged ROM (>24h)[e] (n) (%)	39 (21.8)
Intrapartum antibiotics[f] (n) (%)	35 (17.4)
Smoking during pregnancy[g] (n) (%)	16 (8.4)
SAMPLE COLLECTION[h]	
At the maternal ward (no neonatal ward)	58 (24.2)
Samples collected within 12 hours of birth	189 (80.4)

data only known for: [a]236 cases, [b]217 cases, [c]112cases, [d]239 case, [e]112 cases, [f]201 cases, [g]190 cases, [h]237cases.

Table 4.3 Comparisons between detection of broad-range bacteria and demographical, clinical and sample's data.

	MOLECULAR DETECTION OF BACTERIA		
DEMOGRAPHICAL DATA	**positive (n=98)**	**negative (n=142)**	**p-values***
NEONATE'S GENDER			
Female (n) (%)	48 (49.0)	56 (39.7)[a]	0.185
Male (n) (%)	50 (51.0)	85 (60.3)[a]	
MATERNAL AGE[b]; mean (SD) range	28.5 (5.8)[b]	28.7 (5.8)[c]	0.822
MATERNAL ETHNIC ORIGIN			
Asian-Bangladeshi/Indian/Pakistani (n) (%)	47 (48.5)[b]	70 (49.3)	1.000
Asian-other (n) (%)	7 (7.2)[b]	9 (6.3)	0.798
Black African/Caribbean/other (n) (%)	11 (11.3)[b]	19 (13.4)	0.695
White British/Irish/other (n) (%)	26 (26.8)[b]	42 (29.6)	0.664
Other-mixed (n) (%)	6 (6.2)[b]	2 (1.4)	0.065
CLINICAL CHARACTERISTICS			
Multiple delivery (n) (%)	5 (5.1)	14 (9.9)	0.227
Vaginal delivery/ non-Caesarean (n) (%)	62 (63.3)	55 (39.9)[d]	**0.001****
Primiparous (n) (%)	47 (52.2)[e]	73 (56.6)[f]	0.582
Parity (median) (range)	1 (0- 8)	1 (0- 13)	0.577
Previous complications (n) (%)	22 (48.9)[g]	41 (61.2)[h]	0.245
Gestational age (wk); mean (SD)	35.3 (5.1)	34.6 (4.8)	0.308
Preterm birth (n) (%)	53 (54.1)	87 (61.3)	0.288
Birthweight (g); mean (SD)	2405.1 (944.4)[b]	2265.3 (973.0)	0.271
Low birthweight (n) (%)	47 (48.5)[b]	84 (59.2)	0.113
Time of ROM (h) (median) (range)	11 (0- 2184)[i]	1 (0- 720)[j]	0.111
Prolonged ROM (>24h) (n) (%)	26 (34.7)[i]	13 (12.5)[j]	**<0.001****
Intrapartum antibiotics (n) (%)	16 (20.3)[k]	19 (15.6)[l]	0.448
Smoking during pregnancy (n) (%)	9 (12.3)[g]	7 (6.0)[m]	0.178
SAMPLE COLLECTION			
At the maternal ward (no neonatal ward)	27 (27.6)[b]	31 (21.8)[c]	0.358
Samples collected within 12 hours of birth	77 (81.1)	112 (80.0)	0.869

data only known for: [a]141 cases, [b]97 cases, [c]140 cases, [d]138 cases, [e]90cases, [f]127 cases, [g]73 cases, [h]39 cases, [i]75 cases, [j]105 cases, [k]79 cases, [l]122 cases, [m]122 cases.
*p-values determined using Fisher's Exact test for qualitative variables and 2-tailed Independent-samples T-test for quantitative variables.
** Significant difference as determined by p<0.005.

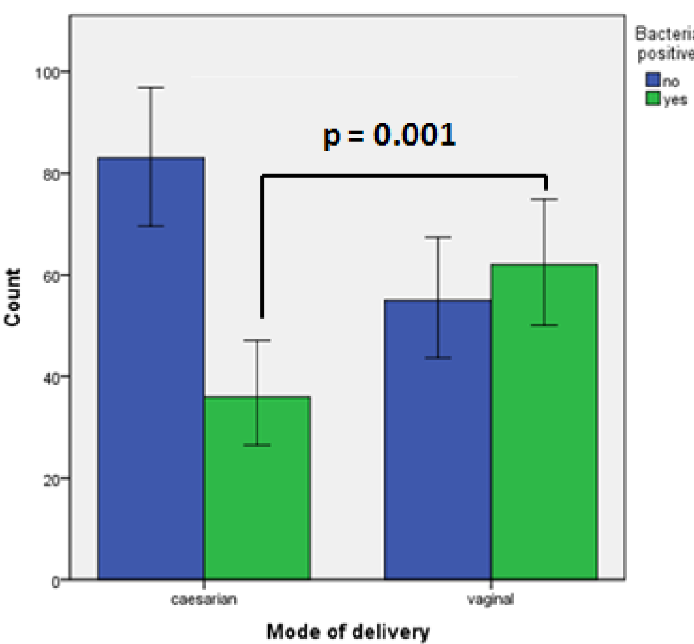

Figure 4.1 Clustered bar diagram representing prevalence of bacteria comparing Caesarean section and vaginal delivery.

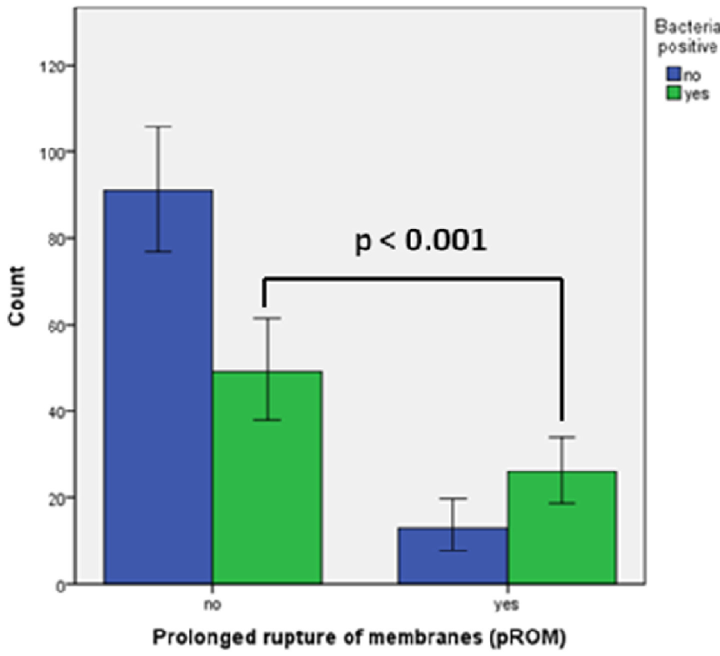

Figure 4.2 Clustered bar diagram representing prevalence of bacteria comparing presence and absence of pROM (>24h).

4.4 Comparisons between detection of most prevalent bacteria and demographical/clinical data.

Interestingly, when the presence of specific bacteria was compared with demographical characteristics (Table 4.4), *S. mitis* group members were less prevalent in mothers originally from South Asian countries (Bangladesh, India and Pakistan) (p= 0.024) and of Black ethnic origin (p= 0.031). Also, an inverse association between *U. urealyticum/parvum* and other-mixed ethnic origin (p= 0.016) was observed.

Presence of the most prevalent species was also compared with vaginal delivery, primiparity, history of previous complications, PTB, LBW, presence of pROM, administration of iATB, smoking during pregnancy, collection of the samples at the maternal wards and samples collected within 12 hours post-delivery (Table 4.5). Bivariate analysis showed associations between vaginal delivery and presence of *S. pneumoniae/mitis/oralis/infantis* (p= 0.025), *U. urealyticum/parvum* (p= 0.003), *Lactobacillus* spp. (p= 0.014), *E. coli* (p= 0.001) and *S. sanguinegens/amnionii* (p= 0.018). Also, presence of *Lactobacillus* spp. was significantly higher in term deliveries (≥37 weeks) (p= 0.011); and *Lactobacillus* spp. (p= 0.006) and G. *vaginalis* (p= 0.046) were more prevalent in non-low birthweight. Moreover, women that presented with pROM were more likely to present members of the *S. mitis* group (p= 0.046) and *E. coli* (p= 0.006). It was also observed that women who received antibiotics during delivery presented with a significantly higher prevalence of *S. agalactiae* (p= 0.009). Similarly, presence of *U. urealyticum/parvum* (p= 0.018), *Lactobacillus* spp. (p= 0.018), G. *vaginalis* (p= 0.002) and *S. sanguinegens/amnionii* (p= 0.040) was associated with collection of the samples at a maternal ward rather than during neonate hospitalization in the neonatal wards. Finally, an association was shown between *Pseudomonas* spp. and samples collected more than 12 hours post-delivery (p= 0.042). Comparisons of variables that showed associations are represented in clustered bar diagrams in Figure 4.3.

Table 4.4 Comparisons between presence of specific bacteria and demographical characteristics (p-values).

DEMOGRAPHICAL DATA	GENERAL BACTERIA	*S. mitis* group	*Ureaplasma* spp.	*Lactobacillus* spp.	*E. coli*	*Pseudomonas* spp.	*S. agalactiae* (GBS)	*G. vaginalis*	*Sneathia* spp.
NEONATE'S GENDER (male or female)[a]	0.185	0.306	1.000	0.807	1.000	0.135	1.000	0.338	0.735
MATERNAL AGE[b]	0.822	0.159	0.181	0.514	0.442	0.809	0.981	0.829	0.075
MATERNAL ETHNIC ORIGIN[a]									
Asian-Bangladeshi/Indian/ Pakistani	1.000	0.024**α	0.808	1.000	0.279	0.564	0.765	1.000	0.500
Asian-other	0.798	1.000	1.000	1.000	1.000	0.573	0.541	0.507	1.000
Black African/Caribbean/other	0.695	0.031**α	1.000	1.000	1.000	1.000	1.000	1.000	1.000
White British/Irish/other	0.664	0.825	0.786	1.000	0.122	0.517	0.733	0.729	0.123
Other-mixed	0.065	0.602	0.016**α	0.471	0.073	0.342	0.318	0.293	1.000

[a] data not known for 1 case, [b] data not known for 3 cases; p-values determined using Fisher's Exact test; **Significant difference as determined by $p<0.05$.
α Prevalence associated with no observation of the clinical variable.

Table 4.5 Most prevalent bacteria in NGA and associations with clinical variables.

	Bacteria most prevalent in NGA	No. (%)^	COMPARISONS with clinical variables (p-values)*									
			vaginal delivery	**pROM**	**primi-parous**	**previous complic.**	**PTB**	**LBW**	**iATB**	**smoking**	**maternal ward**	**collected more 12h post-delivery**
1	*Streptococcus pneumoniae/mitis/ oralis/infantis*	27 (28)	**0.025****	**0.046****	0.672	0.360	0.148	0.064	0.376	1.000	0.814	0.287
2	*Ureaplasma urealyticum/parvum*	18 (18)	**0.003****	0.106	0.804	1.000	1.000	1.000	0.410	1.000	**0.018****	1.000
3	*Lactobacillus spp.*	18 (18)	**0.014****	0.647	0.629	1.000	**0.011**α**	**0.006**α**	0.192	1.000	**0.018****	0.208
4	*Escherichia coli*	14 (14)	**0.001****	**0.006****	0.259	0.084	0.269	0.053	1.000	0.414	0.109	0.723
5	*Pseudomonas* spp.	12 (12)	0.251	0.613	0.772	0.166	1.000	0.773	0.351	0.465	0.168	**0.042****
6	*Streptococcus agalactiae*	11 (11)	0.539	1.000	0.352	0.384	0.056	0.070	**0.009****	1.000	0.305	0.454
7	*Gardnerella vaginalis*	10 (10)	0.058	0.389	1.000	1.000	0.098	**0.046**α**	0.008	1.000	**0.002****	0.690
8	*Sneathia sanguinegens/amnionii*	9 (9)	**0.018****	0.176	0.473	1.000	0.739	0.518	0.608	0.465	**0.040****	1.000

^ Determined as percentage of the samples positive to the universal bacterial 16S rDNA primers used (out of 98). NGA, Neonatal gastric aspirates; pROM, Prolonged rupture of membranes; PTB, Preterm birth; LBW, Low birthweight; iATB, intrapartum antibiotics. *p-values determined using Fisher's Exact test, **Significant difference as determined by $p<0.05$. α Prevalence associated with no observation of the clinical variable.

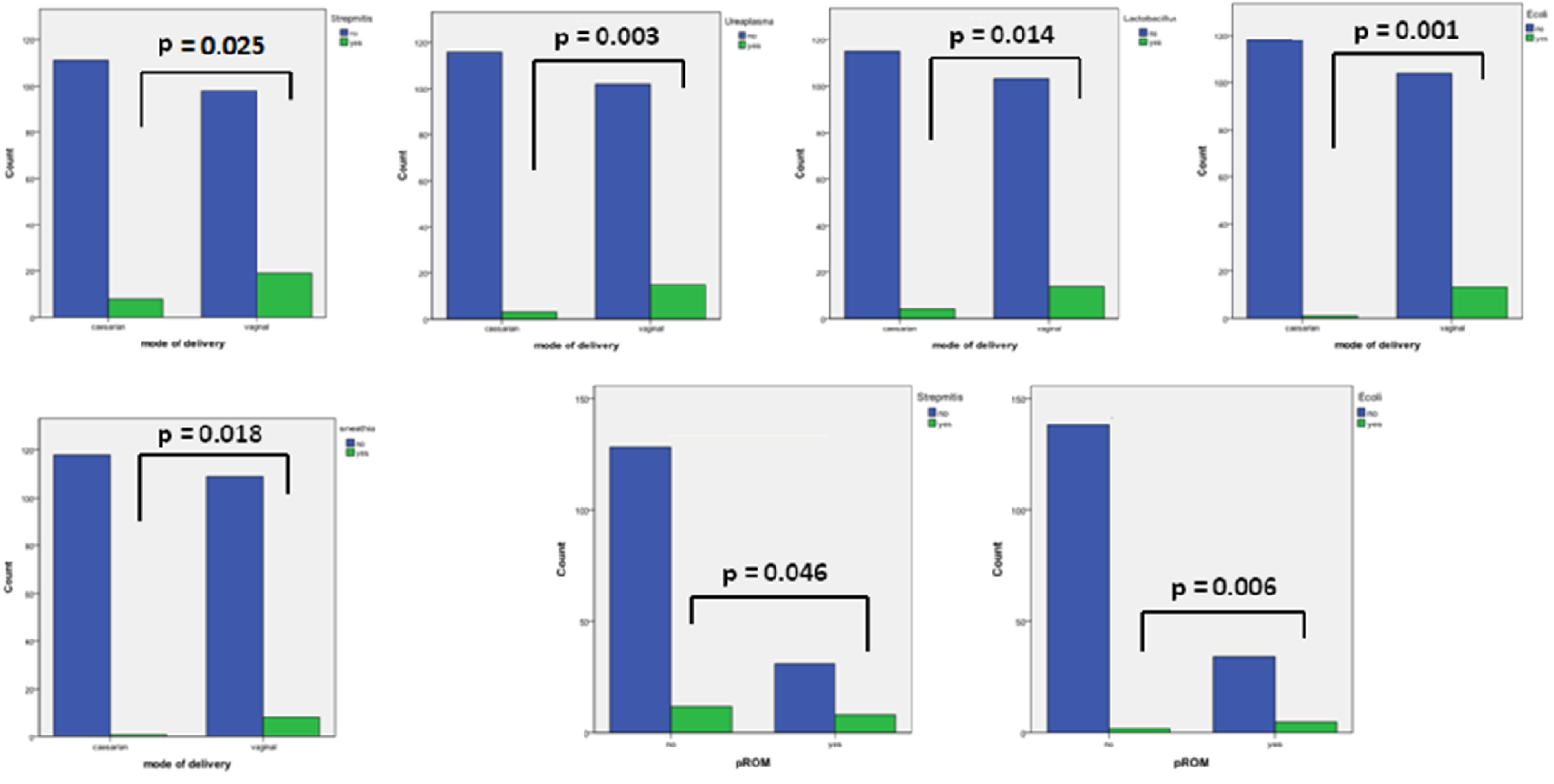

Figure 4.3 Clustered bar charts representing associations between most prevalent bacteria in NGA and clinical variables (only those showing significant associations are represented here). Strepmitis, *Streptococcus pneumoniae/mitis/oralis/infantis*; Ureaplasma, *Ureaplasma urealyticum/parvum*; Lactobacillus, *Lactobacillus* spp.; Ecoli, *Escherichia coli*; Sneathia, *Sneathia sanguinegens/amnionii*.

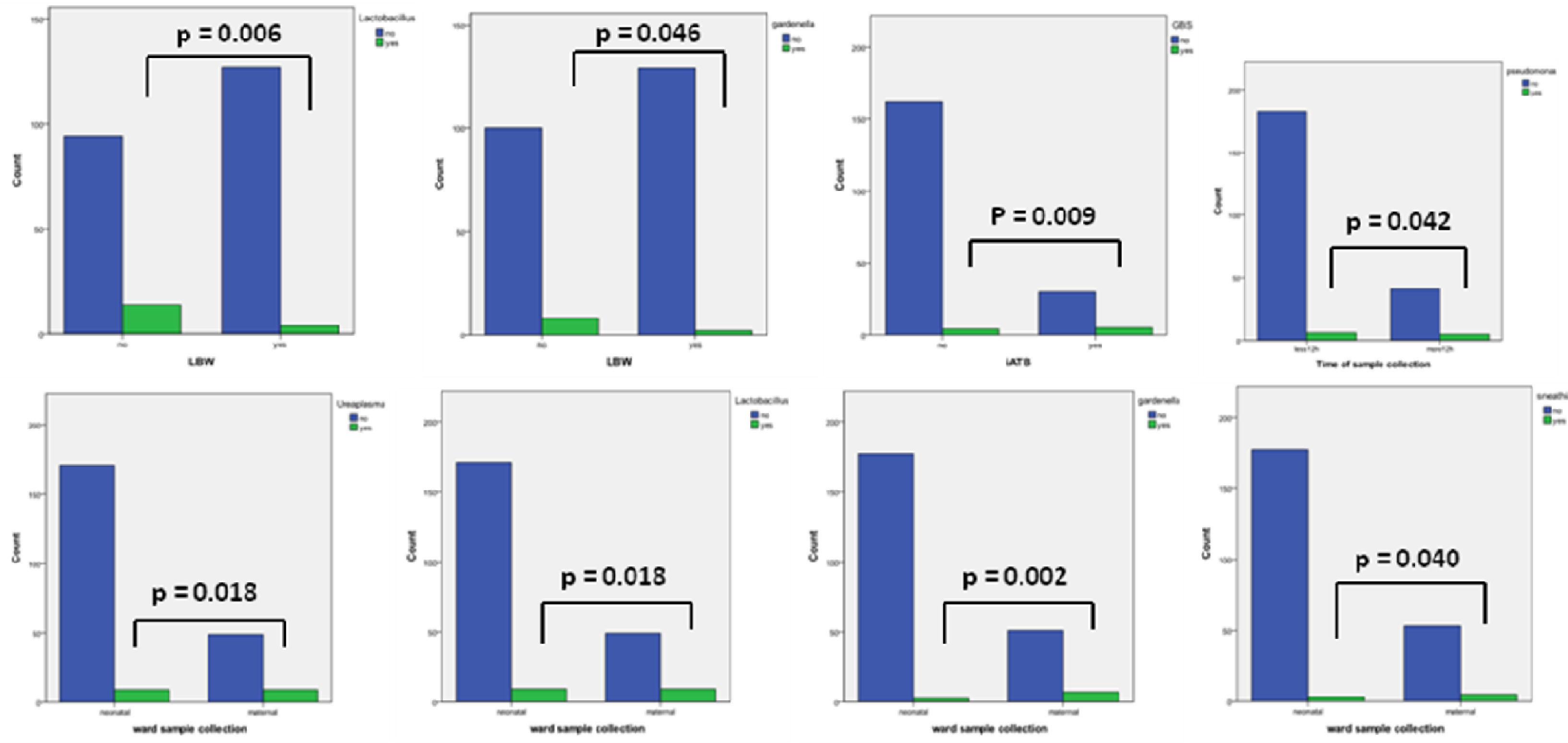

Figure 4.3 (cont.) Lactobacillus, *Lactobacillus* spp.; gardenella, *Gardnerella vaginalis*; GBS, *Streptococcus agalactiae*; pseudomonas, *Pseudomonas* spp.; Ureaplasma, *Ureaplasma urealyticum/parvum*; Sneathia, *Sneathia sanguinegens/amnionii*.

4.5 Multivariate analyses.

When analyzing demographical and clinical variables using a multivariate logistic regression, few of the associations observed with the bivariate analyses remained. A non-white variable was included in the logistic regression model. Gestational age was also included in this model, instead of PTB, since this variable also showed association with the *S. mitis* group not observed with the binomial PTB; and also, to avoid conflicts with LBW because most of the LBW may also be PTB. Tables 4.6 and 4.7 summarize the results of the multivariate logistic regression including in the model those variables that showed associations with the bivariate tests.

The multivariate analysis confirmed the associations observed for molecular detection of broad-range bacteria in NGA. Detection positive showed a significant association with both vaginal delivery (OR= 2.3, 95% CI:1.2- 4.5) and pROM (OR=4.3, 95% CI: 1.7- 10.3) (Table 4.6; Figure 4.4).

Due to the low number of cases for each specific bacterium, only certain species could be analyzed by logistic regression (Table 4.7). Associations were observed between vaginal delivery and pROM and presence of *S. pneumoniae/mitis/oralis/infantis* (OR=3.23, 95% CI: 1.0- 10.3 and OR=5.17, 95% CI: 1.6- 16.8 respectively) (Figure 4.5). Also, administration of iATB was predictive for detection of *S. agalactiae* in the samples (OR=7.27, 95% CI: 1.6- 32.7). Finally, even not statistically significant (p= 0.062), *U. urealyticum/parvum* was eight times more likely to be observed in neonates that were delivered vaginally (95% CI: 0.9- 73.2).

Table 4.6 Multivariate logistic regression for detection of bacteria in NGA using broad-range molecular techniques.

VARIABLES	DETECTION OF BACTERIA			
	p-value	OR	95% C.I.	
			Lower	Upper
non-white	0.275	0.66	0.31	1.39
vaginal delivery	**0.015****	**2.31**	**1.17**	**4.55**
gestational age (wk)	0.360	1.06	0.94	1.19
LBW	0.825	0.88	0.29	2.69
pROM	**0.002****	**4.23**	**1.73**	**10.34**
iATB	0.543	1.30	0.56	3.03
maternal ward	0.256	0.53	0.18	1.59
sampled>12h	0.601	0.79	0.32	1.92

OR= odds ratio; LBW, Low birthweight; pROM, prolongued of membranes; iATB, intrapartum antibiotics. **Significant difference as determined by $p<0.05$.

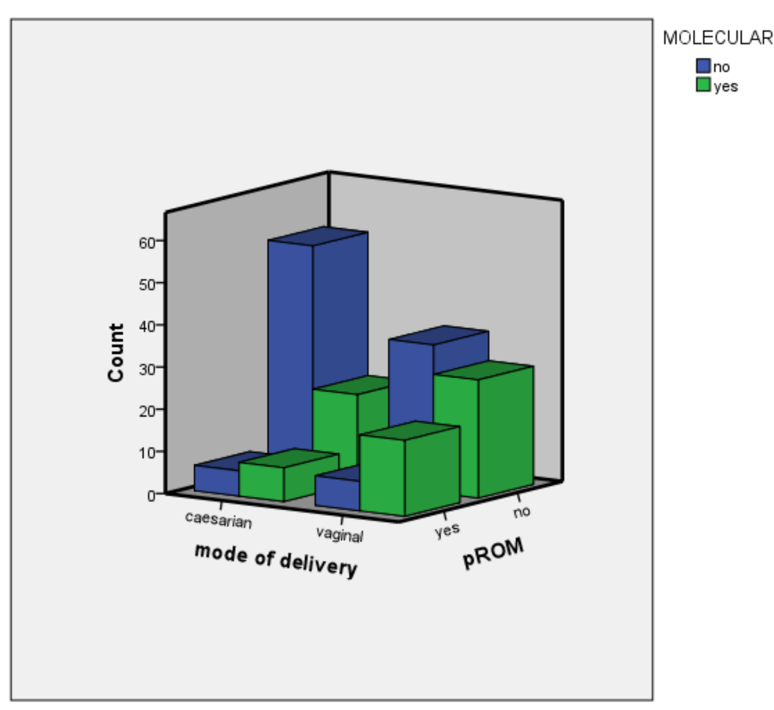

Figure 4.4 Clustered bar chart representing prevalence of bacteria using molecular techniques comparing mode of delivery and presence of pROM.

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Table 4.7 Multivariate logistic regression for identification of species-specific bacteria in NGA.

VARIABLES	***S. mitis* group**				***U. urealyticum/parvum***				***S. agalactiae***			
	p-values	OR	95% C.I.		p-values	OR	95% C.I.		p-values	OR	95% C.I.	
			Lower	Upper			Lower	Upper			Lower	Upper
non-white	0.880	0.91	0.28	2.99	0.776	1.29	0.23	7.26	0.271	0.42	0.09	1.95
vaginal	**0.048****	**3.23**	**1.01**	**10.35**	**0.062**	**8.11**	0.90	73.16	0.327	0.46	0.10	2.15
gestational age (wk)	0.180	1.15	0.94	1.42	0.881	0.98	0.78	1.24	0.712	1.06	0.76	1.48
LBW	0.801	1.26	0.21	7.68	0.645	1.81	0.14	22.90	0.611	0.46	0.02	9.29
pROM	**0.006****	**5.17**	**1.58**	**16.85**	0.890	1.14	0.18	7.08	0.727	0.71	0.11	4.72
iATB	0.280	1.96	0.58	6.66	0.545	1.71	0.30	9.69	**0.010****	**7.27**	**1.62**	**32.66**
maternal ward	0.170	0.21	0.02	1.95	0.323	2.84	0.36	22.44	0.528	1.79	0.29	11.04
sampled>12h	0.822	1.16	0.31	4.33	0.507	1.83	0.31	10.79	0.676	0.61	0.06	6.27

OR= odds ratio; LBW, Low birthweight; pROM, prolonged rupture of membranes; iATB, intrapartum antibiotics

**Significant difference as determined by $p<0.05$.

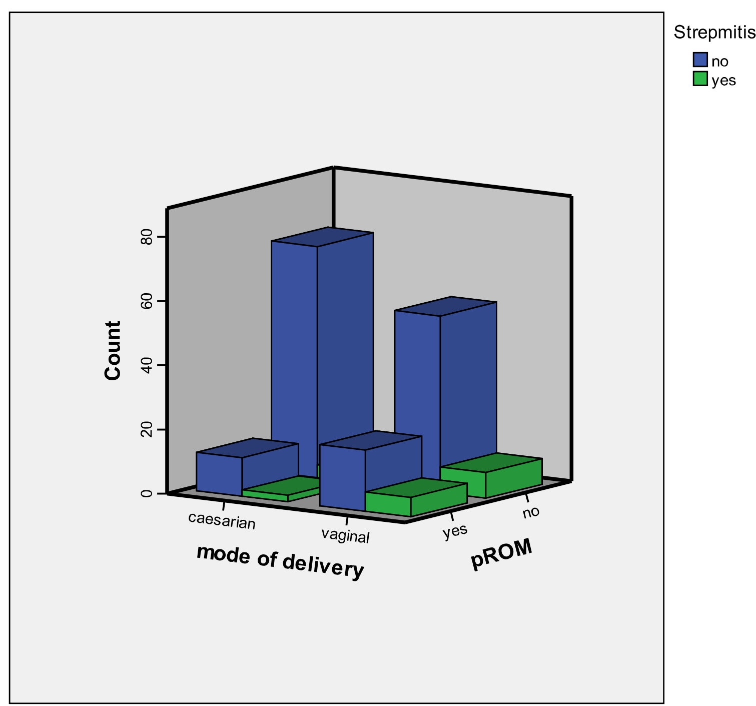


Figure 4.5 Clustered bar chart representing prevalence of *S. mitis* group comparing mode of delivery and presence of pROM.

4.6 Discussion.

The bivariate comparative analysis performed between prevalence of the broad range of bacteria present in the samples and the clinical and non-clinical variables supported the hypothesis that many of the samples may contain transient bacteria probably acquired from the birth canal during vaginal delivery or as a result of the prolonged exposure to the external environment. Nevertheless, even though bacteria were more prevalent in vaginal deliveries, most of the species identified in this study have also been observed in Caesarean sections, the only exceptions being *S. epidermidis* (n=6) and *A. christensenii* (n=4), which are frequent colonizers of the female genito-urinary tract (Collins et al., 1999). Similarly, few bacteria were observed only in cases of pROM (*S. constellatus/intermedius/anginosus* and *P. aeruginosa/otitidis*), while the opposite was observed for *S. epidermidis*, *Peptoniphilus* sp., *P. fluorescens*, *S. salivarius/vestibularis* and *F. nucleatum* which were only present when rupture of membranes occurred at less than 24 hours before

delivery. The former contradicts the observation made by Cahill *et al.* (2005) who reported a high prevalence of *F. nucleatum* in cases of pROM. However, due to their low prevalence in the samples, it is not possible to make any accurate conclusion.

Further analysis was also performed to compare prevalence of specific bacteria with the clinical observations and sample characteristics. Numerous bacteria showed association with vaginal delivery when using the bivariate analysis. However, similar species were also prevalent in the samples collected at the maternal ward, indicating possible contamination due to the difficulty of achieving sterile conditions during sample collection at these wards. After adjusting for a range of variables, statistical associations with vaginal delivery and pROM could only be confirmed for the members of the *S. pneumoniae/mitis/oralis/infantis*. Also, although not statistically significant, *U. urealyticum* was observed to be eight times more prevalent in vaginal deliveries.

In this study, *S. pneumoniae/mitis/oralis/infantis* were the most prevalent bacteria (28%) in samples of NGA. Unfortunately, members of the *S. mitis* group are genetically closely related to each other and they were not able to be differentiated in this study. Although '*viridans*' *streptococci* (*S. mitis* and *S. oralis*) are of low pathogenity, *S. mitis* has been reported to cause early-onset neonatal infection and neonatal death as a result of its resistance to antibiotics (Adams and Faix, 1994). High prevalence of *S. mitis* group components associated with the female genito-urinary tract, the amniotic environment, or early-onset neonatal infections has not been reported before. On the other hand, *S. pneumoniae* is one of the major bacterial pathogens worldwide causing bacteraemia and community-acquired infections in infants (Selva et al., 2010). *U. urealyticum* has been previously implicated in several forms of APO (Waites et al., 2005). However, carriage of *U. urealyticum* in the women's genitourinary tract will not necessarily trigger an APO (Waites et al., 2005, Govender et al., 2009), nor will treatment of the infection alter the occurrence of prematurity (Romero et al., 1989a). Prevalence of *Ureaplasma* species in NGA is likely to derive from the birth canal during delivery. Although these bacteria may originate from the vagina, their possible contribution to neonatal infections should be

further analyzed. Interestingly, the non-white ethnic origin group showed a protective effect for presence of *S. pneumoniae/mitis/oralis/infantis* and *U. urealyticum*, as most individuals presenting these pathogens were from white ethnic origin.

Also, a significantly higher prevalence of *S. agalactiae* (GBS) was observed in relation to the administration of iATB. Logistic regression analysis confirmed this observation (OR=7.3; 95% C.I.: 1.6- 32.7). One explanation might be that samples of NGA are routinely taken from those cases with a history of infection with GBS. Intrapartum antibiotic prophylaxis has been instituted as a routine hospital procedure since the mid-1990s, successfully decreasing the incidence of early-onset GBS septicaemia in more than 65% of neonates (Schrag et al., 2000, Ohlsson and Shah, 2009). However, iATB administration may disrupt the perinatal microbiota, allowing *S. agalactiae* antibiotic-resistant strains and other pathogenic bacteria to proliferate.

Another interesting observation was the presence of *Lactobacillus* spp. in association with neonates delivered at term and presenting a birthweight ≥ 2500 g. *Lactobacillus* spp. are usually observed dominating the microbiota in the vagina and cervix of the healthy female reproductive system and may prevent colonization of more pathogenic species (Boris and Barbés, 2000, Wilson, 2008). Similarly, significantly higher presence of *G. vaginalis* was observed when the birthweight was ≥ 2500 g and in samples collected at the maternal ward. Unfortunately, these associations were not able to be analyzed in a multivariate analysis due to the low number of positive samples.

Moreover, the most common pathogens recognized as causing nosocomial bloodstream infection in NICU (*E. coli, Staphylococcus* spp., *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*) have also been identified in the samples. *E. coli* was associated with both vaginal delivery and pROM. It is one of the most common bacterial species responsible for neonatal infections and is increasingly implicated in nosocomial outbreaks in NICU due to multidrug-resistant strains. Finally, an association between *Pseudomonas* spp. in NGA samples collected >12 hours post-

delivery was observed.

Although no associations were observed between general or specific infection and PTB, LBW, or previous complications, identification of the bacteria in the samples may still indicate colonization of the amniotic cavity and fetus, and therefore identify a potential infectious agent. In general, the observations in this study agree with the ones previously reported, which contributes to the plausibility that the samples of NGA could represent a valuable specimen in well-controlled studies. In particular, these samples may be suitable to investigate the presence of potential oral pathogens in association with perinatal infections and APO. However, clinical observations should be carefully considered when analysing the results.

CHAPTER 5

QUANTITATIVE ANALYSIS OF PERIODONTAL PATHOGENS IN NEONATAL GASTRIC ASPIRATES

5.1 Introduction.

Maternal periodontal infection has been associated with APO in several epidemiological and interventional studies (Offenbacher et al., 1996, Dasanayake, 1998, Jeffcoat et al., 2001, López et al., 2002a, Offenbacher et al., 2006a, Jeffcoat et al., 2010). Clinical findings have also reported presence of possible oral bacteria in the amniotic environment of women who present with an APO, suggesting the possibility of an oral-uterine translocation (Douvier et al., 1999, Andrés et al., 2002, Cahill et al., 2005, León et al., 2007, Han et al., 2010). However, this area remains controversial since other studies have not found the same association (Mitchell-Lewis et al., 2001, Davenport et al., 2002, Michalowicz et al., 2006). The reasons for this variability may to be due to the great differences between study designs and the definitions used for PD and APO (Manau et al., 2008). Undoubtedly, the strongest evidence supporting the possible association between oral bacteria and APO has been provided by animal studies in which oral bacteria were shown to be able to translocate via bloodstream, cross the placental membranes, and specifically invade the amniotic cavity resulting in preterm delivery, fetal growth restriction, and fetal death (Collins et al., 1994, Han et al., 2004, Liu et al., 2007, Fardini et al., 2010).

Periodontitis is a common inflammatory disease initiated primarily by a small subset of opportunistic gram-negative pathogen inhabitants of the subgingival plaque. The microorganisms implicated in PD possess numerous potent virulence factors that may allow them to survive, translocate, and colonise distant non-oral sites. Oral pathogens that are able to translocate and invade the amniotic cavity may be present in the samples of NGA investigated in this study. Hence, a quantitative analysis of selected periodontal bacteria was performed. The quantitative-PCR analysis ensures a higher sensitivity which can detect even those species present in low levels. Moreover, the levels of bacteria would provide an estimate of the clinical relevance regarding their presence in the samples.

The main periodontal pathogens that have been associated with severity and progression of PD, as defined by the World Workshop in Clinical Periodontics

(1996), were included in the analysis (*A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia*). These species have shown mechanisms of invasion and translocation that may facilitate spread through the bloodstream. Besides, they may be able to avoid the immune response, and/or have been detected in sites distant from the oral cavity. For example, *A. actinomycetemcomitans* has been implicated in the aetiology of infective endocarditis, and translocation may occur due to its ability to attach to collagen (Tang et al., 2008). *P. gingivalis* has been involved in cardiovascular diseases and is able to inactivate Toll-like receptors by the release of gingipains which inhibit the host cell recognition (Kishimoto et al., 2006).

Also, quantification of potential oral pathogens that have been identified in this study in the samples of NGA (*F. nucleatum* and *G. elegans*) is reported here. *F. nucleatum* is one of the most prevalent species in the oral cavity in health and disease. This pathogen is also one of the most common bacterial species isolated from AF in cases of PTL and intact membranes, which explains the great interest this microorganism have received in several studies investigating its contribution to preterm delivery and other APO (Hill, 1998, Madianos et al., 2001, Han et al., 2004, Liu et al., 2007, Han et al., 2009). *G. elegans* was detected in the samples of NGA in this study. This bacterium is a member of the human oral microbiota and also thought to be a potent pathogen in endocarditis (Ohara-Nemoto et al., 2005). Moreover, *G. elegans* has shown to inhibit proliferation of human peripheral blood mononuclear cells and this mechanism appears to enable the microorganism to cause infective endocarditis (Kanamoto et al., 2007).

5.2 Selection of species-specific primers for q-PCR analysis.

Species-specific forward and reverse primers were used if possible, although in some cases a combination of specific and universal primer was preferred to ensure technique efficiency by amplifying short length fragments. In all the cases, specificity and sensitivity of each set of primers was confirmed against a list of pathogens as previously detailed (Section 2.14). The set of primers used for universal amplification have a demonstrated advantage over other possible options as shown in

a previous study (Yu and Morrison, 2004). Also, in this study, it was demonstrated that the primer set 357F-518R was more efficient than the primer set 27F-357R.

5.3 Growth of bacteria, identification, counts and determination of culture absorbance, and DNA extraction.

A. actinomycetemcomitans, P. gingivalis, T. forsythia, F. nucleatum, and *G. elegans* were grown in this study as previously described (Section 2.6). A colony obtained from the pure culture was subjected to end-point PCR using the set of primers detailed in Table 2.5 (Chapter 2) and successfully identified using direct sequencing. The bacterial counts and culture absorbance were determined in order to obtain a stock of cells from known concentration. Table 5.1 shows the OD600 corresponding to a pre-determined bacterial count (in CFU/ml) for each strain. DNA extraction was then performed on one aliquot containing 200 µl of the stock using the same protocol described for the samples without the need for mucolyse digestion. Remaining stocks were stored frozen at -70ºC.

Table 5.1 Bacteria grown in this study and corresponding OD600 values for predetermined CFU/ml.

Bacterial strains	CFU/ml	OD600
F. nucleatum	10^9	1.756
A. actinomycetemcomitans	10^9	0.237
P. gingivalis	10^{10}	0.287
T. forsythia	10^9	1.510
G. elegans	10^8	0.368

CFU, colony-forming units; OD600, optical density at a wavelength of 600 nm.

5.4 Dilution series of template and construction of standard curves for q-PCR.

Standard curves for absolute quantification were prepared from a dilution series of template of known concentration. Genomic DNA from each target bacteria was used as a template for the construction of standard curves. The standards were run following the protocol described below for q-PCR analysis (Chapter 2). For the construction of standard curves the LightCycler® 480 software was used. All the standard curves included dilutions from 10^2 to 10^7. In all cases the slopes were within the acceptable range (between -3.0 and -3.6; –3.3 for 100% PCR efficiency (Efficiency = [10(-1/slope)] – 1)). Standard curves characteristics are detailed in Table 5.2.

One difficulty to accurately determine the levels of general bacteria in the samples was related to the differences in numbers of *rrn* operons per bacteria. The number of operons per bacterial genome has been demonstrated to vary from one to 15, and this value is only known for a few species (Lee et al., 2009). This limitation could be successfully avoided by constructing standard curves using known concentration of bacteria grown in pure culture to calculate levels of specific bacteria. However, it was not possible to calculate the levels of general bacteria due to the diversity of the samples (unknown numbers of species and their relative abundance). In this study, in order to overcome this limitation, an average number of five copies of the gene were allocated to calculate general bacteria numbers in the samples. This was calculated as the average copy number of *rrn* encoding-genes in the range of bacteria observed in NGA (only the bacteria with known operon number were included). The operon copy number was obtained from The Ribosomal RNA Operon Copy Number Database (Lee et al., 2009) and varied between one and 10.

Table 5.2 Characteristics of the standard curves constructed in this study for quantitative analysis of oral bacteria.

BACTERIA	PRIMERS	Efficiency	Slope	Error
General bacteria	357F-518R	1.964	-3.411	0.0233
F. nucleatum	Fn1F-Fn2R	1.951	-3.445	0.0119
A. actinomycetemcomitans	AaF-C11R	1.989	-3.348	0.0069
P. gingivalis	Pg2F-Pg2R	1.944	-3.465	0.0166
T. forsythia	Tf2F-357R	1.938	-3.480	0.0100
G. elegans	GeF-357R	1.959	-3.424	0.0467

5.5 Absolute amounts of general bacteria and specific periodontal pathogens using q-PCR.

A total of 201 samples of NGA were included. The number of samples analysed per bacterium targeted varied mainly due to availability of the sample. One hundred and seventy-five samples were analysed with the universal primer set, 10 of which did not produce a result. Samples consistently not producing a result after three repetitions were considered negative and were not included in the analysis. Similarly, the number of samples evaluated for each specific bacterium was as follows (the numbers of negative samples is shown in brackets): *F. nucleatum*, 191 (28); A. *actinomycetemcomitans,* 101 (4); *P. gingivalis,* 145 (45); *T. forsythia*, 99 (34); *G. elegans*, 102 (44).

Figure 5.1 illustrates absolute levels of the bacteria in the samples of NGA. The levels of general bacteria ranged between 4.15E-01 and 5.18E+06 cells/ml; the mean level was calculated as 1.04E+05 cells/ml (median= 1.51E+03). It could be observed that in all the cases, levels of general bacteria were higher than the levels of each specific bacterium tested. *A. actinomycetemcomitans* and *T. forsythia* were observed at very low levels in the samples evaluated, reaching a maximum of 4.65E+01 and 1.57E+01 cells/ml respectively. These levels may represent the background of the reaction since similar levels were also observed in the controls (both H_2O and PCR

mix only). *P. gingivalis* (mean= 9.33E+00, median= 5.22E-02) was also present at low levels, although two of the samples produced high detection levels of the bacteria in the sample (2.98E+02 and 1.75E+02 cells/ml). Interestingly, the sample that contained the highest level of *P. gingivalis* also harboured similar levels of *F. nucleatum* (1.51E+02 cells/ml). Overall, the samples analysed with primers specific for *F. nucleatum* contained high levels of these bacteria, reaching up to 2.32E+03 cells/ml. The mean level of *F. nucleatum* was determined as 1.09E+01 cells/ml (median= 2.17E+01). Also, few samples contained high levels of *G. elegans*, with a maximum of 5.83E+02 cells/ml (mean= 4.04E+01; median=5.48E+00) being observed. Bacterial identity was confirmed using direct sequencing of the end-point PCR products for *F. nucleatum, P. gingivalis* and *G. elegans*.

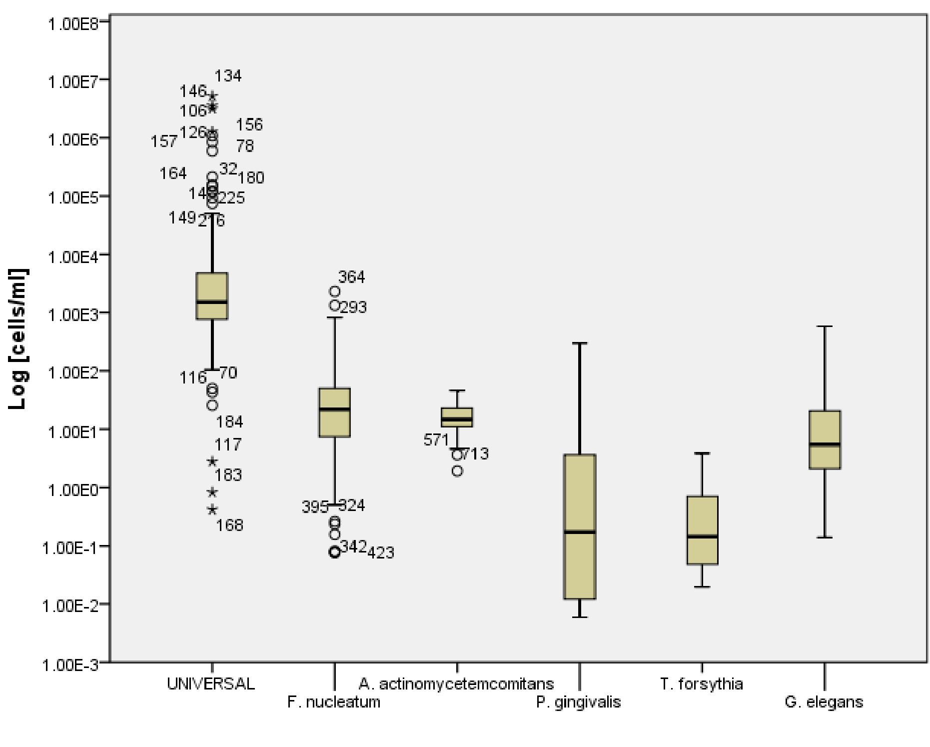


Figure 5.1 Box-plots representing absolute levels of universal and specific bacteria in neonatal gastric aspirates as determined using q-PCR analysis.

5.6 Relative amounts of potential oral bacteria in neonatal gastric aspirates.

Levels of general (universal) bacteria were used to determine relative amounts of specific bacteria in the samples. Relative amount was calculated as a percentage of the specific bacteria out of the total bacterial load. Percentage of *F. nucleatum* ranged from less than 0.01 to 49.5% of the total bacterial load (mean= 5.1%; median= 1.2%). *P. gingivalis* was observed in high proportion in two samples representing 11.7% and 20.3%; the percentage of *P. gingivalis* in the remainder of the samples analysed ranged from <0.01% (in 48 samples) to 4.5% with a mean value of 0.6% (median= <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 to the rest of the samples whose relative amounts were not higher than 0.2%. Percentages of *T. forsythia*, however, were always <0.01%. Finally, the percentage of *G. elegans* in the samples was determined as being up to 34.8% (mean= 3.0%; median= 0.3%) (Figure 5.2).

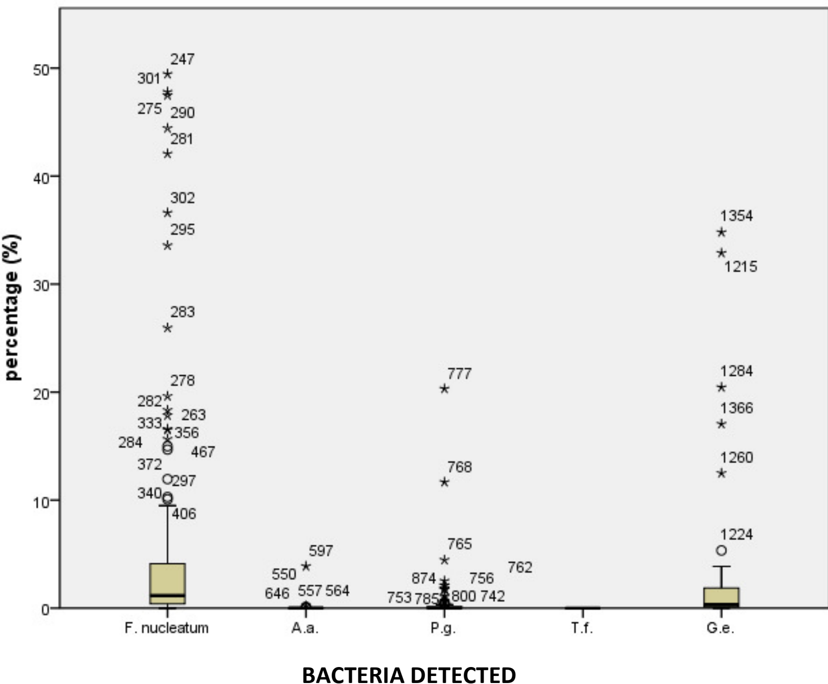


Figure 5.2 Box-plots representing relative amount of periodontal bacteria in NGA calculated as percentage of the total bacterial load in the samples.

5.7 Prevalence of potential oral bacteria in neonatal gastric aspirates using q-PCR analysis.

Prevalence was determined using a designated detection limit, which was determined by comparisons with the end-point PCR results. Determination of prevalence should allow confirmation of the associations observed with the initial PCR analysis using a more sensitive technique. It was mainly performed in order to identify those cases to be included in downstream analysis, in which end-point PCR was necessary.

Prevalence was determined based upon detection limits observed with the end-point PCR results. In general, end-point PCR was positive when samples contained more than 100 cells/ml of a specific bacterium by q-PCR. A sensitivity and specificity test demonstrated that presence of *F. nucleatum* was, respectively, 90% and 91% accurate when using the end-point PCR results as the gold standard. One hundred bacterial cells has also been determined previously as the detection limit for identification of periodontal bacteria by real-time PCR (meridol®Perio Diagnostics) (Jervøe-Storm et al., 2005). Therefore, samples that contained levels higher than 100 cells/ml were considered positive in this study. Nevertheless, the same consistency was not observed with the results obtained with the universal primers. This may be due to the higher sensitivity inherent to the q-PCR technique compared to the end-point PCR method; and also because of the limitation derived from the *rrn* operon number. In this study, detection limit was selected as 500 cells/ml since the highest sensitivity (87.8%) was observed when comparing the data with the end-point PCR results as the gold standard (specificity was 17.6%).

Using this criterion, general bacteria were considered to be present in 140 of 175 (80%) samples. An average of 1.23E+05 cells/ml (median= 1.87E+03) was observed in the positive samples, compared to 2.03E+02 cells/ml (median= 1.31E+02) in the negative samples. Specific bacteria that were considered positive using the 100 cells/ml detection limit were *F. nucleatum*, *P. gingivalis* and *G. elegans*. Twenty-six samples out of 163 (15.9%) were positive for *F. nucleatum* (mean= 4.41E+02; median= 2.51E+02). Two samples out of 100 (2.0%) were positive for *P. gingivalis*

(mean= 2.37E+02; median= 2.37E+02) and five samples out of 58 (8.6%) were positive for *G. elegans* (mean= 3.46E+02; median= 2.63E+02). Levels of bacteria have been illustrated by prevalence in Figure 5.3.

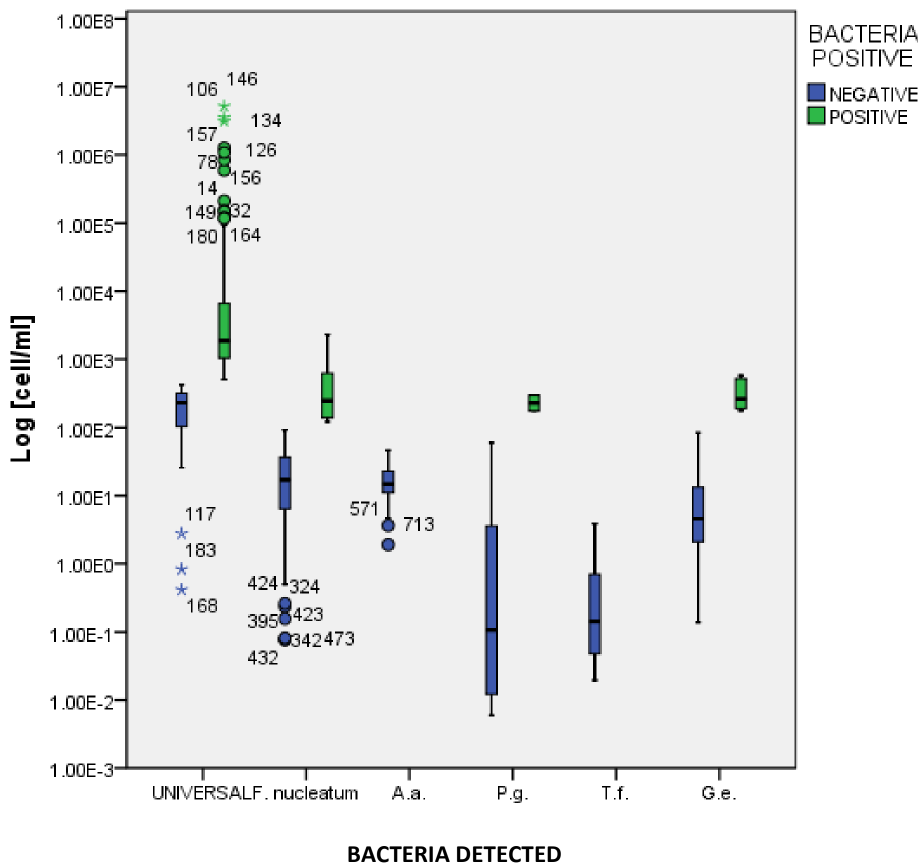


Figure 5.3 Clustered box-plot representing levels of bacteria in positive and negative samples as determined by the detection limit set in this study.

5.8 Statistical analyses.

The following tables and figures summarize the statistical analyses performed with the data obtained with the quantitative analysis. Comparisons between prevalence of bacteria and demographical and clinical data were only available for general bacteria and *F. nucleatum* when using the q-PCR analysis (Table 5.3). Interestingly,

associations with presence of general bacteria (> 500 cells/ml) were observed with both PTB (p= 0.002) and LBW (p= 0.004) (Table 5.3 and Figure 5.4). This was confirmed with an inverse association when comparisons were made with gestational age (p= 0.001) and birthweight (p= 0.006). The Fisher's exact test and the independent t-test were used to analyse the qualitative and the quantitative data respectively (Table 5.3). Furthermore, associations were performed for the type of PTB and LBW presented. Type of complication was evaluated using ANOVA followed by Tuckey analyses. Preterm birth was classified as mild PTB (32-36 weeks), very PTB (28-31 weeks), and extremely PTB (< 28 weeks). Low birthweight was classified as mild LBW (1500-2499 g), very LBW (1000-1499 g) and extremely LBW (< 1000 g) (Figure 5.4). Associations were observed only between term delivery and very PTB (p= 0.050); and term delivery and extremely PTB (p= 0.010). These results demonstrate that term and mild preterm neonates present higher prevalence of bacteria compared to the very preterm and term neonates.

Contrary to the observations made with the broad-range PCR analyses, associations between prevalence of general bacteria and mode of delivery or pROM were not observed with the q-PCR analysis (Table 5.3). However, when absolute levels of general bacteria rather than prevalence was evaluated a significant association with vaginal delivery (p = 0.037) could be observed but not with pROM. Finally, a multivariate analysis including gestational age and LBW and other important variables, confirmed association between presence of bacteria using q-PCR approach and gestational age (p= 0.009; OR=1.17; 95% C.I.: 1.0-1.3) (Table 5.4).

Associations between prevalence of *F. nucleatum* in the samples and clinical and non-clinical variables are detailed in Table 5.3. An association with multiple deliveries (twins) was observed (p= 0.037). Interestingly, associations with non-white origin (p= 0.062) and LBW (p= 0.065) were close to significance; these are represented in Figure 5.5. However, none of these was maintained using a multivariable approach. Absolute and relative levels of *F. nucleatum* in NGA also showed few associations using the bivariate analyses. Absolute amounts were associated with twin delivery (p= 0.019) and samples collected at the maternal ward

(p= 0.033), and relative amounts were associated with women presenting pROM (p= 0.001) (Tables 5.5 -5.6).

Table 5.3 Comparisons between prevalence of general bacteria and *F. nucleatum* in NGA as determined by q-PCR analysis with demographical and clinical data.

COMPARISONS BETWEEN PREVALENCE AND VARIABLES (p-values)*

DEMOGRAPHICAL DATA	**GENERAL BACTERIA (> 500 cells/ml)**	***F. nucleatum* (>100 cells/ml)**
Neonate's gender (M or F)[a]	0.791	0.531
Maternal age at risk (<18 and >35 years)[b]	0.566	0.762
Non-white maternal's ethnic origin[a]	0.194	**0.062**
CLINICAL CHARACTERISTICS		
Multiple delivery	0.340	**0.037****
Vaginal delivery/ non-Caesarean section[c]	0.432	0.407
Primiparous[d]	0.891	1.000
Previous complications[e]	0.702	0.746
Gestational age (wk)¶	**0.001** α**	0.461
Preterm birth	**0.002****	0.412
Birthweight (g)¶[a]	**0.006** α**	0.642
Low birthweight[a]	**0.004****	**0.065**
Prolonged ROM (>24h)[f]	0.471	1.000
Intrapartum antibiotics[g]	0.714	0.311
Smoking during pregnancy[h]	0.431	1.000
SAMPLE COLLECTION[b]		
At the maternal ward (or neonatal ward)	0.359	0.091
Samples collected within 12 hours of birth	0.620	1.000

data not known for: [a]1 case, [b]3cases, [c]4 cases, [d]23 cases, ; data only known for [e]112cases, [f]112 cases, [g]201 cases, [h]190 cases. * p-values determined using Fisher's Exact test for qualitative variables and 2-tailed Independent T-test for quantitative data (¶). ** Significant difference as determined by p<0.005. α Inverse association with the clinical variable.

Finally, even though very low amounts of A. *actinomycetemcomitans, P. gingivalis, T. forsythia*, and *G. elegans* were observed in the samples, few associations were

found with certain demographical and clinical data as observed in Tables 5.5 and 5.6 and represented in Figures 5.6 and 5.7.

5.9 Discussion.

The use of species-specific primers has been demonstrated to be a more sensitive technique to identify bacteria in a defined sample compared to traditional (Jervøe-Storm et al., 2005, Melendez et al., 2010) and broad-range techniques (Payne et al., 2010). In this study, this was clearly conducted for *F. nucleatum*, which allowed identification of a high prevalence of this species in the samples. *F. nucleatum* was detected in 26 (15.9%) of the NGA analysed, compare to its presence in only two samples (2.0%) using end-point PCR. *G. elegans* was also identified at a higher prevalence (5 samples; 8.6%) compared to the initial approach (2 samples; 2%). Also, these methods allowed identification of *P. gingivalis*, which was not detected previously with the other techniques.

This is the first time a study has used a predetermined detection limit to indicate prevalence in the samples. The main reason for using this approach was to avoid false positives (type I error), which represent a common drawback inherent in the use of SYBR-Green based detection chemistry. SYBR-Green binds to any double stranded DNA, including non-specific reaction products, which may result in an overestimation of the target concentration. This was also applied for general bacteria using a detection limit of 500 cels/ml. Contrary to the results obtained with the broad-range techniques, associations were not observed with vaginal delivery and pROM. On the other hand, a strong inverse association with PTB and LBW could be observed with the bivariate analysis. Significantly higher levels of bacteria were also observed using as the variables the qualitative measures of gestational age and birthweight.

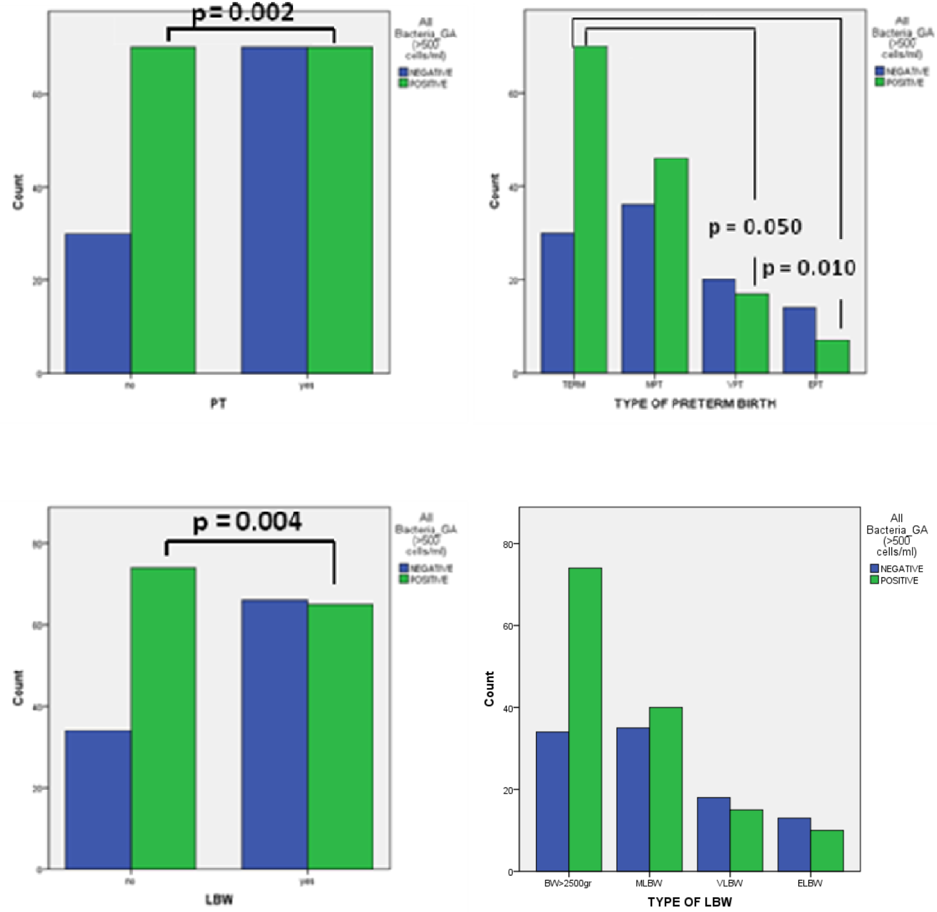


Figure 5.4 Clustered bar charts of the associations observed between prevalence of general bacteria and preterm birth (PT) and low birthweight (LBW). Fisher's Exact test and ANOVA followed by Tuckey HDS statistical analyses were performed to determine associations. MPT, mild PTB; VPT, very PTB; EPT, extremely PTB; BW, birthweight; MBW, mild LBW, VLBW, very LBW; ELBW, extremely low LBW.

Table 5.4 Multivariable analysis model to determine associations between prevalence of general bacteria using q-PCR analyses and demographical and clinical characteristics.

VARIABLES	Prevalence of bacteria using q-PCR			
	p-values	OR	95% C.I.	
			Lower	Upper
non-white	0.320	1.48	0.68	3.21
vaginal	0.151	1.65	0.83	3.26
pROM	0.677	0.84	0.36	1.95
gestational age (wk)	**0.009****	**1.17**	**1.04**	**1.32**
LBW	0.186	2.12	0.70	6.48
smoking	0.856	0.89	0.26	3.03
iATB	0.936	1.04	0.45	2.41
sampled>12h	0.100	0.47	0.19	1.16

OR= odds ratio; LBW, Low birthweight; pROM, prolonged rupture of membranes; iATB, intrapartum antibiotics. ** Significant difference as determined by $p<0.05$.

Interestingly, the association with gestational age remained after adjusting for important clinical factors such as maternal origin, mode of delivery, smoking, pROM, iATB and samples taken more than 12 hours post-delivery. It has been suggested that uterine contractions may exert a suction-like effect where vaginal fluid is introduced into the uterine cavity (Seong et al., 2008). Contractions occur in higher intensity as the infant is ready to be delivered at term, and also an increased cervical dilatation is required for delivery of a larger infant. This may explain the higher rates of bacteria at term observed in this study. Conversely, when analyzing results using the absolute levels of bacteria, an association with vaginal delivery was observed. The use of a detection limit may have avoided detection of those transient low levels of bacteria acquired from the birth canal during delivery. It is likely that the higher levels of bacteria in the samples originated from vaginal fluids aspired during labour.

Table 5.5 Comparisons between absolute amounts of periodontal bacteria in NGA determined by q-PCR analysis with demographical and clinical data.

	COMPARISONS BETWEEN ABSOLUTE AMOUNTS (cells/ml) OF BACTERIA AND DEMOGRAPHICAL AND CLINICAL VARIABLES (p-values*)					
DEMOGRAPHICAL DATA	**General Bacteria**	***F. nucleatum***	***A. actinomyce-temcomitans***	***P. gingivalis***	***T. forsythia***	***G. elegans***
Neonate's gender (M or F)[a]	0.449	0.342	0.141	0.611	0.131	0.587
Maternal age at risk (<18 and >35 years)[b]	0.441	0.386	0.061	0.766	0.167	0.768
Non-white maternal's ethnic origin[a]	0.582	0.190	0.597	0.742	0.734	0.907
CLINICAL CHARACTERISTICS						
Multiple delivery	0.396	**0.019****	0.161	0.913	0.921	0.155
Vaginal delivery/ non-Caesarean section[c]	**0.037****	0.848	0.315	0.751	0.741	0.739
Primiparous[d]	0.509	0.902	0.932	0.882	0.449	0.507
Previous complications[e]	0.886	0.963	0.770	0.581	0.639	0.573
Preterm birth	0.185	0.934	0.573	0.157	0.537	0.978
Low birthweight[a]	0.494	0.411	0.916	0.050	0.575	0.881
Prolonged ROM (>24h)[f]	0.113	0.130	0.617	0.927	**0.019****	0.297
Intrapartum antibiotics[g]	0.896	0.488	0.995	0.982	0.882	0.889
Smoking during pregnancy[h]	0.702	0.549	0.354	0.258	0.836	0.276
SAMPLE COLLECTION[b]						
At the maternal ward (or neonatal ward)	0.676	**0.033****	**0.004****	0.232	**0.010****	0.472
Samples collected within 12 hours of birth	0.717	0.904	0.124	0.507	0.634	0.707

data not known for: [a]1 case, [b]3cases, [c]4 cases, [d]23 cases, ; data only known for [e]112cases,[f]112 cases, [g]201 cases, [h]190 cases. * p-values determined using Mann Whitney U Test. ** Significant difference as determined by $p<0.05$.

Table 5.6 Comparisons between relative amounts of periodontal bacteria in NGA determined by q-PCR analysis with demographical and clinical data.

	COMPARISONS BETWEEN RELATIVE AMOUNTS (%) OF BACTERIA AND DEMOGRAPHICAL AND CLINICAL VARIABLES (p-values*)				
DEMOGRAPHICAL DATA	***F. nucleatum***	***A. actinomyce-temcomitans***	***P. gingivalis***	***T. forsythia***	***G. elegans***
Neonate's gender (M or F)[a]	0.846	0.263	0.552	1.000	0.823
Maternal age at risk (<18 and >35 years)[b]	0.874	0.645	0.696	1.000	0.229
Non-white maternal's ethnic origin[a]	0.260	0.830	0.227	1.000	1.000
CLINICAL CHARACTERISTICS					
Multiple delivery	0.171	0.281	0.390	1.000	**0.043****
Vaginal delivery/ non-Caesarean section[c]	0.146	**0.028****	0.837	1.000	0.720
Primiparous[d]	0.173	0.789	0.718	1.000	0.717
Previous complications[e]	0.866	0.635	0.618	1.000	0.272
Preterm birth	0.607	0.890	0.189	1.000	0.914
Low birthweight[a]	0.875	0.776	**0.029****	1.000	0.690
Prolonged ROM (>24h)[f]	**0.001****	0.074	0.667	1.000	**0.052**
Intrapartum antibiotics[g]	0.633	0.585	0.175	1.000	0.533
Smoking during pregnancy[h]	0.351	0.711	0.290	1.000	0.586
SAMPLE COLLECTION[b]					
At the maternal ward (or neonatal ward)	0.153	0.967	0.467	1.000	0.265
Samples collected within 12 hours of birth	0.732	0.671	0.715	1.000	0.907

data not known for: [a]1 case, [b]3cases, [c]4 cases, [d]23 cases, ; data only known for [e]112cases,[f]112 cases, [g]201 cases, [h]190 cases. * p-values determined using Mann Whitney U Test. ** Significant difference as determined by p<0.05.

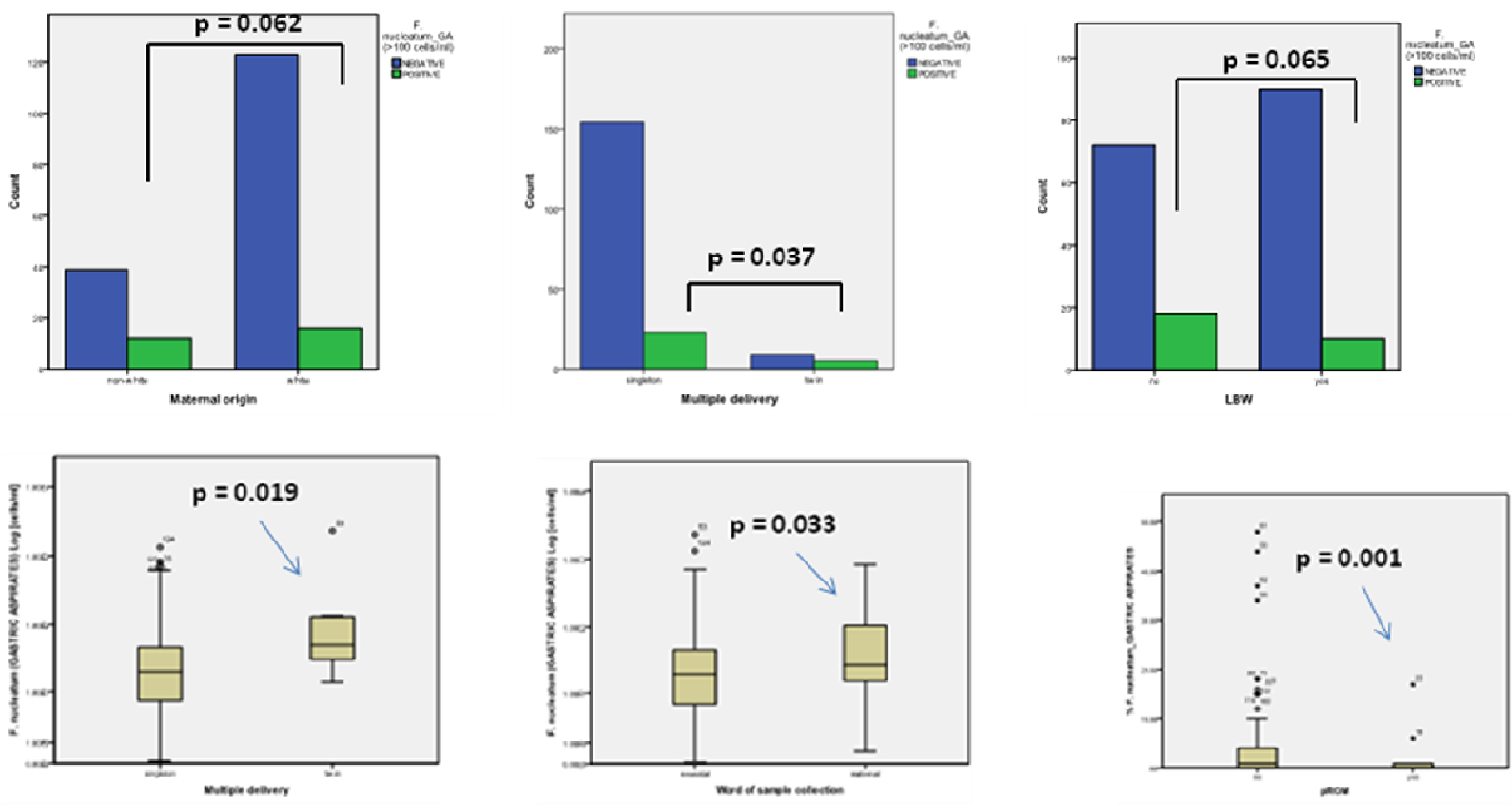


Figure 5.5 Bar charts and box-plots describing prevalence, absolute and relative amounts of *F. nucleatum* in association with clinical variables. Only statistically significant associations ($p<0.05$) or close to significance are represented here.

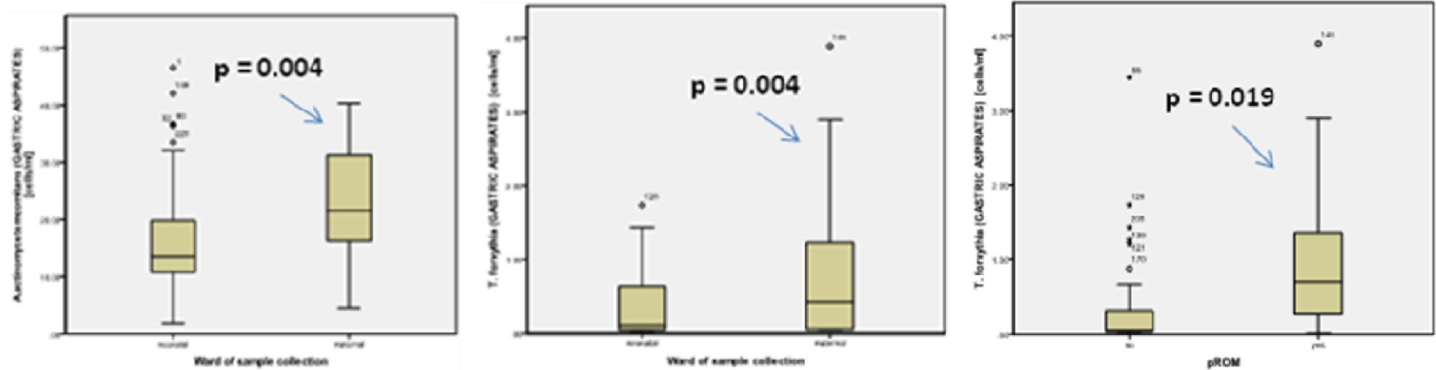


Figure 5.6 Box-plots comparing absolute amount of specific bacteria (except *F. nucleatum*) where a statistical association with a clinical variable was observed.

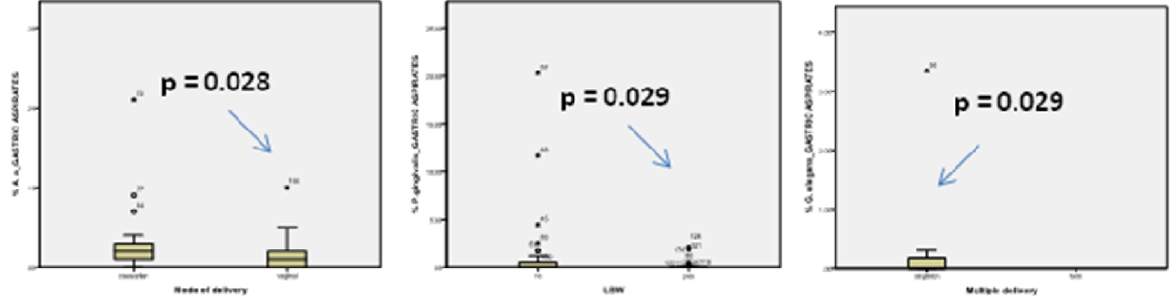


Figure 5.7 Box-plots comparing relative amount of specific bacteria (except *F. nucleatum*) where a statistical association with a clinical variable was observed. In box-plot representing *% A. actinomycetemcomitans*, one result indicating 3.9% has been omitted from the vaginal cases.

Preliminary work using q-PCR in a small set of samples of NGA (n=43) has been reported by Jones *et al*. (2010). Bacterial load ranged from 3.00E+03 to 1.74E+08 cells/ml (average= 3.10E+05) using the TaqMan® method. Contrary to the results observed in the 175 samples analysed in this study, they demonstrated an association with pROM. It is not clear if the association with pROM varied due to the number of samples included or the technique used. TaqMan® chemistry benefits from the use of a probe that hybridizes to an internal region of the PCR product and may provide higher specificity. Nevertheless, the levels of bacteria reported were very similar albeit through the use of different techniques. Another observation is that vaginal delivery, even though not significant, had a higher DNA load than those born by Caesarean section, although this association did not remain after adjusting for pROM.

P. gingivalis has been previously identified at high prevalence (30.8%) in the AF of woman with a diagnosis of threatened PTL (León et al., 2007). This species was identified by colony morphology and biochemical tests only; and its identity was not confirmed by sequencing analysis. 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* was present in the samples. In this study, *P. gingivalis* was found to be present in only two samples (2.0%). When analysing the clinical information from these two cases, they did not show any consistency; the cases differed in gender, maternal origin, and ward where the sample was collected. Both mothers were primiparous, one infant was delivered preterm (36 weeks) and vaginally, while the other was term (40 weeks) and delivered by Caesarean section. Contrary to the q-PCR analysis, both samples were negative with gram staining, culture, and broad-range molecular techniques. Using q-PCR, the total bacterial load in the samples was relatively low (1.47E+03 and 1.50E+3 cells/ml), indicating a high percentage in the samples (11.7 and 20.3%). The relative levels could be overestimated due to the difficulty to calculate precisely the levels of general bacteria in the NGA as explained previously (Section 5.4). Similarly to the absolute levels of *P. gingivalis* observed in this study (2.98E+02 and 1.75E+02

cells/ml), Barak *et al*. (2007) reported an average level of 3.61E+02 cells/cm^3 in homogenated placenta tissue of women with pre-eclampsia and PTB.

Barak *et al*. (2007) also identified *T. forsythia* (2.31E+02) at significantly high levels compared with their controls with no pre-eclampsia (5.10E+01 and 1.70E+01 for *P. gingivalis* and *T. forsythia,* respectively). *A. actinomycetemcomitans* (1.88E+01) and *F. nucleatum* (3.30E+01) were also observed only in the cases group. Similar to their results, *A. actinomycetemcomitans* was identified at very low levels in the current study (max: 4.65E+01 cells/ml). These low levels did not allow identification by sequencing even when performing a nested PCR (two successive PCR runs, the first one using universal primers and the second one using specific primers that target a sub-fragment within the first PCR product). Furthermore, in contrast to the current study, *T. forsythia* was identified at high levels and *F. nucleatum* was identified at low levels in the placental samples. One reason for the differences between levels of bacteria may be the samples analysed, and the fact that the gene copy number was not considered before hand, so numbers may have been miscalculated (Rajendhran and Gunasekaran, 2010). There is not sufficient information provided as regards the methods used, therefore it is not possible to draw further conclusions. No other study has identified periodontal bacteria in an amniotic environment using a quantitative-PCR approach.

Interestingly, the sample that contained the highest level of *P. gingivalis* (2.98E+02 cells/ml) also harboured similar levels of *F. nucleatum* (1.51E+02 cells/ml). Possible co-migration mechanisms have been proposed in a previous *in vitro* study, in which co-infection with *F. nucleatum* increased 20-fold the invasion ability of *P. gingivalis* in both gingival epithelial and aortic endothelial cells (Saito et al., 2008). Fardini *et al*. (2010) observed, in an animal model, that *F. nucleatum* but not *P. gingivalis* or any other member of the red-complex (*P. gingivalis*, *T. forsythia* and *T. denticola*) was able to invade the murine placenta, although it was present in the plaque sample that was inoculated directly into the bloodstream. The possible co-migration properties should be further investigated in experimentally controlled studies.

The relative amount of bacteria was also analysed in this study. Indeed, relative levels provided a better understanding of the potential role of periodontal bacteria in APO. With a few exceptions, the percentage of oral pathogens in NGA was very low. Therefore, their contribution to the APO may be minimal. Bacteria may have translocated from the oral cavity to the amniotic environment during an episode of bacteraemia. However, there is also a need to evaluate other possible means whereby an oral pathogen might be transferred to the samples, such as from medical staff during delivery, and earlier colonization through oral-genital transmission from the partner and subsequent spread through the ascending route. It should be considered that the relative amount of bacteria, calculated from the total bacterial load, may be inaccurate since it was not possible to calculate the precise number of *rrn* operons per bacterial species. This number varies between one and 15; therefore, it can be anticipated that the discrepancy would not be higher than a factor of 10.

Few associations were observed following statistical analysis. These varied between bacteria and for prevalence, absolute or relative amounts. *F. nucleatum*, *A. actinomycetemcomitans*, and *T. forsythia* showed association with maternal ward. Nevertheless, these associations were not confirmed with the multivariable analysis. In most cases, levels of the bacteria were too low and therefore not able to be amplified with specific primers for identification. The presence of oral bacteria in the amniotic environment (AF, placenta) has usually been noted in case reports only; which indicates it is relatively rare that translocation of periodontal bacteria to the amniotic environment can cause an APO. This suggests that other factors, such as gene-environmental or gene-gene factors may be involved (Romero et al., 2006).

Conversely, *F. nucleatum* and *G. elegans* have been observed at high prevalence and, in some cases, as an elevated percentage of the total bacterial load. *F. nucleatum* is one of the most common species isolated from AF in cases of PTL and intact membranes (Romero et al., 1989b, Hill, 1998). However, *Fusobacterium* spp., although at low levels, are inhabitants of the vagina (Hillier et al., 1993, Bennett and Eley, 1993), although their precise origin needs to be further investigated.

CHAPTER 6

PERIODONTAL BACTERIA IN THE MATERNAL ORAL CAVITY AND THE VAGINA AS THE POSSIBLE ORIGIN OF THE BACTERIA IN NEONATAL GASTRIC ASPIRATES

6.1 Introduction.

This study has demonstrated the presence of potential oral bacteria in the samples of NGA including *F. nucleatum*, *P. gingivalis* and *G. elegans*. The findings suggest the possibility that these bacteria may have translocated from the oral cavity to the amniotic environment. Studies in animal models support this hypothesis, since *F. nucleatum* and *P. gingivalis* have both demonstrated the ability to translocate through the bloodstream and invade the fetal tissues (Lin et al., 2003b, Han et al., 2004, Boggess et al., 2005). Presence of certain oral pathogens and no other prevalent oral inhabitants might be explained by specific mechanisms that allowed these opportunistic species to cross the placental membranes and invade the amniotic cavity, while other oral pathogens may not possess the same ability (Fardini et al., 2010). The specific ability to invade the placental tissues and no other organs has also been demonstrated in animals (Han et al., 2004).

Nevertheless, presence of potential oral bacteria in the samples does not indicate their source. It is possible that the bacteria observed in the samples may have originated from other body sites. The oral cavity and the vagina harbour similar microbiota. Species such as *Prevotella*, *Veillonella*, *Megasphera, Peptostreptococcus, Lactobacillus, Leptotrichia* and *Streptococcus* are common to both sites. To date, no species of the genus *Aggregatibacter* (*Actinobacillus*), *Porphyromonas*, *Tannerella* and *Granulicatella* have been reported as common inhabitants of the woman's genitourinary tract in humans, even though *Porphyromonas* spp. and *G. elegans* have been identified in the vagina previously (Hill et al., 2005, Yamamoto et al., 2009). However, there is no report of the use of species-specific and sensitive techniques to investigate the prevalence of these pathogens in the women's genitourinary tract.

On the other hand, *Fusobacterium* spp. has been reported as a common inhabitant of the vagina (Hillier et al., 1993). The maternal vagina could therefore represent a reservoir for this species. Previously, Hill (1993) suggested that *F. nucleatum* subspecies (*F. nucleatum* subsp. *vincentii*, subsp. *nucleatum* and subsp.

polymorphum) found in the AF from women with intact membranes match more closely to the strains present in subgingival sites. However, Han *et al*. (2010) demonstrated a sequence match between the subgingival *F. nucleatum* subsp. *animalis* strain and the strain isolated from the fetus in a case of a term stillbirth; the same strain was not found in the vagina, rectum or supragingival sample of the same woman. *F. nucleatum* subsp. *animalis* has also been reported as commonly prevalent in the human gut (Strauss et al., 2008). There is a need to further investigate prevalence of *Fusobacterium* spp. at different body sites and the potential sources of the bacteria associated with APO.

This study aimed to determine the presence of periodontal bacteria in the maternal oral cavity and the vagina, and to compare the prevalence, absolute and relative levels of the bacteria in the mothers with the levels in samples of NGA. Also, similarities and differences in species and subspecies of *Fusobacterium* among the three sites were investigated.

6.2 Participant's recruitment.

Women were recruited during an initial five month period from January to May 2009 (16 mothers), and later by post from January to March 2010 (3 mothers). The rate of acceptance was 69.6% (16 of 23) for women approached at the hospital, and 23.1% (3 of 13) for the women invited by post. Women invited by post were limited to those whose infant's sample was known to contain any of the bacteria of interest *(F. nucleatum, P. gingivalis* and *G. elegans*) and from whom there was a remaining sample of NGA or DNA from their respective infant for further experiments. Of the 19 women included in the study, 13 (68.4%) agreed to provide a vaginal swab.

6.3 Demographical and clinical characteristics of the women included in the study.

The 19 women included in this part of the study constituted a representative subset of the initial group of cases. Maternal ethnicity comprised five Asian/Bangladeshi, two Asian-Indian, one black African, 10 white British/Irish, and one white-other. The mean age was 28 years with only two subjects who were over 35 years of age (age at risk of PTB). Ten women were primigravidae (56%) and in one case parity was unknown. Mode of delivery included 10 vaginal deliveries (52.6%), eight emergency caesareans (42.1%), and one elective caesarean (5.3%). The mean gestational period was 35 weeks and ranged from 24 to 41 weeks. The number of PTB was 11; seven cases were mild PTB (32-36 weeks), two cases were very PTB (28-31 weeks), and two cases were extremely PTB (<28 weeks). The mean weight at delivery was 2360 g with the lowest measure observed being 700 g and the highest being 3848 g. Nine women delivered LBW infants, three were cases of pROM, and four women received iATB. One of the women had twins and they both provided a sample of NGA at the NICU. Finally, 14 (73.7%) of the samples were collected in the neonatal ward, and five (26.3 %) in the labour wards.

6.4 Bacterial profiles in the neonates whose mothers were included in the study.

The profile of bacteria observed in the NGA acquired from the respective neonates of those mother's included in this part of the study is described here. Using the broad-range molecular analysis, 10 (52.6%) samples were positive for presence of bacteria when including only this samples; three samples were shown to contain *S. pneumoniae/mitis/oralis/infantis*; one sample was positive for other members of the S. *mitis* group (*S. sanguinis/gordonii* and *S. sinensis/parasanguis*) and *S. salivarious/vestibularis*. Two samples contained *U. urealyticum*. Two samples were positive for *E. coli*; and the other two samples contained *S. agalactiae*. *F. necrophorum* was identified in one sample and *S. epidermidis* and *H. influenza* were both present in one sample. Most of these bacteria were observed in vaginal deliveries with only one exception (*S. pneumoniae/mitis/oralis/infantis*) which was

also obtained from a case of caesarean section. Bacteria in NGA from women that received iATB were observed as *U. urealyticum, S. agalactiae* and *F. necrophorum*and were all present in samples obtained in the labour ward. No other bacteria were identified in this group of samples with the broad-range methods used. Species from the genera *Lactobacillus*, *Pseudomonas*, *Gardnerella*, *Sneathia, Veillonella*, and *Prevotella* were not observed in these group of infants. In general, this group presented with low prevalence and richness of potential bacterial contaminants as compared to the original group.

On the other hand, using the q-PCR analysis, the average level of general bacteria for this group was 2.65E+05 cells/ml; 15 samples (79.0%) were considered positive with the 500 cells/ml detection limit. Positive samples were obtained from both cases of vaginal (52.6%) and caesarean deliveries (47.4%). Using species-specific primers for periodontal bacteria, four samples were considered positive (>100 cells/ml) for *F. nucleatum* (mean= 8.07E+02 cells/ml) in the 18 samples able for analysis (one vaginal and three Caesarean deliveries). Interestingly, both NGA samples obtained from the twins included in this part of the study were positive for *F. nucleatum.* Samples of NGA which presented results for the quantification of bacteria were: *A. actinomycetemcomitans* (11), *P. gingivalis* (8), *T. forsythia* (6) and *G. elegans* (2). As determined previously, all the samples were negative for *A. actinomycetemcomitans* and *T. forsythia*. One of the cases included in this analysis was positive for both *P. gingivalis* and *F. nucleatum*. No positive cases of *G. elegans* were included.

6.5 Periodontal examination and other clinical data.

Results from the periodontal examination and clinical variables collected using the questionnaires are detailed in Table 6.1. In most of the cases, the woman presented with few localized periodontal pockets (between 4 mm and 6 mm). In only one of the cases the number of sites with pockets was 18 (10% of the sites), distributed in eight different teeth. Presence of pockets in at least one site and number of pockets were therefore included in this report.

Table 6.1 Data obtained from performing a periodontal examination and from the questionnaires provided to the participants.

PERIODONTAL STATUS	**TOTAL (%) (N=19)**
Presence of pockets >4mm (n) (%)	13 (68.4)
Number of sites with pockets >4mm (median) (range)	2 (0-18)
DATA OBTAIN FROM QUESTIONNAIRE	
Drinking habits (n) (%)	1 (5.3)
Smoking during pregnancy/ smoker (n) (%)	2 (10.5)
Infections/antibiotic during pregnancy (n) (%)	3 (15.8)
Regular dental care (n) (%)	10 (52.6)
Antenatal care (n) (%)	6 (31.6)
Stress during pregnancy (n) (%)	7 (36.8)
Married or living together (n) (%)	17 (89.5)
Father's age[a] (mean) (range)	31 (20-40)
Education: college/university[a] (n) (%)	13 (72.2)
Sexual relations during pregnancy[a] (n) (%)	16 (88.9)
Oro-genital contact[a] (n) (%)	11 (61.1)

[a]data not known for 1 case

6.6 Quantitative analysis of periodontal bacteria in the maternal oral cavity and vagina.

The q-PCR analysis was performed directly from the genomic DNA extracted from the samples. Levels of *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *G. elegans* and total bacterial load were determined from the maternal oral cavity and vagina samples. Absolute amounts, relative amounts, and prevalence of bacteria were calculated as detailed before.

Absolute amounts of bacteria were obtained from all the available samples of the mother's oral cavity and vagina. All the samples were positive for presence of bacteria (>500 cells/ml). The minimum and maximum levels of general bacteria in the samples from the oral cavity were 3.47E+04 and 1.09E+07 cells/ml, respectively, with the mean amount determined as 1.65E+06 cells/ml (median=8.41E+05 cells/ml). The vaginal samples contained a minimum of 3.49E+03 cells/ml, and a

maximum of 1.98E+06 cells/ml (mean= 6.00E+05; median= 2.00E+05). Using a detection limit of 100 cells/ml, *F. nucleatum* was present in 19/19 (100%) samples of the oral cavity and 7/13 (53.8%) samples from the vagina. *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *G. elegans* were present in four (21.1%), four (21.1%), 19 (100%) and two (10.5%) of the oral samples respectively, but were not present in the vagina. Absolute and relative amount of bacteria in the oral cavity and vaginal samples are detailed in Table 6.2 and Table 6.3, respectively. Figure 6.1 represents the absolute amounts of bacteria in the maternal oral and vaginal samples. A red horizontal dashed line has been placed at the 100 cells/ml detection limit to indicate presence or absence of specific bacteria in the samples. Figure 6.2 allow comparisons between absolute levels of bacteria in the neonatal and maternal sites.

Table 6.2 Levels of periodontal bacteria in the maternal oral cavity.

	ORAL CAVITY					
	Absolute amount (cells/ml)				Relative amount (%)	
	min	max	average	median	max	average
F. nucleatum	5.43E+02	2.66E+06	2.44E+05	2.14E+04	32.4	9.6
A. actinomyce-temcomitans	3.63E+00	4.35E+03	5.83E+02	6.88E+00	5.2	0.4
P. gingivalis	1.08E+00	2.47E+05	2.03E+04	7.07E+00	10.9	1.2
T. forsythia	1.26E+02	5.68E+05	8.41E+04	1.22E+04	22.2	5.7
G. elegans	1.82E-05	2.33E+04	2.69E+03	1.82E-05	0.6	0.1

Table 6.3 Levels of periodontal bacteria in the maternal vagina.

	VAGINA					
	Absolute amount (cells/ml)				Relative amount (%)	
	min	max	average	median	max	average
F. nucleatum	8.00E+00	8.18E+03	1.53E+02	1.74E+02	3.68	0.67
A. actinomyce-temcomitans	3.63E+00	1.97E+01	1.03E+01	9.37E+00	0.27	0.03
P. gingivalis	5.42E-01	8.96E+00	3.77E+00	2.96E+00	0.03	0.01
T. forsythia	2.50E-02	2.21E+00	6.11E-01	2.77E-01	0.01	0.00
G. elegans	1.82E-05	4.95E+01	1.66E+01	1.79E-01	0.00	0.00

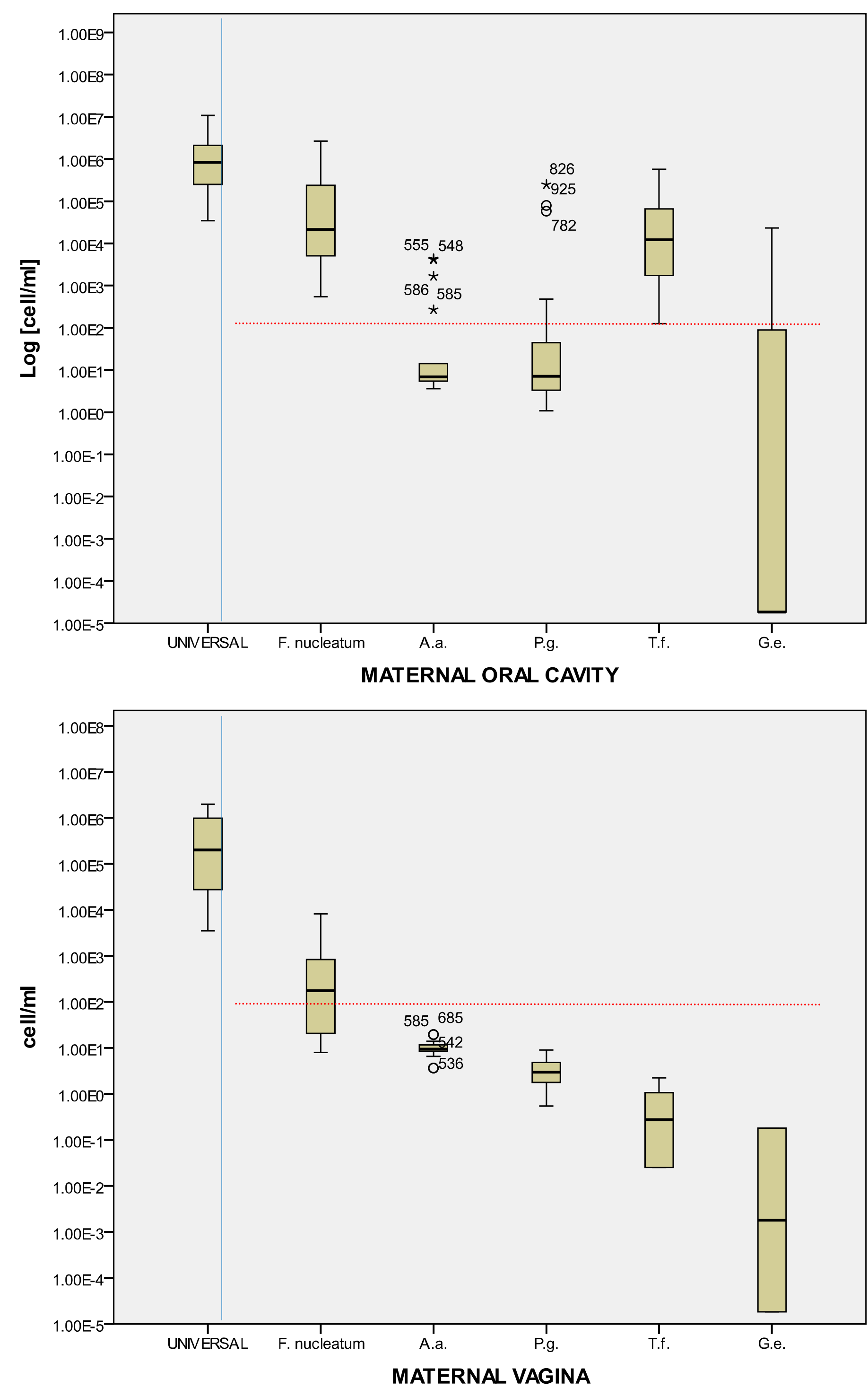


Figure 6.1 Box-plots representing absolute amounts of general and periodontal bacteria in the maternal oral cavity and vagina. Red dashed line placed at 100 cells/ml detection limit.

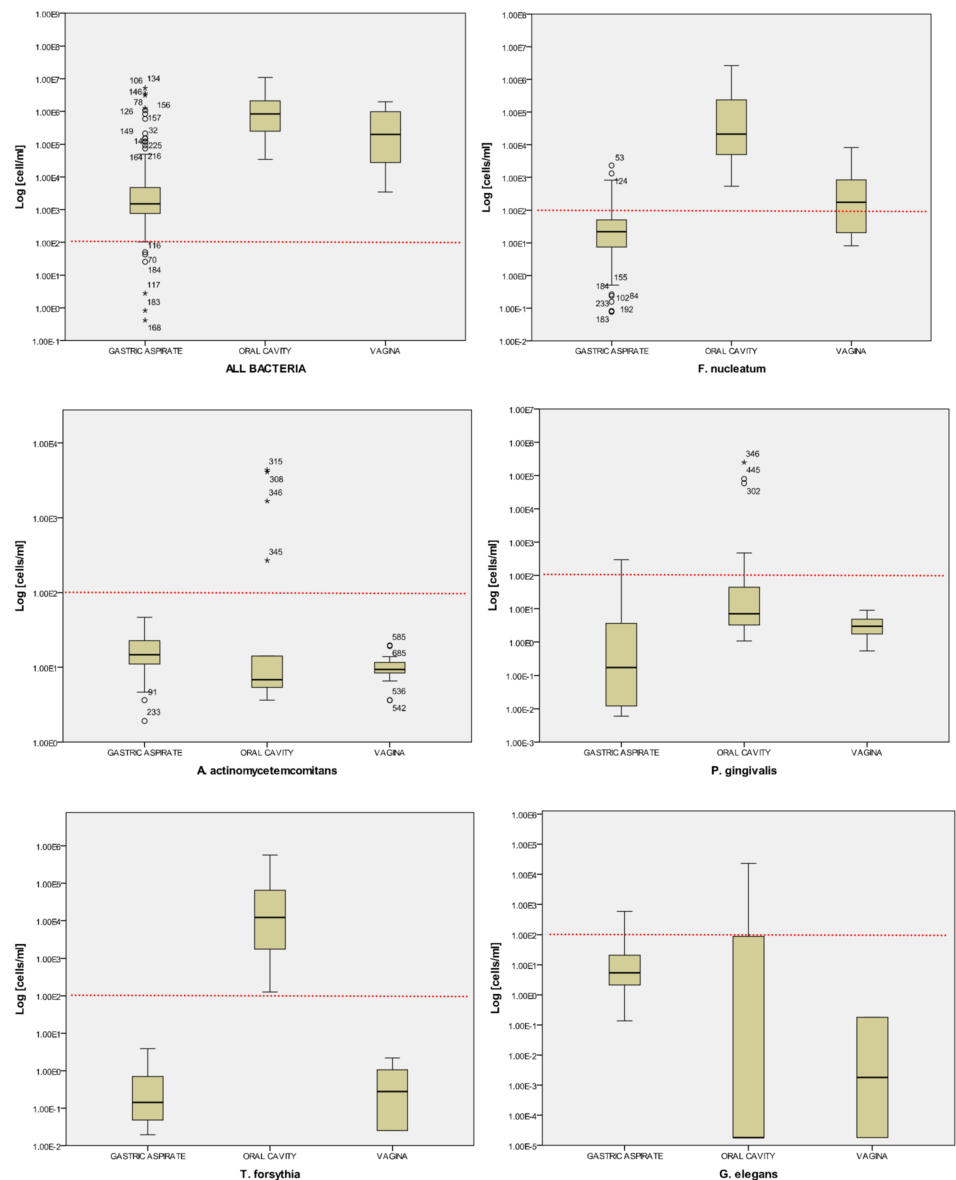


Figure 6.2 Box-plots comparing levels of general bacteria and periodontal pathogens in three sites: NGA, maternal oral cavity and maternal vagina. Red dashed line placed at 100 cells/ml detection limit.

6.7 Statistical analyses and observations.

Where possible, statistical associations were performed, although due to the limited number of samples limited observations could be made from the data. Few associations were observed between levels of periodontal bacteria in the oral and vaginal samples and demographical and clinical data. Higher amounts of general bacteria in the oral cavity was associated with white maternal origin (p= 0.048) and smoking (p= 0.035), while low levels of general bacteria in the vagina was associated with pROM (p= 0.039). *F. nucleatum* in the oral cavity was also associated with smoking (p= 0.025). Relative amount of *A. actinomycetemcomitans* in the oral cavity was associated with non-white maternal origin (p= 0.015); particularly women from Asian/Bangladeshi origin. Even though levels may be negative, higher levels of *A. actinomycetemcomitans* and lower levels of *P. gingivalis* in the vagina was associated with pROM (p= 0.031 and 0.021, respectively), and high levels and percentage of *A. actinomycetemcomitans* in the oral cavity was associated with sexual relations during pregnancy (p= 0.026 and 0.010 respectively). Table 6.4 represent the p-values observed when levels of bacteria were compared with the demographical and clinical data using a bivariate analysis. However, the associations did not remained using the multivariable analysis.

It was also observed that women who presented with periodontal inflammation (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 NGA samples. Interestingly, no sample of NGA contained *F. nucleatum* when women did not present with periodontal pockets, they also presented the lowest levels of *F. nucleatum* in the oral cavity, while the prevalence in the vagina was mixed.

Table 6.4 Comparisons between absolute and relative amounts of periodontal bacteria in the maternal oral cavity and vagina determined by q-PCR analysis with demographical and clinical data.

	COMPARISONS BETWEEN LEVELS OF PERIODONTAL BACTERIA AND DEMOGRAPHICAL AND CLINICAL DATA (p-values*)										
	ORAL CAVITY										
DEMOGRAPHICAL AND CLINICAL DATA	**Absolute amount (cells/ml)**						**Relative amounts (%)**				
	UNIV.	**F.n.**	**A.a.**	**P.g.**	**T.f.**	**G.e.**	**F.n.**	**A.a.**	**P.g.**	**T.f.**	**G.e.**
Non-white ethnic origin	**0.048****	0.069	0.062	0.457	0.680	0.200	0.213	**0.015****	0.271	0.563	0.297
Vaginal delivery	0.102	0.102	0.656	0.369	0.288	0.592	0.460	-	0.060	0.438	0.383
Preterm birth	0.322	0.869	0.213	0.680	0.869	0.283	0.263	0.200	0.161	0.680	0.467
Low birthweight	0.253	0.414	0.401	0.870	0.683	1.000	0.460	0.903	0.322	0.540	0.662
Prolonged ROM	0.248	0.564	0.585	0.386	0.564	0.453	0.942	0.922	1.000	0.885	0.568
Intrapartum antibiotics	0.332	0.182	0.089	0.716	0.808	-	0.464	0.111	0.768	0.544	-
Smoking during pregnancy	**0.035****	**0.025****	0.371	0.888	0.092	0.352	0.139	0.501	0.374	0.399	0.453
Sexual relationship	0.726	0.257	**0.026****	0.487	0.397	0.756	0.327	**0.01****	0.272	0.129	0.490
Oro-genital contact	0.187	0.073	0.172	0.545	0.724	0.856	0.145	0.230	0.413	0.659	0.297
	VAGINA										
DEMOGRAPHICAL AND CLINICAL DATA	**Absolute amount (cells/ml)**						**Relative amounts (%)**				
	UNIV.	**F.n.**	**A.a.**	**P.g.**	**T.f.**	**G.e.**	**F.n.**	**A.a.**	**P.g.**	**T.f.**	**G.e.**
Non-white ethnic origin	0.176	0.091	0.498	0.866	0.893	0.317	0.304	0.154	0.115	0.157	1.000
Vaginal delivery	0.558	0.464	0.341	0.464	0.522	0.317	0.843	0.757	0.688	0.264	1.000
Preterm birth	0.758	0.440	0.122	0.355	0.686	0.317	0.211	1.000	0.933	0.480	1.000
Low birthweight	0.775	0.317	0.352	0.668	0.371	0.317	-	0.880	0.876	0.264	1.000
Prolonged ROM	**0.039****	0.071	0.302	0.197	0.593	-	0.289	**0.031****	**0.021****	0.248	-
Intrapartum antibiotics	0.794	0.794	0.117	0.602	0.845	-	0.335	1.000	0.778	0.114	-
Smoking during pregnancy	0.076	0.114	0.075	0.430	-	-	0.505	0.145	0.194	-	-
Sexual relationship	0.789	1.000	0.789	0.789	0.746	0.533	0.588	0.778	0.558	0.724	-
Oro-genital contact	0.758	0.643	0.279	1.000	0.628	0.186	0.917	0.464	0.612	0.264	-

* p-values determined using Mann Whitney U test. UNIV., General bacteria; F.n. , *F. nucelatum*; A.a., *A. actinomycetemcomitans*; P.g., *P. gingivalis*; T.f., *T. forsythia*; G.e., *G. elegans*. ** Significant difference as determined by $p<0.05$.

6.8 Comparison between prevalence, absolute and relative amounts of *F. nucleatum* in the three sites.

Figure 6.3 illustrates the comparison between prevalence, absolute and relative levels of *F. nucleatum* between the three sites. Overall, prevalence and levels of the bacteria in the oral cavity was high (100% prevalence; median= 2.14E+04 cells/ml, 7.0%) compared to the equivalent in the vagina (53%; 1.13E+02 cells/ml, 0.05%), whereas in the NGA the levels of *F. nucleatum* were in average low and only a few cases showed high levels (16%; 2.17E+01, 1.2%). Comparisons between absolute and relative amounts of *F. nucleatum* between the three sites comparing individual cases are also illustrated in Table 6.5.

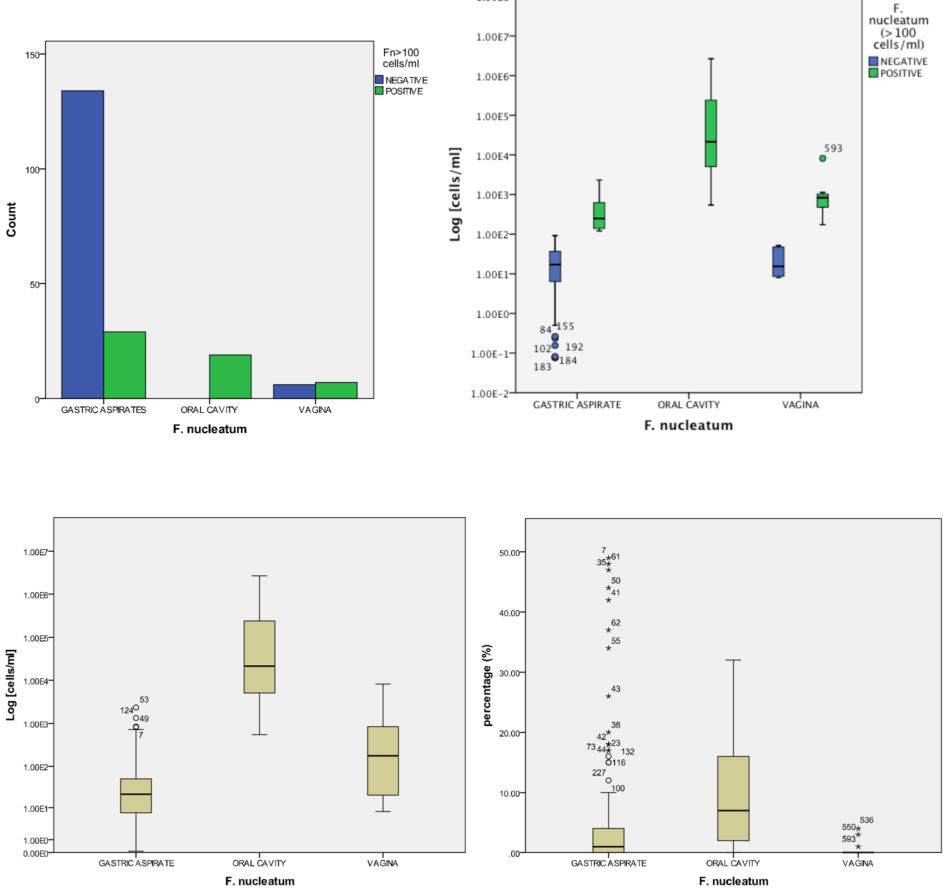


Figure 6.3 Diagrams representing comparisons between the prevalence, absolute and relative amount of *F. nucleatum* between the neonatal and maternal samples.

6.9 Comparison between *Fusobacterium* species/subspecies in the neonatal and maternal sites.

All the available samples from the NGA (n=201), oral cavity (n=19) and vagina (n=13) were included in the analysis. The identification of the *Fusobacterium* species was performed using species-specific primers in an end-point PCR. Primers Fn1F and Fn2R (Table 2.4, Chapter 2) would amplify the 16S rRNA gene of all members of the genus *Fusobacterium*. The products were cleaned and sequenced directly for identification. The primer Fn1F was used for sequencing. Sequences were verified using Chromas software. The sequences were finally identified by comparisons against the HOMD database. Usually, the species with the highest similarity was selected to identify the taxa. However, some samples identified more than one species with the same percentage of nucleotides matches in which case both were included. This was observed in two samples of NGA and one sample of the oral cavity. Also, similarity was >97% in most of the cases except for two samples from the vagina which similarity with the closest relative was 94.4% with *F. nucleatum* subsp. *nucleatum* (case 1) and 89% *F. nucleatum* subsp. *animalis* (case 55).

Identification of *F. necrophorum* in one of the samples of NGA confirmed the result obtained using the broad-range approach. *F. necrophorum* was also observed in the vagina, but not detected in the oral cavity of any women. *F. naviforme* was only observed in the NGA while *F. periodonticum* was only observed in the oral cavity. Table 6.6 summarizes the results obtained.

The results obtained with this technique were also evaluated to compare the species and subspecies identified between samples of each individual case available. Table 6.7 summarises the findings. Figure 6.6 represents the sequence comparisons by alignment of case 54. Sequences were aligned using Multalign and the chromatograms of the bases showing differences 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.

The results from the respective mother were available for all the samples of the oral cavity and only two samples of the vagina. In four cases, bacteria from the vaginal samples were not able to be amplified; this may be due to either low amount of bacteria in the sample (cases 5 and 65) or due to low percentage of the total load (cases 5, 10 and 65) as observed with the q-PCR analysis. In two cases, samples were not available.

Table 6.5 Absolute and relative amount of *F. nucleatum* comparing levels between the neonatal and maternal sites from each individual case.

	NGA		**ORAL CAVITY**		**VAGINA**	
CASE ID	Absolute amount (cells/ml)	Relative amount (%)	Absolute amount (cells/ml)	Relative amount (%)	Absolute amount (cells/ml)	Relative amount (%)
1	**2.32E+03**	n/a	**1.22E+05**	17.0	**8.35E+02**	3.7
4	4.10E+01	2.0	**2.14E+04**	2.4	**3.55E+02**	0.0
5	**1.51E+02**	10.3	**4.46E+03**	0.2	4.73E+01	0.0
10	**6.24E+02**	36.6	**1.67E+05**	4.5	**1.13E+03**	0.0
18	1.74E+00	3.5	**1.89E+03**	0.4	**9.25E+02**	3.3
53	4.51E+01	0.4	**1.59E+05**	7.0	8.65E+00	0.2
54	2.07E+01	1.8	**2.66E+06**	24.4	**6.43E+02**	0.8
55	4.66E+01	0.0	**2.71E+05**	10.6	**1.74E+02**	0.3
62	6.65E+00	0.4	**3.97E+05**	15.1	**8.18E+03**	0.1
65	6.46E+00	15.0	**3.06E+05**	23.7	2.05E+01	0.0
162	**1.34E+02**	5.3	**2.35E+05**	32.4	5.19E+01	0.0

NGA; neonatal gastric aspirates; n/a, sample not available; amounts in **bold** indicate samples considered positive (>100 cells/ml).

Table 6.6 Detection and identification of *Fusobacterium* spp. amplified in the neonatal and maternal sites using a direct sequencing approach.

***Fusobacterium* spp. and *F. nucleatum* subspecies**	**NGA**	**ORAL CAVITY**	**VAGINA**
Number of samples (N)	201	19	13
***Fusobacterium* spp. primers-positive N(%)**	18 (8.9)	19 (100)	5 (38.5)
F. necrophorum	1 (0.5)	--	1 (7.7)
F. periodonticum	--	3 (15.8)	--
F. naviforme	2 (1.0)	--	--
*F. nucleatum**	17 (8.5)	16 (84.2)	4 (30.8)
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)

NGA; neonatal gastric aspirates. * Percentages of each subspecies of *F. nucleatum* were calculated from the total of *F. nucleatum* species.

6.10 Discussion.

Most of the studies reporting presence of an oral pathogen in the amniotic environment have not confirmed the origin of the bacteria. Animal studies support the possibility that oral bacteria can translocate through the bloodstream and specifically invade the amniotic tissues. However, in these experiments bacteria are usually inoculated directly into the bloodstream, which does not entirely support the oral-utero translocation hypothesis. In this chapter the possibility that potential oral bacteria present in the NGA may have originated from a non-oral site was evaluated.

The results presented demonstrate that the most common bacteria associated with PD (*A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia*) are not normally present in the vagina. It has been suggested that oral bacteria may reach the female genito-urinary tract during oro-genital sex (Edwards and Carne, 1998, Saini et al., 2010). Nonetheless, in this study, oro-genital contact did not show any influence as regards the presence and levels of these bacteria in the vagina.

Table 6.7 *Fusobacterium* spp. identified with species-specific primers directed to the 16S rRNA gene in an end-point PCR followed by direct sequencing in neonatal and maternal sites.

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, *F. nucleatum* subsp. *polymorphum*; Fna, *F. nucleatum* subsp. *animalis*; Fnv, *F. nucleatum* subsp. *vincentii*; Fnn, *F. nucleatum* subsp. *nucleatum.*

CASE 54

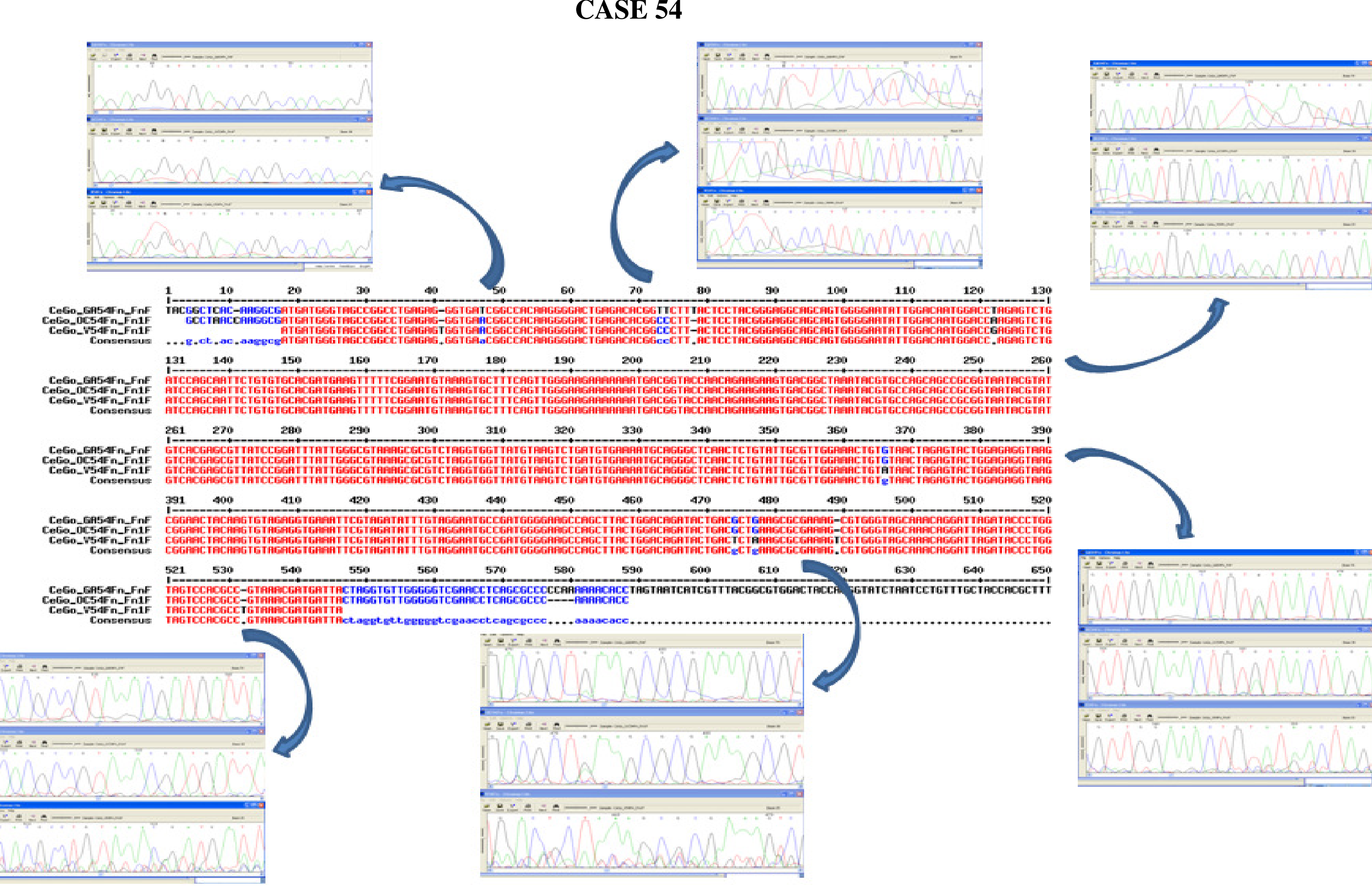


Figure 6.4 Diagram representing the alignments of the *Fusobacterium* spp. amplified from the NGA and their respective counterpart in the maternal oral cavity and vagina of CASE 54. Each mismatch has been amplified to show differences in the sequencing chomatograms.

In one of the cases, *P. gingivalis* was observed in the neonatal (NGA) samples of a woman delivering a preterm infant vaginally; however, the same pathogen was not present in the woman's oral cavity or vaginal sample. The participant confirmed having sexual relationships and performing oro-genital contact during pregnancy which, in this particular case, may explain presence of the bacteria in the neonate. However, the partners' periodontal bacteria were not evaluated. Also, other potential routes of transmission should be explored, e.g. contamination acquired from other members of the family or the medical staff.

Levels of periodontal bacteria in the pregnant female oral cavity have been studied previously (Persson et al., 2008, Urbán et al., 2010, Ryu et al., 2010). Interestingly, in this study, *T. forsythia* was not associated with PD as demonstrated previously (Mineoka et al., 2008). It was observed in all the maternal oral samples at a relatively high percentage (mean= 8.41E+04 cells/ml, median= 1.22E+04 cells/ml, average relative amount= 5.7%) compared to the other common periodontal bacteria (*A. actinomycetemcomitans* and *P. gingivalis*). These other bacteria were in contrast observed in high levels in a few samples and in association with the presence of periodontal pockets. This is the first study reporting high levels of *T. forsythia* in the oral cavity of pregnant women, and a more extensive analysis should be performed to investigate this observation. Furthermore, this is the first study that has measured levels of *G. elegans*. However, this pathogen was observed in low amounts and less than 1% relative levels in the oral cavity and in very low levels in the vagina (less than 0.01%).

The results of this study confirm that *F. nucleatum* is highly prevalent in the oral cavity (Moore and Moore, 1994) and is also present in the vagina, though at a much lower prevalence and at very low levels and relative amounts. Therefore, the female genitourinary tract may as well represent a reservoir for the pathogen. Interestingly, in all the four cases that were shown to contain *F. nucleatum* and *P. gingivalis* in the newborn's gastric aspirates, women presented with at least one pocket of 4 mm to 6 mm, with three of the cases being emergency Caesarean sections. Conversely, mothers who did not have periodontal pockets did not haurbour *F. nucleatum* in the

corresponding NGA. However, all had sexual relationships during pregnancy and, except for one woman, all performed oro-genital contact during pregnancy and some cases were shown to contain *F. nucleatum* in the vaginal sample. Levels of this pathogen in the vagina, when comparing samples for each individual case, supported a potential vaginal origin since every case was shown to contain the bacterium, although in some cases this was observed at non-detectable levels (<100 cells/ml) and at low prevalence in the samples.

To help confirm the precise source of the bacteria present in the NGA further analysis of the species/subspecies was performed by 16S rRNA gene sequence analysis. The PCR primers used were designed to amplify the 16S rRNA gene of bacteria from genus *Fusobacterium* (*F. canifelinum, F. equinum, F. gonidiaformans, F. mortiferum, F. naviforme, F. necrogenes, F. necrophorum, F. nucleatum, F. perfoetens, F. periodonticum, F. pseudonecrophorum, F. russii, F. simiae, F. ulcerans* and *F. varium*). In this study, those of most clinical significance were identified in the samples (*F. nucleatum*, *F. periodonticum*, *F. necrophorum* and *F. naviforme*). *F. periodonticum* was limited to the oral cavity, even though it has been reported not to be restricted to the oral niche (Strauss et al., 2008). *F. 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 and Eley (1993), they observed that *F. necrophorum* is more commonly found in the lower part of the body and genitals, whereas *F. nucleatum* is more commonly found in the upper body and oropharynx.

The most common subspecies of *F. nucleatum* in the samples of NGA was *F. nucleatum* subsp. *polymorphum* (72% of *F. nucleatum* species). Other subspecies observed were subsp. *vincentii* (18%) and subsp. *animalis* (11%). Similar to what has been previously suggested (Hill, 1993), the range of subspecies observed in the infants resemble more closely those observed in the oral cavity of the mothers, which showed a higher prevalence of *F. nucleatum* subsp. *vincentii* (37%), followed by subsp. *polymorphum* (32%), subsp. *nucleatum* (16%) and subsp. *animalis* (5%). On the other hand, the subspecies of *F. nucleatum* observed in the vagina were mostly *F. nucleatum* subsp. *animalis* (16%) and only one case showed the presence of *F.*

nucleatum subsp. *nucleatum* (20%). Previous evidence confirmed that the subsp. *animalis* causing a stillbirth originated from the oral cavity (Han et al., 2010). *F. nucleatum* subsp. *animalis* has also been reported as an uncommon oral strain but significantly more prevalent in necrotizing ulcerative gingivitis in a Chinese population (Gmür et al., 2006), and is also common inhabitant of the human gut (Strauss et al., 2008).

Finally, the comparisons performed between samples of individual cases showed that the species observed in the NGA most closely related with those in the oral cavity than with those in the vagina. Specifically, the alignment of the sequences of *F. nucleatum* for case 54 demonstrated eight sites in the sequences that do not match with the vaginal but do match with the oral strain. In three remaining sites, the oral and vaginal strain sequences do match, while in one case there is no match among any of the strains. These variations might be due to the presence of a range of different species/subspecies since the samples are likely to carry a mixture of the bacteria. No member of the subsp. *fusiforme* was identified.

CHAPTER 7

THE 16S-23S rRNA INTERGENIC SPACER REGION TO DETERMINE PRECISE ORIGIN OF *F. NUCLEATUM* IN NEONATES

7.1 Introduction.

In previous chapters, the results of investigations were based on the ability of the 16S rRNA gene to identify and quantify bacteria. Samples sequencing of the 16S rRNA gene has been established as the gold standard for identification and taxonomic classification of bacterial species. It has been previously demonstrated that all the *Fusobacterium* species present with unique sequences of the 16S rRNA gene and substantial intrageneric heterogeneity (Lawson et al., 1991, Gmür et al., 2006, Strauss et al., 2008). This was also observed for *F. nucleatum* subspecies, even though they exhibited relatively high levels of sequence similarity. However, the disadvantage of using the 16S rRNA gene for discrimination is the lack of sufficient resolution to enable a precise differentiation between strain variations within a species. Identification to a species level may be insufficient proof of translocation of bacteria from one site to another. More precise techniques are necessary to compare the strains of *F. nucleatum* observed in the NGA with strains present in the maternal oral and vaginal samples in order to determine the origin of the bacteria.

The 16S-23S rDNA ISR is increasingly being used for the analysis of phylogenetic relationships among species and subspecies of the same genus (Barry et al., 1991, Tyrrell et al., 1997, Sadeghifard et al., 2006) due to its more divergent sequence as compared to the 16S rDNA (Söller et al., 2000). The heterogeneity of the ISR in terms of both copy number and length has raised the possibility of using this methodology for bacterial identification and typing purposes (Gürtler and Stanisich, 1996). Previously, the ISR located between the 16S and the 23S rRNA genes has been used to examine inter- and intraspecies relationships of the *Fusobacterium* species demonstrating a high phylogenetic resolution (Conrads et al., 2002).

As observed in this report, the subspecies of *F. nucleatum* identified in the NGA samples seemed to present a better match with oral stains than with vaginal strains. Although the match was closer with the oral clones, there were still mismatches at the 16S rDNA level. A 100% match between the site of origin and the recipient site would be expected in order to determine origin. Variability of the ISR has been

previously exploited in a case report of a 39th week stillbirth to determine the origin of *F. nucleatum* (Han et al., 2010). *F. nucleatum* was, for the first time, demonstrated to originate from the maternal oral cavity since the same clone was identified in the placenta/fetus and the mother's subgingival plaque but not in maternal supragingival plaque, vagina or rectum. However, the match was performed through identification of an end-point PCR positive band using *Fusobacterium*-specific primers directed to the ISR (constructed from the fetal *F. nucleatum* strain). Little information was provided regarding the construction of the primers and the primer detection limits were not mentioned and probably not considered in this study. It is possible the same species might be present in the vagina or gut at levels that were undetectable using end-point PCR, as observed in the current study.

After a thorough evaluation of the literature, a lack of information regarding the ability of this genomic region to discriminate between species and strains of the genus *Fusobacterium* was noted. Therefore, the first part of this chapter presents an assessment of the ISR as a tool to discriminate strains of *F. nucleatum* and the second part includes the use of this methodology to precisely identify the origin of *F. nucleatum* in NGA.

7.2 Specific amplification of the intergenic spacer region of *Fusobacterium* species/subspecies origin.

Unlike the 16S rDNA sequences, the 23S rDNA has not been extensively studied and therefore there is still very little information about this gene. Hence, a species-specific primer for the genus *Fusobacteria* targeting the 16S rRNA gene was used in combination with a universal primer in the 23S rRNA gene to amplify the complete ISR from both reference strains and samples. The 456R primer has been suggested as the most highly conserved universal primer in the 23S rDNA for amplification of most bacteria (Gürtler and Stanisich, 1996). Primer 456R were therefore used in this study. Exceptional cases where the primer 456R did not amplify a product, primer 129R was used.

Conrads *et al*. (2002) previously determined that the ISR of *Fusobacterium* strains ranged in length between 108 bp (*Leptotrichia buccalis*) and 262 bp (*Fusobacterium prausnitzii*). It was therefore expected that the forward primer Fn1F in the 16S rDNA, and the reverse primer 456R in the 23S rDNA would amplify a fragment with a length range between ca. 1860 and 2020 bases. The proposed amplicon would consist of: i) 1290 bases of the 16S rRNA gene, ii) the ISR between the 16S and 23S rRNA genes, and iii) the first 460 bases of the 23S rRNA gene (Figure 7.1).

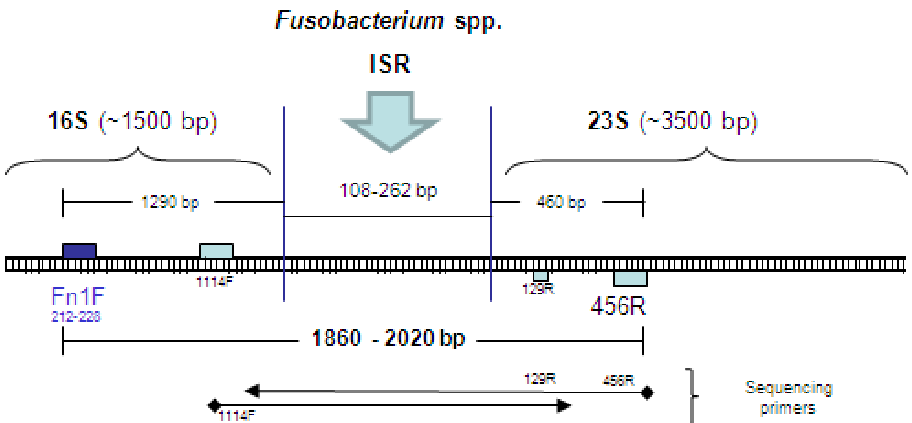


Figure 7.1 Schematic representations of the 16S, the ISR, and the 23S rDNA portions of the *rrn* operon including the primers used for amplification and sequencing in this study.

7.3 Cloning and analysis of fragment lengths in agarose gels.

The ISR was amplified from the five different subspecies of *F. nucleatum* grown in pure culture in this study (Table 2.2 Chapter 2). The products were then subjected to cloning and 10-20 randomly selected clones were amplified following the same protocol as the initial amplification. This was followed by the use of an extended agarose gel analysis to resolve the products. Different fragment lengths could be clearly observed in all the strains, evaluated suggesting this variation might be due to differening numbers of *rrn* operons. Figure 7.2 represents fragment lengths from clones of the *F. nucleatum* subsp. *polymorphum* strain NCTC 10562. At least three different fragment sizes could be differentiated in the gel for this strain. Two fragment sizes were observed for the other subspecies of *F. nucleatum*. As observed

in the gels, fragment lengths ranged between ca. 1900 and 2300 bp.

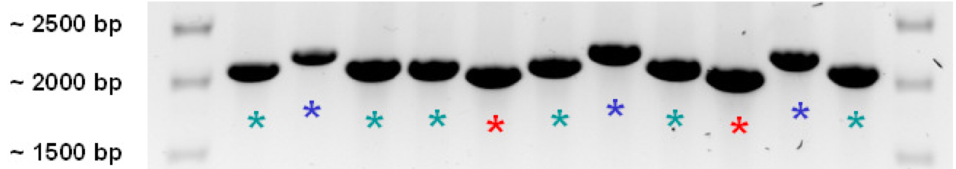


Figure 7.2 Picture of agarose gel showing three different fragment sizes of clones amplified from *F. nucleatum* NCTC 10562 using primers Fn1F (16S) and 456R (23S). Stars in different colours indicate possible different fragment lengths. Blue star, long fragments; green star, medium size fragment; red star, short fragments.

7.4 Sequencing and alignment of clones.

A library of clones was constructed from the reference strains. This was later performed for the clinical samples of NGA, maternal oral cavity, and vagina in order to individualize species, subspecies, and operons within them. Basically, representative clones observed at variable levels in the extended agarose gel analysis were sequenced for analysis. The total number of clones analysed from each subspecies were: nine *F. nucleatum* subsp. *animalis*, 10 *F. nucleatum* subsp. *fusiforme*; 10 *F. nucleatum* subsp. *nucleatum*, 11 *F. nucleatum* subsp. *polymorphum*, and six *F. nucleatum* subsp. *vincentii*.

Each clone was sequenced with the forward primer 1114F and the reverse primer 456R (Table 2.7, Chapter 2). The sequences were analysed using the software Chromas and aligned using Multalign. Whenever a mismatch was observed between the sequences produced with the forward primer and the reverse complement of the sequences obtained with the reverse primer, this was corrected by looking at the sequencing chromatograms. Finally, identification of the species/subspecies was performed through sequence comparison against BLAST, the RDP, and HOMD libraries of sequences.

7.5 Determination of the intergenic spacer region sizes.

The ISR lengths of the *F. nucleatum* species were determined using the sequences obtained from the reference clones of the five subspecies of *F. nucleatum*. Alignment of tclone sequences against published data of the 16S, the ISR, and the 23S rDNA of *F. nucleatum* ATCC 25586 were performed using Multalign. By comparisons with these data, the limits of the ISR fragments were identified in the clones and the lengths of ISR calculated.

7.6 Identification of the five subspecies of *F. nucleatum* using standard sequence comparison methods.

Variable results were observed when using different sequence libraries sites to identify the clones of the subspecies of *F. nucleatum* grown and analysed in this study. The data was analysed with HOMD, RDP, and BLAST. In general, all the strains were identified as *Fusobacterium* spp. In the case of *F. nucleatum* subsp. *animalis* and subsp. *nucleatum*, both were successfully identified by all three databases. *F. nucleatum* subsp. *polymorphum* was identified with RDP and HOMD, but the HOMD also identified these clones as *F. periodonticum*. On the other hand, BLAST identified the strain of *F. nucleatum* subsp. *polymorphum* as both subsp. *nucleatum* and *F. periodonticum*. *F. nucleatum* subsp. *fusiforme* was only identified by BLAST; HOMD and RDP identified this strain as *F. nucleatum* subsp. *vincentii* or *F. naviforme*. Finally, *F. nucleatum* subsp. *vincentii* was identified only by HOMD, while the RDP and BLAST identified it as *F. nucleatum* subsp. *fusiforme*.

7.7 Analysis of the intergenic spacer region by sequence comparisons and phylogenetic analysis.

The sequence data was used to analyse differences among the five different subspecies of *F. nucleatum,* as well as the five *rrn* operons of *F. nucleatum* ATCC 25586 published in the Oral Pathogen Sequence Database, Los Alamos National Laboratory website. The published data was also used to investigate the efficacy of

the methodology used in this study to make comparisons with the equivalent data obtained from the *F. nucleatum* ATCC 25586 grown in this study. Finally, sequences of clinical strains were compared with their corresponding counterpart in the oral and vaginal samples to confirm origin. Phylogenetic trees were constructed with the clone sequences to investigate relationships between the samples' *F. nucleatum* strains found in the samples.

7.7.1 Sequence comparisons between the five operons of *F. nucleatum* ATCC 25586 using published data of the complete genome.

Figure 7.3 represents a sequence comparison between the five different operons of the *F. nucleatum* subsp. *nucleatum* ATCC 25586. Data was obtained from the Oral Pathogen Sequence Database, Los Alamos National Laboratory (http://www.oralgen.lanl.gov/). The data indicates that this subspecies possesses four short identical operons and one long operon due to size variation of the ISR fragment (155 and 330 bp). Only three mismatches were observed when comparing the short and the long fragment. It could also be observed that the long fragment of the ISR contained an extra 9 bp fragment, followed by one $tRNA^{Ala}$ and one $tRNA^{Ile}$ (each 77 bp in length and separated by 3 bp), and another 9 bp fragment in the ISR (Figure 7.4).

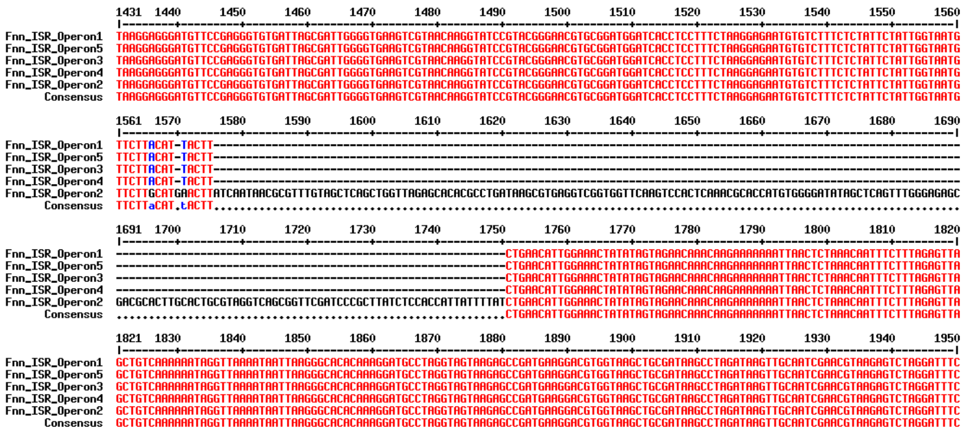


Figure 7.3 Sequence comparison of the 16S-23S ISR between the five different operons of *F. nucleatum* ATCC 25586, Oralgen, Los Alamos National Laboratory, New Mexico. The ISR is located between base 1503 and 1835.

>FnnATCC25586_ISR_Operon2
…TCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTTGCACCTGAAGTAGCAGGCCT
AACCGTAAGGAGGGATGTTCCGAGGGTGTGATTAGCGATTGGGGTGAAGTCGTAACAAG
GTATCCGTACGGGAACGT**NGCGGATGGATCACCTCCTTTCTAAGGAGAATGTGTCTTT**
CTCTATTCTATTGGTAATGTTCTTGCATGAACTTATCAATAACGCGTTTGTAGCTCA
GCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCACTCAAA
CGCACCATGTGGGGATATAGCTCAGTTTGGGAGAGCGACGCACTTGCACTGCGTAG
GTCAGCGGTTCGATCCCGCTTATCTCCACCATTATTTTATCTGAACATTGGAAACTA
TATAGTAGAACAAACAAGAAAAAAATTAACTCTAAACAATTTCTTTAGAGTTAGCTG
TCAAAAAATAGNGTTAAAATAATTAAGGGCACACAAAGGATGCCTAGGTAGTAAGAGC
CGATGAAGGACGTGGTAAGCTGCGATAAGCCTAGATAAGTTGCAATCGAACGTAAGAGT
CTAGGATTTCCGAA…

Figure 7.4 Sequence deduced from the published data of *F. nucleatum* ATCC 25586, long fragment (Operon 2). Ns were placed at the beginning and end of the ISR (in bold). Bases in blue indicate the sequence contained only in the long fragment of the ISR. Section highlighted in green represents the $tRNA^{Ala}$ and the section in yellow represents $tRNA^{Ile}$.

7.7.2 Sequence comparisons between published data of *F. nucleatum* ATCC 25586 and clones produced in this study.

In order to evaluate the data obtained in this study against the data already published in the literature, a comparison between known sequence data and data produced in this study for the ATCC 25586 strain of *F. nucleatum* were performed. Figure 7.5 demonstrates that the methodology used in this study, successfully amplify the two different sized *rrn* operon fragments from the *F. nucleatum* spp. *nucleatum* and these matched 100% with the published data.

7.7.3 Sequence analysis of the intergenic spacer region of the five subspecies of *F. nucleatum.*

The clones obtained from each strain of *F. nucleatum* grown in this study were aligned to identify different lengths of the ISR. This demonstrated that subspecies *animalis*, *fusiforme*, *nucleatum*, and *vincentii* possess ISR of two different sizes, while *F. nucleatum* subsp. *polymorphum* contained three different ISR fragment lengths. A total of five different lengths of the ISR were identified in the five strains:

331, 322, 222, 156 and 147 bases in length. The ISR fragments present in each subspecies varied: *F. nucleatum* subsp. *polymorphum* contained ISR 331, 222, and 156 bp lengths; subsp. *animalis* and subsp. *nucleatum* presented fragments sizes of 331 and 156 bp; subsp. *fusiforme* and subsp. *vincentii* presented fragment sizes of 322, and 147 bp. It could be observed that the long ISR fragments length (331 and 322) contained both $tRNA^{Ala}$ and $tRNA^{Ile}$. Figure 7.6 shows alignment of the clones of the different *F. nucleatum* strains. Identical sequences were identified and eliminated. Sequences presenting at least one mismatch in any region were kept and included in the analysis. An N was introduced at the beginning and at the end of one representative sequence for each length for identification. It could be observed that the variability in length of ISR was due to the presence or lack of certain regions (of different lengths) along the ISR. It is also evident that the five subspecies present few mismatches along the 16S, ISR, and 23S rDNA.

7.7.4 Phylogenetic analysis of the intergenic spacer region of the five subspecies of *F. nucleatum.*

Figure 7.7 demonstrates the ability of 16S, ISR, and 23S to discriminate bacteria at the subspecies level, and also possibly at the strain level. The variability in fragments of 16S and 23S rRNA genes were important to allow discrimination between subspecies, since when the ISR alone was used the same discrimination could not be observed (Figure 7.8). Clinical strain identified as *S. sanguinegens* and *L. buccalis* were included as outgroups with and confirmed distant relation with both fragments of the *rrn* operon (not included in the graphs).

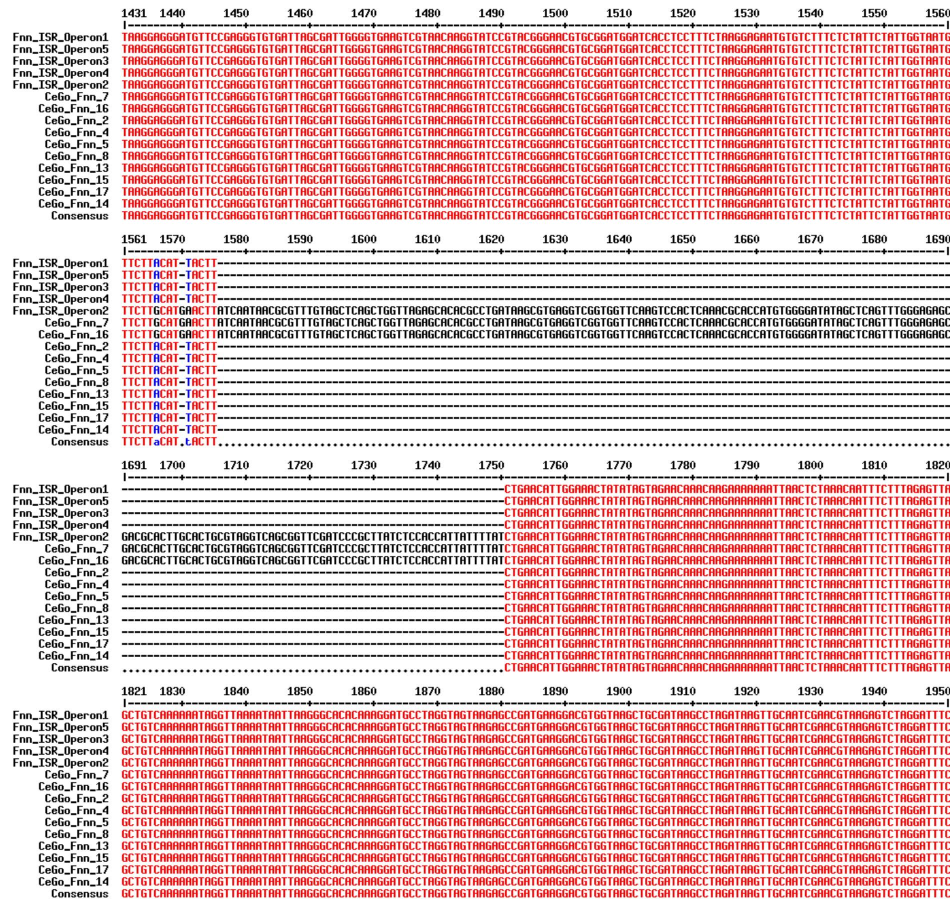


Figure 7.5 Sequence comparison of the ISR between operons of *F. nucleatum* ATCC 25586 (Fnn_ISR_Operon#) obtained from the Oral Pathogen Sequence Database, and the sequences obtained in this study from a pure culture of *F. nucleatum* ATCC 25586 (CeGo_Fnn_#).

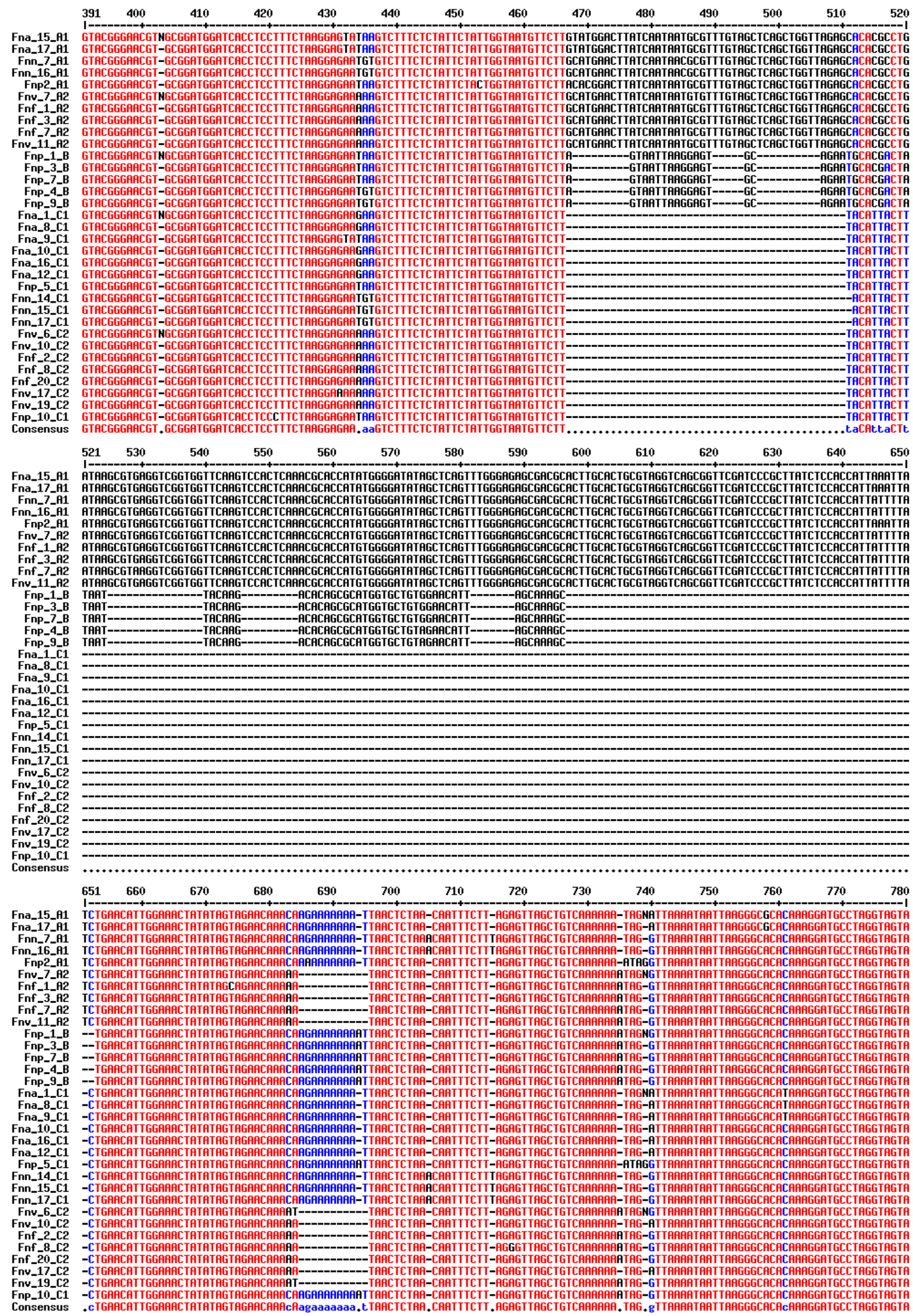


Figure 7.6 Sequence comparison of the 16S-23S ISR of *F. nucleatum* subspecies grown in this study. Fna, *F. nucleatum* subsp. *animalis*; Fnf, *F. nucleatum* subsp. *fusiforme;* Fnn, *F. nucleatum* subsp. *nucleatum;* Fnp, *F. nucleatum* subsp. *polymorphum*; Fnv, *F. nucleatum* subsp. *vincentii*. Different fragment sizes were identified as: A1, 331 bases; A2, 322 bases; B, 222 bases; C1, 156 bases; and C2, 147 bases.

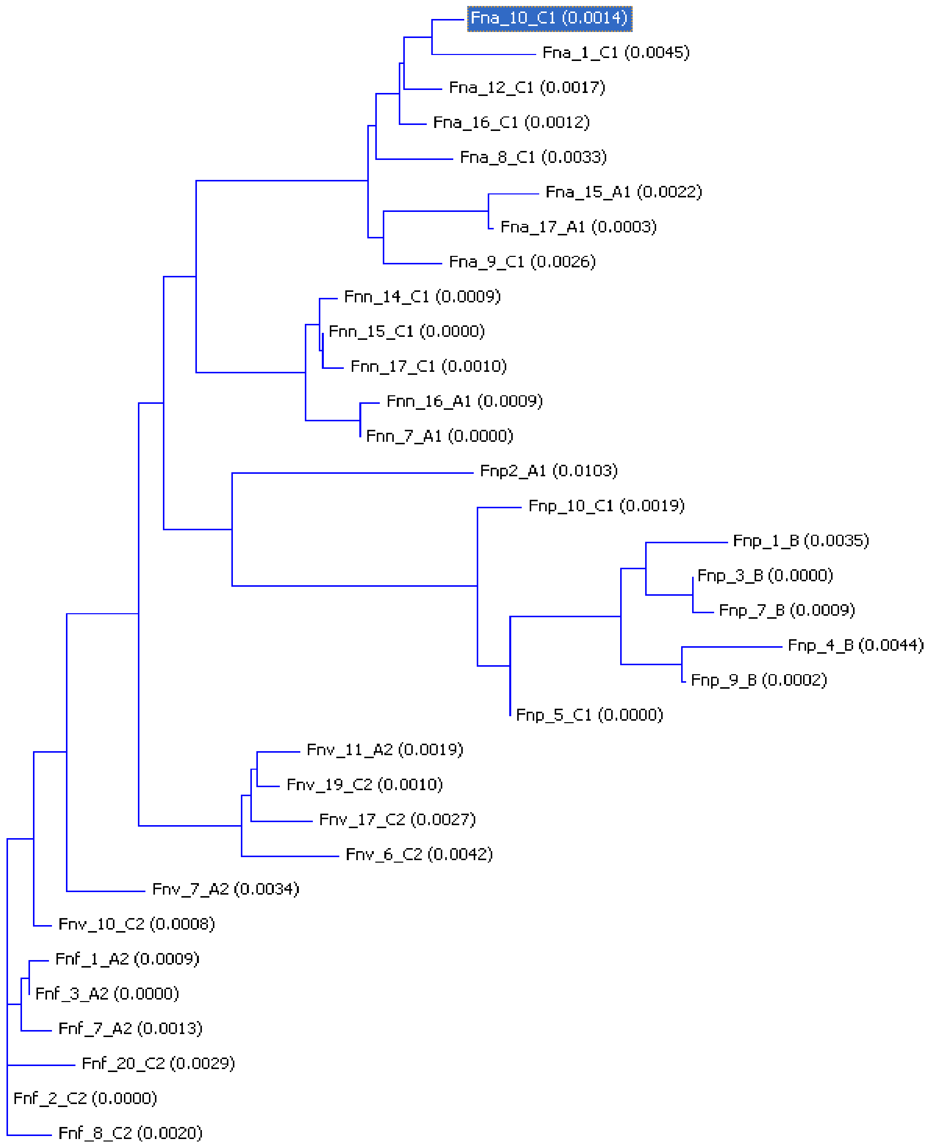


Figure 7.7 Phylogenetic relationships between the five subspecies of *F. nucleatum* clones constructed using fragments of DNA composed of 1290 bases of the 16S rDNA, the complete ISR, and the first 460 bases of the 23S rDNA of the *rrn* operon. Fna, *F. nucleatum* subsp. *animalis*; Fnf, *F. nucleatum* subsp. *fusiforme;* Fnn, *F. nucleatum* subsp. *nucleatum;* Fnp, *F. nucleatum* subsp. *polymorphum*; Fnv, *F. nucleatum* subsp. *vincentii.*

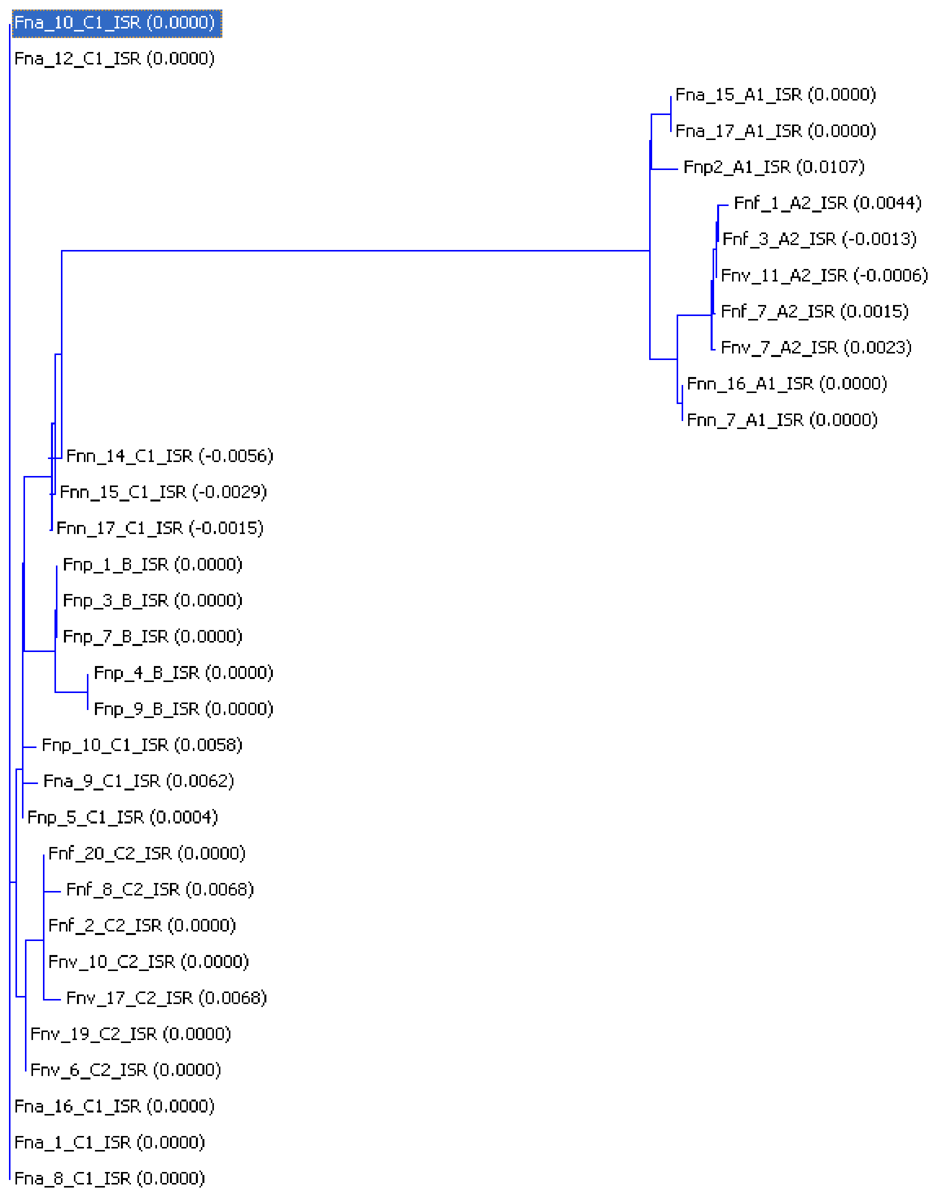


Figure 7.8 Phylogenetic tree showing genetic relationships between the five subspecies of *F. nucleatum* clones using the complete ISR of the *rrn* operon only. Fna, *F. nucleatum* subsp. *animalis*; Fnf, *F. nucleatum* subsp. *fusiforme;* Fnn, *F. nucleatum* subsp. *nucleatum;* Fnp, *F. nucleatum* subsp. *polymorphum*; Fnv, *F. nucleatum* subsp. *vincentii.*

7.7.5 Sequence analysis of *F. nucleatum* clones from samples of NGA, oral cavity and vagina from a single case.

Clones obtained from an individual sample of NGA were compared against the corresponding samples of the maternal oral cavity and vagina. Clones amplifying other non-*Fusobacterium* species were also observed in the samples; seven clones from the NGA sample were identified as *S. sanguinegens* and the remaining seven clones were confirmed as *F. nucleatum.* Two *Leptotrichia* spp. and two *L. buccalis* clones were identified in the oral cavity and the vagina, respectively, and these clones were excluded from the analysis. Consequently, 20 clones from the oral cavity and 14 clones from the vagina identified as *F. nucleatum* were included.

A 100% match between none of the clones of the NGA and either the oral cavity or the vagina was observed in case 10 (Figure 7.9 A-D). To facilitate analysis, the number of mismatches was calculated and the data grouped by size of ISR. Clones of the NGA were aligned against each clone of the oral cavity and the vagina and mismatches were counted. For the 331 bp ISR, the clone with the lowest number of mismatches in the 16S-ISR-23S rDNA fragment was observed with an oral clone (n= 14), compared to 19 in the vaginal clone (mismatches in the ISR were eight for both) (Figure 7.9 A). For the 222 bp ISR (Figure 7.9 B), no vaginal clones were identified and the minimum number of mismatches was 21 between the NGA and the oral clones (17 mismatches in the ISR only). For the 156 bp ISR clones, a minimum number of mismatches was observed with clones from the oral cavity (10 mismatches, only one in the ISR), while a minimum of 12 mismatches (3 in the ISR) were observed between the NGA and the vaginal clones (Figure 7.9 C). No clones from the NGA were identified of sizes 322 and 147 bp. In conclusion, the least number of mismatches were observed with the oral cavity clones.

7.7.6 Phylogenetic analysis to compare clones from samples of NGA, oral cavity and vagina from a single case.

Figure 7.10 represents the phylogenetic tree produced with clones obtained from the NGA and maternal samples (oral cavity and vagina) from a single case. Interestingly, it can be seen that clones from the NGA appear to originate from the same ancestor as clones the oral cavity, whereas clones from the vagina originate from a more distant ancestor. The data is also represented by grouping the clones by ISR length together with the clones of the five strains of *F. nucleatum* grown in this study (Figure 7.11 A-E). Figure 7.11 D clearly shows that the *F. nucleatum* strain (s) in the NGA may have originated from the oral cavity of the respective mother.

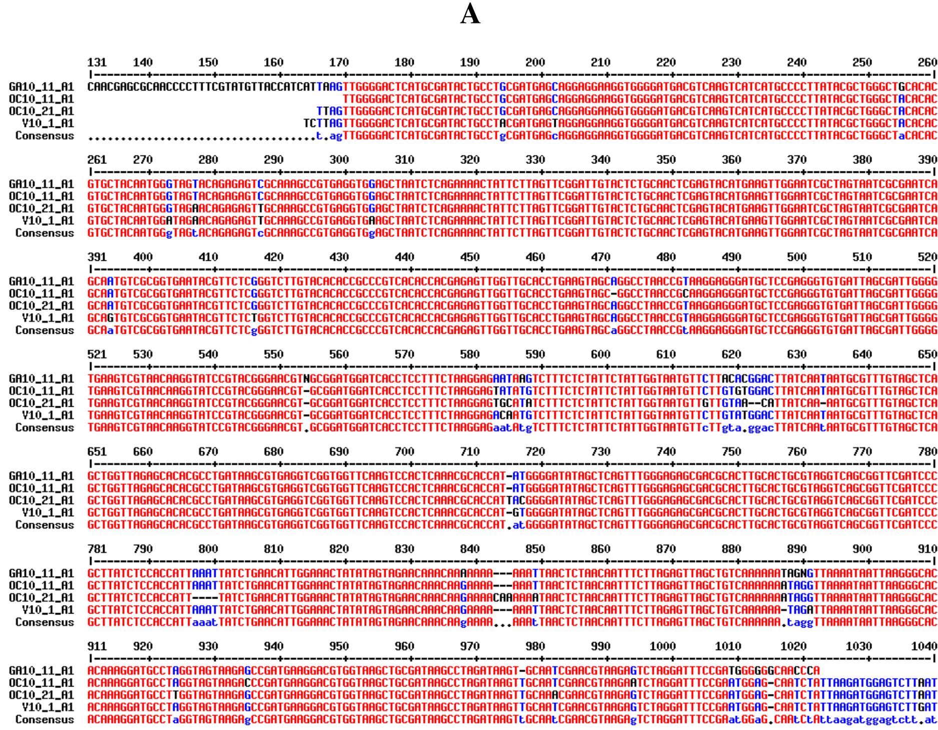


Figure 7.9 Comparison of aligned sequences of the 16S-ISR-23S rDNA of those clones obtained from the neonatal and maternal sites from **CASE 10**. Sequences from each ISR size are represented separately in figures A, B and C. GA10; neonatal gastric aspirate case 10; OC10, maternal oral sample case 10; V10, vaginal sample case 10. **A.** ISR fragment size 331 bp (A1).

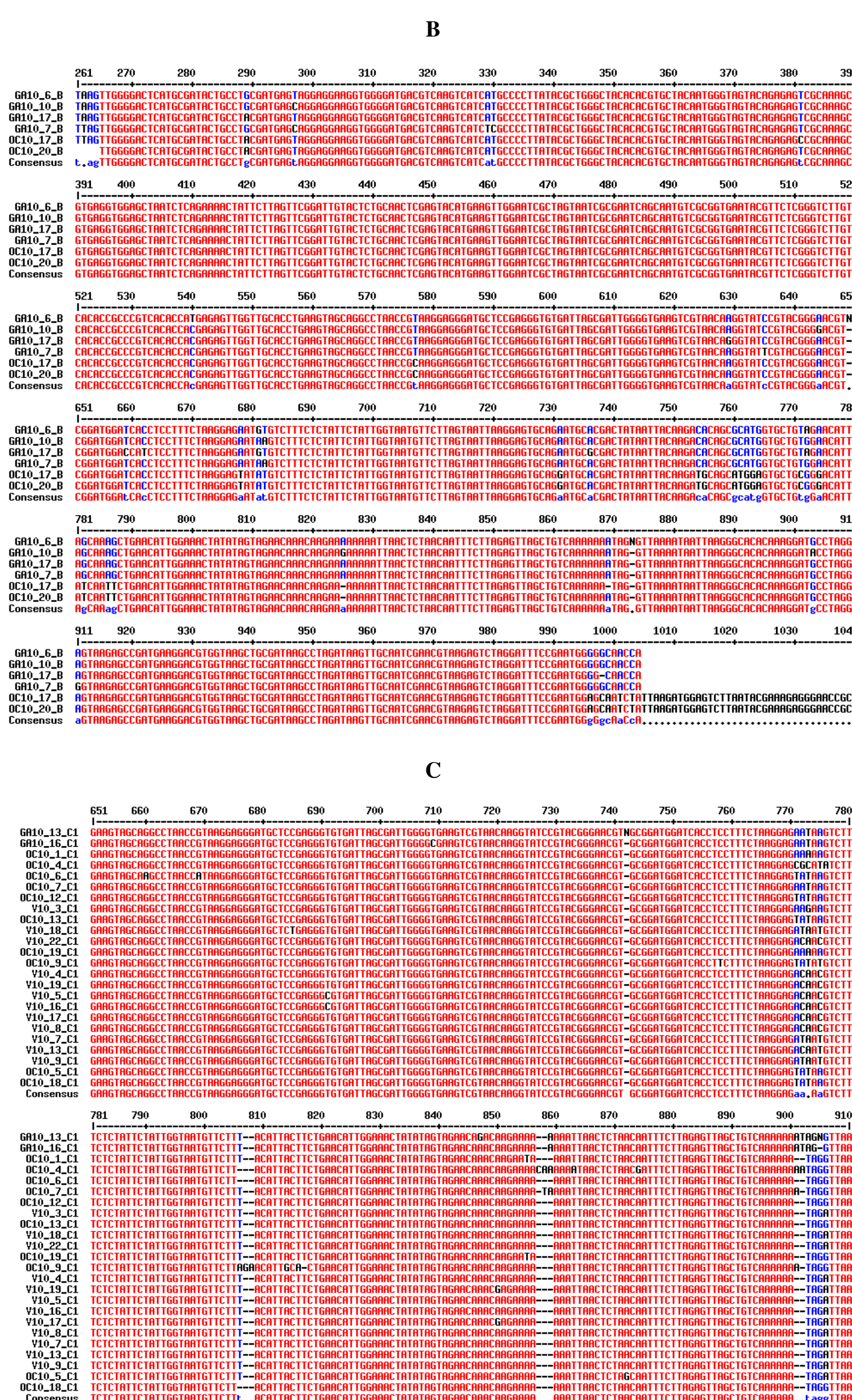


Figure 7.9 (cont.) **B.** ISR fragment size 222 bp (B). **C.** ISR fragment size 156 bp (C1).

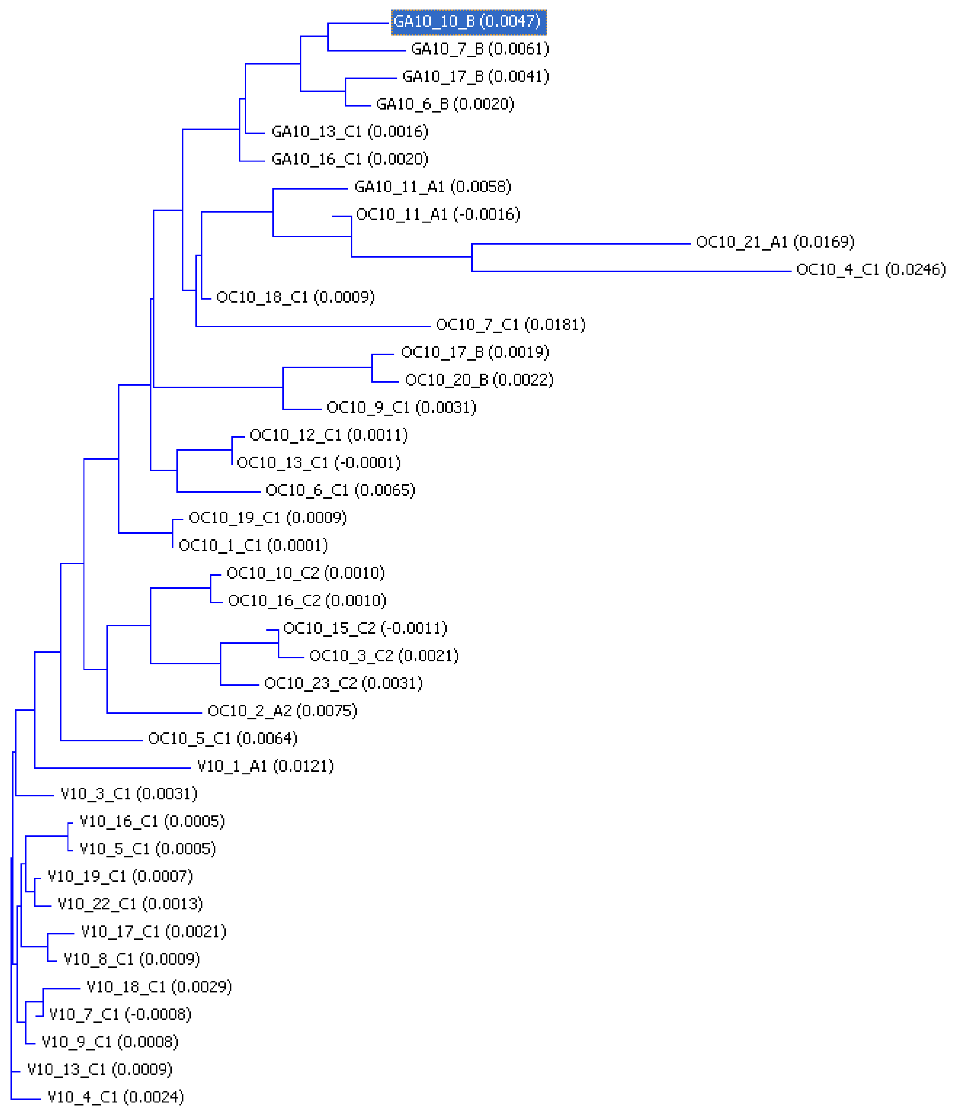
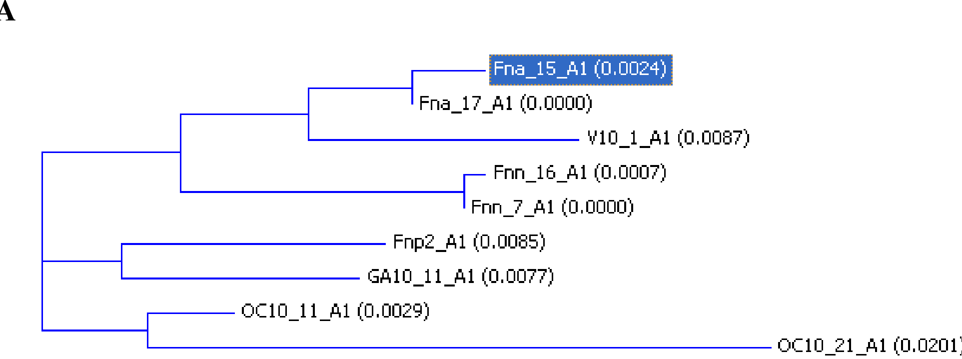
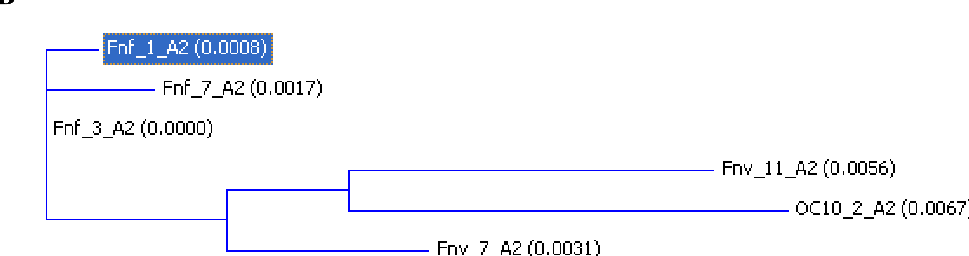


Figure 7.10 Phylogenetic tree showing genetic relationships amongst clones of *F. nucleatum* strains amplified from the neonatal and maternal samples from a single case study (**CASE 10**). GA10, clones of NGA from case 10; OC10, clones from the maternal oral cavity from case 10; V10, clones from the maternal vagina from case 10.

A



B



C

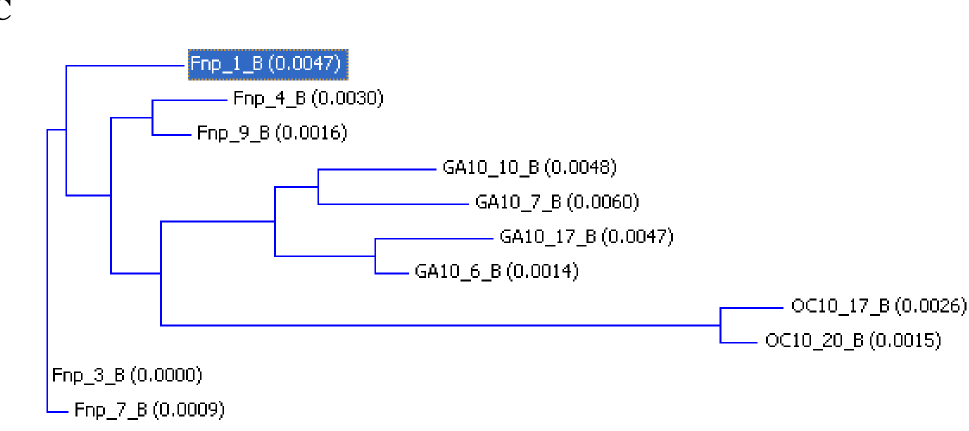
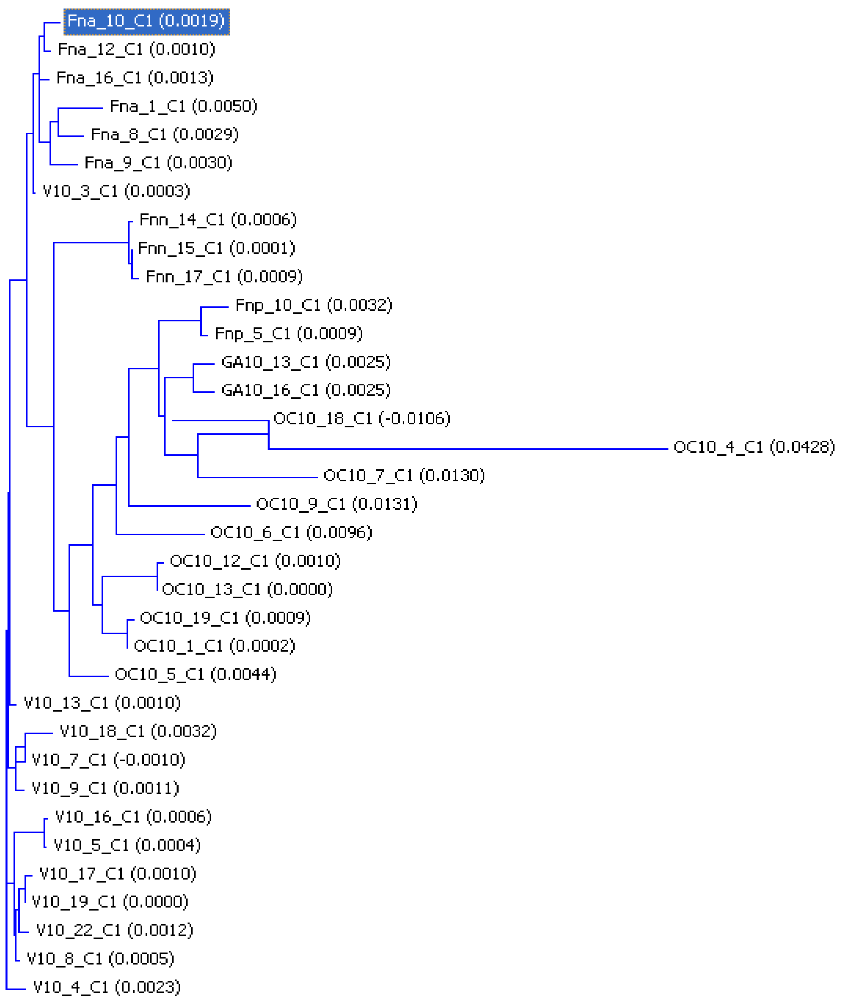


Figure 7.11 Phylogenetic relationships among clones of *F. nucleatum* strains amplified from the neonatal and maternal samples from a single case study (**CASE 10**) and comparisons with the *F. nucleatum* subspecies strains. GA10, clones of NGA from case 10; OC10, clones of the maternal oral cavity from case 10; V10, clones of the maternal vagina from case 10. Fna, *F. nucleatum* subsp. *animalis*; Fnf, *F. nucleatum* subsp. *fusiforme;* Fnn, *F. nucleatum* subsp. *nucleatum;* Fnp, *F. nucleatum* subsp. *polymorphum*; Fnv, *F. nucleatum* subsp. *vincentii.* **A.** ISR fragment size 331 bp (A1). **B.** ISR fragment size 322 bp (A2). **C.** ISR fragment size 222 bp (B).

D



E

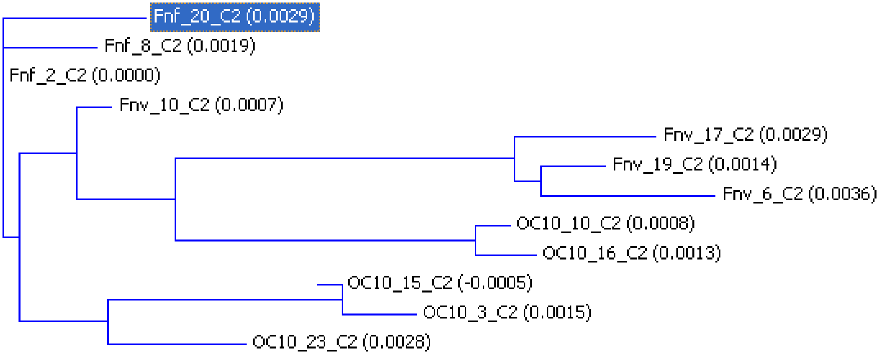


Figure 7.11 (cont.) **D.** ISR fragment size 156 bp (C1). **E.** ISR fragment size 147 bp (E).

7.8 Discussion.

This study aimed to assess the ability of the 16S-23S rDNA ISR to differentiate species of *F. nucleatum* at the strain level and to apply this methodology to determine the precise origin of *F. nucleatum* in NGA. Several studies have been performed to define bacterial species/subspecies and to investigate phylogenetic relationships using the ISR in a range of important clinical strains including *Enterococcus* spp. (Tyrrell et al., 1997), *Campylobacter* spp. (Christensen et al., 1999), *Pseudomonas* spp. (Guasp et al., 2000), strains of *Clostridium difficile* (Gürtler, 1993, Sadeghifard et al., 2006), subspecies and serotypes of *Salmonella enterica* (Christensen), *Streptococcus* spp. (Hassan et al., 2003, Chen et al., 2004), and *P. gingivalis* (Rumpf et al., 1999). In general, these studies demonstrated the markedly higher heterogeneity of this region as compared to the gold standard, the 16S rRNA gene, to differentiate closely related taxa.

Also, the ability of the ISR to allow discrimination of species belonging to the *Fusobacterium* genus has been analysed previously (Narayanan et al., 2001, Conrads et al., 2002, Jin et al., 2002b, Jin et al., 2002a). Only one study has presented data related to the length of the five subspecies of *F. nucleatum* (Conrads et al., 2002). The band sizes were calculated by approximation in an agarose gel and were determined as three to four bands for *F. nucleatum* subsp. *nucleatum*, three bands for *F. nucleatum* subsp. *animalis*, *fusiforme* and *animalis* and one band for *F. nucleatum* subsp. *polymorphum*. The ISR length was calculated for the shorter fragment only and lengths varied between 121 and 151 bp. The results presented here differ greatly with the ones previously published with respect to the number of bands and the length of the ISR.

As observed in this study, a total of five different lengths of the ISR were identified and calculated precisely in the five strains of *F. nucleatum* examined. A clear pattern was observed in terms of ISR length for each strain. Two fragments (a short 156 or 147 bp version, and a long 331 or 322 bp version) were presented in each strain, except for the subsp. *polymorphum* which also contained a third ISR (222 bp). This

variability is known to be due to differences in the *rrn* operons (Sadeghifard et al., 2006). As observed, the short and long fragments were identical in size for subsp. *animalis*, subsp. *nucleatum* and subsp. *polymorphum* (short: 156 bp, long: 331 bp). These differ from the corresponding ones for *F. nucleatum* subsp. *fusiforme* and *vincentii* (short: 147 bp, long: 322 bp). Both long versions contained a $tRNA^{Ile}$ and a $tRNA^{Ala}$ inserted in the ISR as detailed previously.

Differences in the size of the intergenic spacer region are not always suitable for strain identification (McLaughlin et al., 1993, Gill et al., 1994). The results presented here may not allow discrimination of the five subspecies using only a band pattern in a gel electrophoresis based technique. However, this information combined with construction of specific primers, could represent a useful tool for differentiation of the subspecies of *F. nucleatum* and possibly at the strain level. The mosaic pattern described for the *F. nucleatum* subspecies ISR has been previously described for other genera and species (Gianninò et al., 2001, Carr et al., 2004, Osorio et al., 2005, Sadeghifard et al., 2006) in which the presence and absence of one or more regions (also known as block types) were the reason for the length differences and the basis for more sensitive and discriminatory strain typing methods. In this study, a short variable region in the ISR (bases 28 to 32) (see Figure 7.6) could be clearly identified with variations specific for *F. nucleatum* subsp. *nucleatum* (AATGT), subsp. *polymorphum* (AATAA), subsp. *animalis* (TATAA), and *F. nucleatum* subsp. *vincentii* and subsp. *fusiforme* (AAAAA) that could be used for primer design or as a target site for restriction endonucleases. There is an ongoing discussion regarding *F. nucleatum* subsp. *vincentii* and subsp. *fusiforme* as regards classifying these as the same subspecies (Gharbia and Shah, 1992, Kim et al., 2010), which would explain the homogeneity found in this study and the inability to be correctly identified. This may also explain why subsp. *fusiforme* was not identified in the samples of NGA, oral cavity or vagina in this study.

Case 10 was included in this study to test the methodology proposed. This was a preterm infant, delivered by Caesarean section at 35 weeks of gestation; the mother had suffered a previous miscarriage and presented with localized chronic

periodontitis and pockets in four sites (4-5mm depth). As suggested in previous case reports (Han et al., 2006, Han et al., 2010), a 100% match was expected between the site of origin and the recipient site to precisely determine strain origin. However, the results of this study show that the clones did not show a 100% match. Instead, sequence variations were observed all of between the samples. This may be due to the limited number of clones per sample investigated or due to genetic changes within strains. The 16S-23S ISR region possess a non-coding function, therefore is less susceptible to selection pressure and it can therefore accumulate a higher percentage of mutations than the rRNA genes (Tyrrell et al., 1997). However, a certain degree of variation was also observed in the more conserved 16S and the 23S rDNA fragments included. The sequences of multicopy rRNA genes are mostly identical or nearly identical (Rajendhran and Gunasekaran, 2010). Another possibility may be due to PCR or sequencing bias and the formation of chimeric molecules (Wang and Wang, 1997), even though this was demonstrated to be very unlikely since a 100% match was observed when comparing the sequences obtained in this study with published sequence information for strain ATCC 25586. There is a clear need to accumulate more data regarding inter- and intraspecies variation of *Fusobacterium* species and subspecies, specifically of the ISR and the 23S rDNA regions, and to further evaluate the utility of this methodology to confirm translocation in a well designed study with an appropriate number of cases. Nevertheless, the preliminary data presented here demonstrates a high possibility that the strain of *F. nucleatum* present in the NGA originated from the maternal oral cavity strain rather than from the vagina.

CHAPTER 8

FINAL CONCLUSIONS AND SCOPE FOR FUTURE WORK

Samples of NGA contain a highly diverse range of bacteria. These sample have demonstrated a potential suitability to be analysed for investigation of those potential pathogens associated with the occurrence of perinatal infections and APO. Indeed, NGA should contain the bacteria that were able to reach and colonise the amniotic cavity before birth and therefore include those potential pathogens possibly involved in the initiation of the complications or the cause of early-onset infections in neonates. In general, the bacterial species identified in this study were consistent with observations made in previous studies (Oue et al., 2009, Jones et al., 2010). The bacteria identified in NGA also resembled those identified in studies investigating bacteria in the AF (Miralles et al., 2005). For example, high levels of *U. urealyticum* and *S. agalactiae*, as well as bacteria usually implicated in nosocomial outbreaks, have been observed in neonates that presented with a complication (Waites et al., 2009). Conversely, higher prevalence of *Lactobacillus* spp. has been observed in women/infants with no complications and therefore may have contributed to the prevention of adverse outcomes (Verstraelen et al., 2009).

One disadvantage, related to the analysis of these samples, may be associated with the presence of various bacterial contaminants. As observed in this study, bacteria identified in the samples may have originated from a range of external sources including aspiration of vaginal fluids during labour, vaginal and cervical bacteria acquired during vaginal delivery, skin bacteria, and other environmental bacteria obtained during collection and transportation of the samples. These circumstances would effectively make it more difficult to determine the true pathogens associated with pregnancy complications. The results from this study clearly demonstrated the need to control for certain confounding factors when the presence of given bacteria is being assessed. By excluding these interfering variables (i.e. neonates born vaginally, presentation of pROM, women who received iATB, smokers, samples collected at the maternal wards, and samples collected at more than 12 hours post delivery), the profile of bacteria in these samples will be limited to those most likely to be associated with pregnancy complications.

Similarly, the introduction of more advanced technologies for the investigation of complex bacterial communities in this study was of great value for the characterisation and understanding of the samples analysed. The range of taxa identified in NGA using a combination of molecular techniques has not been detailed before. Both clone analysis and DGGE were equally efficient in detecting the most common bacteria in the samples, yet DGGE is less expensive and facilitated a profile of the bacteria contained in the samples which may represent an advantage over clone analysis. Conversely, clone analysis seems to be more effective in identifying less prevalent species which was of a great benefit for the aims of this study. Particularly, the combination of both techniques represented a valuable methodology, specifically due to the great improvement in identifying those less prevalent species, such as potential bacteria from an oral origin.

The advent of new technologies such as next-generation sequencing allows a great leap forward in the analysis of complex microbial ecosystems (Snyder et al., 2009). The use of this technology to evaluate samples of NGA would not only allow the identification of the numbers and types of bacteria present in these complex samples, but also to better understand the pathophysiology of diseases and maternal susceptibility as regards APO. Nevertheless, the adjunctive use of a more sensitive quantitative technique, q-PCR, as used in this study provided an important contribution to the analysis of specific periodontal pathogens in the samples of NGA. In this context, next-generation sequencing technology does not yet provide a quantitative approach. Therefore, quantitative analysis remains the best approach for the measurement of levels of pathogens in neonatal samples (NGA). These estimates are particularly important in evaluating the clinical relevance of the bacteria in the complications since they allow quantification and also determination of the relative amounts of the total bacteria load.

The use of a species-specific and more sensitive technique also allowed a better understanding of the origin of the bacteria present in the samples. For example, using a detection limit of 500 cells/ml for identification of general bacteria, it could be inferred that higher levels of vaginal bacteria present in the samples of NGA were

most likely to be introduced into the uterine cavity during labour due to a suction-like effect rather that from the birth canal (Seong et al., 2008). An association with increase in gestational age at delivery and a lack of association with vaginal delivery when a detection limit was included supports this theory. This observation could explain presence of common vaginal strains in the samples obtained from Caesarean section deliveries, possibly acquired during labour. However, levels of bacteria in the birth canal during diseases such as BV may be altered. Therefore, in order to understand these associations, initiation of labour and diagnosis of BV should be investigated in further studies.

Of particular note, the use of a quantitative approach compared to the other method used allowed periodontal pathogens to be identified at a higher prevalence in the NGA samples. Prevalence of *F. nucleatum* and *G. elegans* was increased when compared to the end-point broad-range approach (clone analysis and DGGE), and *P. gingivalis* was for the first time detected in a relatively high percentage in two samples. In addition, absence of *A. actinomycetemcomitans* and *T. forsythia* in the neonatal samples was demonstrated. In general, even though the main pathogens associated with PD possess the mechanisms and virulence factors to translocate haematogenously, they were not commonly found in the samples of NGA samples. Conversely, *F. nucleatum* was observed at high prevalence and in high levels in the NGA; and *P. gingivalis* was observed in association with *F. nucleatum*. This indicates that *F. nucleatum* may not only be able to translocate haematogenously as extensively demonstrated previously; but may also serve as a bridge to assist other pathogens to invade and proliferate within the amniotic tissues. Co-migration properties between *F. nucleatum* and *P. gingivalis* have been demonstrated previously in cell culture (Saito et al., 2008), and their joint mechanisms in APO should be further investigated in animal studies.

Levels of the periodontal pathogens in the maternal oral cavity and vagina were also measured in this study. Their prevalence varied in the oral cavity: T. *forsythia* was observed in 100% of the oral samples, *A. actinomycetemcomitans* and *P. gingivalis* in 21%; and *G. elegans* in only 10% of maternal oral samples. Only *F. nucleatum*

was found to be present in the vaginal samples from women. *G. elegans* should be further analysed; as this was identified in 5 (8.6%) samples of NGA using both the broad-range and specific approaches. This oral pathogen has not been previously reported as a common inhabitant of any non-oral site in health but has been involved in several cases of infective endocarditis (Ohara-Nemoto et al., 2005). In this study, only a small number of samples could be evaluated for *G. elegans*. As observed, it was presented in low amounts and less than 1% of the total bacterial load in the oral cavity, and also at very low levels in the vagina (less than 0.01%). Further studies should include controlled studies in animal models, as well as using a higher number of neonatal and maternal samples to explore possible translocation of this potential oral pathogen.

Particular attention was given to *F. nucleatum* as this and previous studies strongly support its potential ability to translocate and cause APO (Han et al., 2004, Liu et al., 2007). In this study, the *F. nucleatum* in NGA closely resembled the strain(s) obtained from the oral cavity. This was demonstrated using two different methodologies. First, by means of direct sequencing to compare fusobacterial subspecies amplified from paired samples from the infant and the respective mother. Subspecies of *F. nucleatum* in neonates demonstrated a better agreement with the strains in the maternal oral cavity (*F. nucleatum* subsp. *polymorphum, vincentii,* and *nucleatum*) in contrast to *F. nucleatum* subsp. *animalis* which was more commonly present in the vagina. The sequence alignment comparison between the strains of Case 54 confirmed this. Secondly, with the analysis of the ISR obtained from the different clones/operons of each sample, Case 10 demonstrated sequence agreement between the neonatal and the oral *F. nucleatum* strains. Furthermore, a phylogenetic analysis clearly illustrated that the NGA strain may have originated from the oral cavity and not from the vagina.

It remains to be determined from analysis of a greater number of samples as to whether the vagina may also represent a reservoir for fusobacterial species that are able to translocate to the neonates. Besides, it would be of great interest to determine the proportion of mothers who can transmit to their infants (either oral or vaginal

strains), the proportion of mothers who possess the strains but do not transmit to their infant, and also the proportions of mothers not presenting with the suspected species but with a positive result in the infant. Finally, it will be interesting to explore those factors possibly involved in the association.

It can be inferred from the data that women who smoke during pregnancy may be at a higher risk of an intrauterine fusobacterial infection, since association with levels of general bacteria and *F. nucleatum* was observed with smoking in this study. Presence of periodontal pockets may represent another risk factor, since woman with pockets demonstrated higher levels of *F. nucleatum* in NGA. However, other parameters such as plaque levels or bleeding on probing should be investigated along with translocation, since periodontitis was not found to be a condition required for potential transmission of bacteria. Other potential ways of translocation were evaluated, such as oro-genital contact during pregnancy. Even though there was not an association with the presence of *F. nucleatum* in the vagina, most of the women whose infants contained *F. nucleatum* admitted having oro-genital sexual contact during pregnancy. Nevertheless, no other potential periodontal bacteria were observed in the vagina of women reporting as having had oro-genital contact during pregnancy. Transmission of periodontal bacteria during oro-genital contact is therefore not supported in this study.

Furthermore, it remains unclear if a 100% genetic match between a strain found at two different sites should be expected in order to unequivocally determine origin. In this study a 100% match was never observed between all strains examined. The complexity with respect to the number of *rrn* operons may represent a limiting factor, and therefore requires the need to analyse a greater number of clones. To avoid this disadvantage, analysis of a gene that exists in a single copy should be considered as an alternative. In any case, it is clear that there is a great need to increase generally the sequence information regarding *F. nucleatum* from a range of different strains. It should be advantageous if an agreement of data accessible from the different libraries available could be achieved. The recent developing technologies, such as next-generation sequencing, will help to ensure an exponential

increase in the current data regarding this and other bacterial species. The diversity of *F. nucleatum* subspecies, strains and *rrn* operons could then be favourably used to characterise patterns of strains of *F. nucleatum* in different body sites. For example, the analysis of agarose gel bands could be better evaluated as a tool for typing *F. nucleatum* strains.

Finally, it remains to investigate whether a direct mechanism involving entry of the infectious agents into the amniotic cavity can influence parturition through a local elevation of mediators regardless of the origin of the pathogen, or if the inflammatory response may differ considerably towards the bacterial species involved. It has been suggested that inflammatory responses vary in intensity depending on the pathogen(s) implicated in the intra-amniotic infection (Oh et al., 2010). This is mainly due to their individual differences in structure, pathogenicity, mechanisms of host invasion and colonisation patterns. Hence, it is critically important to understand the individual potential to induce the release of inflammatory mediators, specifically those that have been indicated to play a central role in the induction of parturition (IL-1β, IL-6, IL-8) (Kamel, 2010). For example, in a previous study, the inflammation caused by *F. nucleatum*, rather than the bacterial colonisation, was shown to stimulate the TLR4-mediated placental inflammatory response causing fetal death in mice (Liu et al., 2007). This could be further evaluated in tissue culture studies, and cytokine profile analysis in animal models and clinical cases. It is likely that a combination of pathogens, rather than a single strain increases the risk for APO through an additive effect.

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APPENDIX

1. Ethical approval granted by the Outer North East London Research Committee (formerly Redbridge & Waltham Forest Local Research Ethics Committee) Ref. No. 08/H0701/61 (August 2008).

2. Ethical approval-Amendment 1- Ref. No. 08/H0701/61. Outer North East London Research Committee. 22-July-2009.

3. Consent form.

4. Validated questionnaire.

5. Periodontal chart.

6. How to collect a self-taken vaginal swab.

ANNEX 1

Redbridge & Waltham Forest Local Research Ethics Committee
Board Room A
2nd Floor
Becketts House
2/14 Ilford Hill
Ilford
Essex
IG1 2QX

Telephone: 0208 9265025
Facsimile: 0208 926 5009

22 August 2008

Dr. Cecilia Gonzales-Marin
MPhil/PhD Student. Research in Dental Institute
Queen Mary, University of London
4 Newark Street
London
E1 2AT

Dear Dr. Gonzales-Marin

Full title of study:	**Possible Association between the Oral Microflora and Adverse Pregnancy and/or Neonatal Outcomes**
REC reference number:	**08/H0701/61**

Thank you for your letter of 20 August 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair on the 21st August 2008

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	2.2	21 July 2008
Application	1	24 April 2008
Investigator CV		24 April 2008
Protocol	2.2	21 July 2008
Protocol	2.1	23 April 2008
Covering Letter	1	12 May 2008
Covering Letter	1	20 August 2008
Letter from Sponsor	1	07 May 2008
Peer Review	1	21 April 2008
Statistician Comments	1	14 April 2008
Compensation Arrangements	1	07 May 2008
Questionnaire	1	
Questionnaire	2.2	21 July 2008
Participant Information Sheet	2.2	21 July 2008
Participant Information Sheet	2.1	23 April 2008
Participant Information Sheet	2.2	21 July 2008
Participant Consent Form	2.2	21 July 2008
Participant Consent Form	2.1	23 April 2008
Response to Request for Further Information	1	21 July 2008
Response to Request for Further Information	2	20 August 2008
interactive material	2.2	21 July 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0701/61	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Rev. Dr Joyce Smith
Chair

Email: janet.carter@redbridge-pct.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2 for other studies]*
Site approval form

ANNEX 2

Outer North East London Research Ethics Committee
Board Room A
2nd Floor
Becketts House
2/14 Ilford Hill
Ilford
Essex
IG1 2QX

Tel: 0208 9265025
Fax: 0208 926 5009

29th October 2009

Dr. C. Gonzales-Marin
Research in Dental Institute
Queen Mary University of London
4 Newark Street
LONDON E1 2AT

Dear Dr. Gonzales-Marin

Study title:	**Possible Association between the Oral Microflora and Adverse Pregnancy and/or Neonatal Outcomes**
REC reference:	**08/H0701/61**
Amendment number:	**1**
Amendment date:	**22 July 2009**

Further to my email today. I am pleased to confirm that the Chair approved the amended documents submitted and I all conditions of the favourable opinion for this amendment have been met.

Ethical opinion

The amendment is approved.

The Committee looks forward to receiving a report of your research findings in due course.

With best wishes

Yours sincerely

Janett Carter
Coordinator ONEL RE

CONSENT FORM (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications
REC Number: 08/H0701/61
Investigator: Dr. Cecilia Gonzales-Marin

Patient Identification Number for this trial: | | | | |

Please initial box to indicate agreement

1.	I confirm that I have read and understand the Information Sheet dated 21/07/08 (version 2.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw from the study at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the Barts and the London/ Queen Mary University of London, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I agree to my GP being informed of my participation in the study.	
5.	I give consent to the research group responsible for this study to obtain a surplus of my baby's sample of gastric aspirate to be used for this study.	
6.	I agree to be examined with respect to the health of my mouth and to contribute with samples of dental plaque, tongue surface brushing/ gentle scraping and saliva.	
7.	I agree to contribute a vaginal swab for the study.	
8.	I agree to complete the questionnaire provided and I give consent to the research team to access the information from my medical records as well as from my baby's medical records.	
9.	I agree to the use of an audio record during my evaluation. I understand that this will be only for the evaluator's aid to register my mouth health status information and that the examiner will not record any response, conversation or data using the audio record. I understand that the record will be destructed immediately after the information has been transferred to the appropriate chart.	

CONSENT FORM (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications
REC Number: 08/H0701/61
Investigator: Dr. Cecilia Gonzales-Marin

10.	I agree to take part in the above study.	

Name of Participant	Signature	Date
Name of Interpreter (if required)	Signature	Date
Name of Person taking consent (Investigator)	Signature	Date

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

Patient Identification Number for this trial: ☐☐☐☐

POSSIBLE ASSOCIATION BETWEEN THE ORAL MICROBIOTA AND ADVERSE PREGNANCY AND/OR NEONATAL OUTCOMES

Please answer the following questions **Date: ……………………..**

Please tick the box with a black pen ☑

Smoking and Drinking

1 *Do you smoke cigarettes now?*

Yes ☐ No ☐ (if no please go to Q.3)

2 *If yes, how many manufactured cigarettes do you smoke in a day?*

- Not every day, only occasionally ☐
- Less than 5 per day ☐
- 5-10 per day ☐
- 11-20 per day ☐
- Over 20 per day ☐

(Now go to Q.6)

3 *If not a present smoker, did you smoke in the past?*

Yes ☐ No ☐ (if no please go to Q.7)

4 *If yes, how many manufactured cigarettes did you smoke per day?*

- Not every day, only occasionally ☐
- Less than 5 per day ☐
- 5-10 per day ☐
- 11-20 per day ☐
- More that 20 per day ☐

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

5 *How old were you when you stopped smoking?*

……………… years.

6 *How old were you when you started smoking?*

……………… years.

7 *In the past 12 months have you taken an alcoholic drink?*

- No ☐
- Special occasions only ☐
- Once or twice a month ☐
- Once or twice a week ☐
- Twice a day or more ☐
- Almost daily ☐

8 *Compared to 1 year ago do you now drink:*

- None at all **(non drinker)** ☐
- The same ☐
- A lot less ☐
- A lot more ☐
- A bit less ☐
- A bit more ☐

9 *If you have given up or reduced drinking what was the main reason?*

- Illness/doctor's orders ☐
- Health precautions ☐
- Pregnancy ☐
- Finance ☐
- Other ☐
- Non drinker ☐ (if non drinker go to Q. 12)

Please specify other reasons…………………………………………………

……………………………………………………………………………………

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

10 *When you drink spirits or wine how many drinks do you usually have during the occasion?* (1 unit of alcohol being equal to 1 glass of wine)

- 1-2 units ☐
- 3-4 units ☐
- 5+ units ☐
- I don't drink spirits or wine ☐

11 If you do not drink spirits or wine and maybe drink beer instead, how many pints do you have during one occasion?

- 1-2 pints ☐
- 3-4 pints ☐
- 5+ pints ☐
- I don't drink beer ☐

Illness and medication during pregnancy

12 Have you had any urinary tract infections (e.g. cystitis) during your pregnancy?

Yes ☐ No ☐ (if no please go to Q.15)

No treatment/medication ☐

Treatment with medication ☐

13 Have you received any medication during this pregnancy?

- No ☐
- Once ☐
- 2-4 times ☐
- more that 4 times ☐

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

14 Did you receive any of the following medication during this pregnancy?

No ☐ Yes ☐

- Vitamin/iron supplements ☐
- Antibiotics ☐
- Topical creams ☐
- Other medication ☐

Dental treatment

15 How often do you visit the dentist?

- Never ☐
- Regularly once every six months ☐
- Regularly once a year ☐
- Only when in pain ☐
- Only when pregnant ☐

16 When did you last visit a dentist?

- I have never visit the dentist ☐
- Within the last 3 months ☐
- Within this pregnancy but more than 3 months ago ☐
- More than 1 year ago ☐
- More than 5 years ago ☐

17 The last time you visited the dentist was it for:
(If last visit was during pregnancy, please refer to it and explain reason and treatment received)

- No visit ☐
- A check up ☐
- Some trouble with your teeth ☐
- Some trouble with your gums ☐

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications
REC Number: 08/H0701/61
Investigator: Dr. Cecilia Gonzales-Marin

- Other (please specify) ☐

....

18 How often do you visit the dental hygienist?

- Never ☐
- Regularly once every six months ☐
- Regularly once a year ☐
- I went during this pregnancy ☐

19 What treatment did the dental hygienist give you at the last visit?

- Oral hygiene instruction ☐
- Polish your teeth ☐
- Scale your teeth ☐
- Hygiene instruction + polish ☐
- Scale and polish ☐
- Hygiene instruction + scale ☐
- All ☐
- No visit ☐

20 How often do you clean your teeth nowadays?

- Never ☐
- Less than once a day ☐
- Once a day ☐
- Twice a day ☐
- More than twice a day ☐
- Other (please specify) .... ☐

21 Do you use anything other than an ordinary toothbrush and toothpaste for cleaning your teeth?

Yes ☐ No ☐

- Interdental brush ☐
- Dental floss ☐

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications
REC Number: 08/H0701/61
Investigator: Dr. Cecilia Gonzales-Marin

- Toothpicks ☐
- Chewing sticks ☐
- Mouthwash (please specify) ☐ ……………………………..
- Other (please specify) ☐ ……………………………...

Diet

22 Were there any particular foods you avoided during your pregnancy?

………………………………………………………………………………………

………………………………………………………………………………………

23 Did you eat any particular foods more often than the normal during your pregnancy?

………………………………………………………………………………………

………………………………………………………………………………………

Paan

		Yes	No
24	*Have you ever been a regular paan chewer?*	☐	☐
25	*Do you add tobacco to your paan?*	☐	☐
26	*Have you ever chewed paan with tobacco?*	☐	☐
27	*Do you chew paan with tobacco at all nowadays?*	☐	☐

28 How many paan with tobacco do you usually chew each day?

……………………………………………………………………………

(If not a paan with tobacco chewer, please go to Q. 31)

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

29 How soon after waking do you usually chew your first paan with tobacco of the day?

- Less than 5 minutes ☐
- 5-15 minutes ☐
- 15-30 minutes ☐
- minutes-1 hour ☐
- 1-2 hours ☐
- more than 2 hours ☐

30 How many paan with tobacco have you chewed so far today?

- Less than 5 per day ☐
- 5-110 per day ☐
- 11-20 per day ☐
- Over 20 per day ☐

Ante-natal Care

31 Have you had ante-natal care?

- GMP ☐
- Hospital/GMP ☐
- Hospital only ☐
- Ante-natal classes ☐
- None ☐

32 When did you start ante-natal care?

- Before 20 weeks ☐
- 20-25 weeks ☐
- After 25 weeks ☐
- No care ☐

33 Do you remember your weight before this pregnancy? …………. Kg

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

34 Were you in the unfortunately situation of facing a stressful life event during this pregnancy?

- Domestic violence ☐
- Close family death or serious disease ☐
- Separation/Divorce ☐
- Insecurity over food/home ☐
- Stress at work ☐
- Extent physical activity ☐
- No ☐ (If no please go to Q.35)
- Other ……………………………………………………………………

35 If yes, when during the pregnancy did the situation happen or start?

- During first three months ☐
- After 3rd month and before 6th month ☐
- During last three months ☐

Paternal information

36 How old is this infant's father?

…………………. years.

37 What is your actual relationship with this infant's father?

- Married ☐
- Not-married but live together ☐
- Separated/Divorced ☐
- Not together, I have another partner ☐
- Other

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

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Investigator: Dr. Cecilia Gonzales-Marin

38 During pregnancy, did you have sexual relations with the infant's father/ or actual partner?

No ☐ Yes ☐

39 Did you perform any oral-genital contact during sexual relationship with your partner?

No ☐ Yes ☐

Socio-demographic characteristics

40 Which category best describe you (your race or ethic group)?

- Asian British ☐
- Asian Indian ☐
- Asian Bangladeshi ☐
- Asian Pakistani ☐
- Asian Middle East/Arab ☐
- Asian Chinese ☐
- Asian Japanese ☐
- Black British ☐
- Black European ☐
- Black Caribbean ☐
- Black American ☐
- Latino ☐
- White British ☐
- White West European ☐
- White Mediterranean ☐
- White North American ☐
- White Latin American ☐
- Mixed Asian/White ☐
- Mixed Black/White ☐
- Mixed Asian/Black ☐
- Other Mixed ☐
- Other (specify) ☐

…………………………..

QUESTIONNAIRE (Version 2.2 Dated 21/07/08)

Title of project: Possible Involvement of the Oral Microbiota in Pregnancy Complications

REC Number: 08/H0701/61

Investigator: Dr. Cecilia Gonzales-Marin

41 Which type of schools did you attend?

- Primary ☐
- Secondary ☐
- College/University ☐
- None ☐

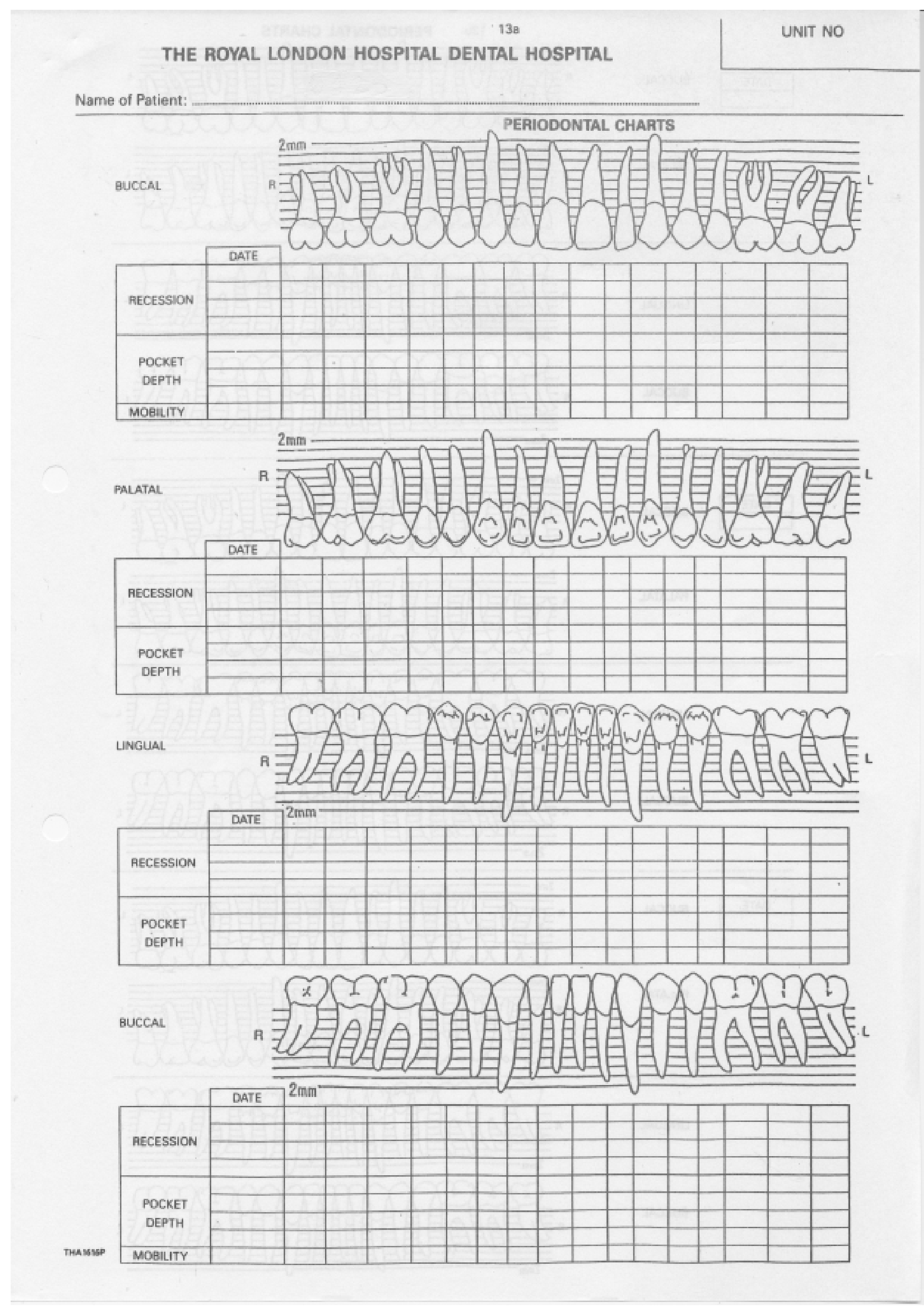
42 Please indicate your date of birth (dd/mm/yyyy)

d	d	m	m	y	y	y	y

Thank you for your help.

Name of Participant	Signature	Date
Name of Interpreter (if required)	Signature	Date
Name of Person taking consent (Investigator)	Signature	Date

ANNEX 5
PERIODONTAL CHART



13B

UNIT NO

THE ROYAL LONDON HOSPITAL DENTAL HOSPITAL

Name of Patient: ....

PERIODONTAL CHARTS

2mm

BUCCAL R L

DATE

RECESSION

POCKET DEPTH

MOBILITY

2mm

PALATAL R L

DATE

RECESSION

POCKET DEPTH

LINGUAL R L

2mm

DATE

RECESSION

POCKET DEPTH

BUCCAL R L

2mm

DATE

RECESSION

POCKET DEPTH

MOBILITY

THA1616P

HOW TO COLLECT A SELF-TAKEN VAGINAL SWAB

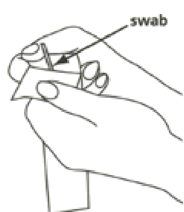
You can take the sample lying down with your legs apart, sitting down with your knees spread apart,
or standing with one foot on a chair.

Please read the following instructions before taking the swab:

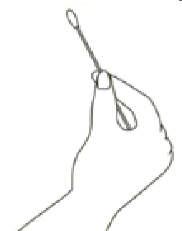
1. **Wash your hands**
Wash your hands thoroughly and rub them with the antiseptic provided, before and after taking your vaginal swab.

2. **If you have never used a tampon…**
You may feel more comfortable if you are familiar with where the swab will be going. Using your fingers, feel your labia (the lips or folds of skin which cover the entrance to your vagina). When you have done this, you can gently part your labia and feel the entrance to your vagina. This is where the swab will be inserted.

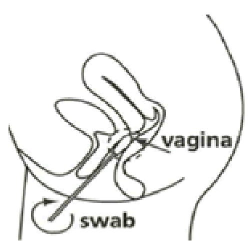
3. **Take the swab out of its packaging**
Peel apart the plastic wrapping from the end that is indicated and take the swab out.
Hold it at the end trying NOT to touch the 'cotton-bud' tip end.
You can ask for another swab if you make a mistake.



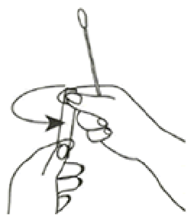
4. **Remove the swab**
Gently remove the swab, trying not to touch any other part of your body, as you may collect other bacteria by mistake!



5. **You are now ready to take the vaginal swab**
Holding the swab by its plastic lid end, gently part your labia. Guide the swab into your vagina. It will easily insert several centimetres – aim for about half of its length. This should not be uncomfortable. Once the swab is comfortable inside, **turn it around five times**.

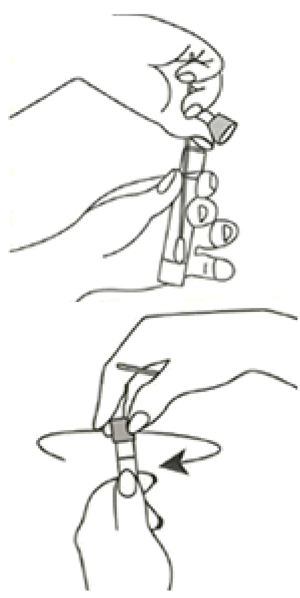


6. **Open the plastic tube**
DO NOT put the swab down –continue holding it



7. Re-insert the swab into the plastic tube.

Take the cap off the clear tube. Immediately, place the swab into the plastic tube with the end sitting in the liquid in the bottom of the tube.



8. Return the swab to the person responsible for the study.

Thank you for your help in this study!