

2016

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This article was originally published as:

Vytla, S., Mendz, G., & Quinlivan, J. (2016). Dental bacterial DNA are present in the amniotic cavity of healthy pregnant women at term. *Translational Medicine*, 6 (4).

Original article available here:

<https://www.omicsonline.org/open-access/dental-bacterial-dna-are-present-in-the-amniotic-cavity-of-healthy-pregnant-women-at-term-2161-1025-1000181.php?aid=77742>

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No changes have been made to this article which was originally published in *Translational Medicine* available at: [10.4172/2161-1025.1000181](https://doi.org/10.4172/2161-1025.1000181)

Vytla, S., Mendz, G., and Quinlivan, J. (2016) Dental bacterial DNA are present in the amniotic cavity of healthy pregnant women at term. *Translational Medicine*, 6(4). Doi: 10.4172/2161-1025.1000181

## Dental Bacterial DNA are Present in the Amniotic Cavity of Healthy Pregnant Women at Term

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Received date: August 01, 2016; Accepted date: August 09, 2016; Published date: August 29, 2016

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### Abstract

**Aims:** To determine if dental bacterial DNA are present in the amniotic cavity of healthy pregnant women undergoing an elective caesarean section at term utilising culture independent techniques.

**Methods:** Pregnant Australian women undergoing an elective caesarean section were recruited. Women completed questionnaires addressing demographics, past and current pregnancies and medical history. One high vaginal swab and three amniotic cavity swabs (amniotic fluid, newborn axilla and placental) were collected under sterile conditions. Samples were analysed using culture-independent techniques to detect the presence of predefined pathogenic bacterial taxa of the oral microbiome. Taxa isolated from the amniotic cavity swabs were compared to those isolated from the vaginal swab.

**Results:** DNA from taxa isolated from the amniotic cavity but not vagina included *A. xylosoxidans*, *A. tumefaciens*, *B. subtilis*, *Bartonella sp*, *Bergeyella sp*, *C. concisus*, *C. curvus*, *C. durum*, *D. microaerophilus*, *G. haemolysans*, *G. morbillorum*, *G. adiacens*, *G. elegans*, *K. pneumoniae*, *L. casei*, *L. paracasei*, *L. fermentum*, *P. aeruginosa*, *P. fluorescens*, *P. pseudoalcaligenes*, *P. stutzeri*, *R. microluginosa*, *S. maltophilia*, *S. pneumoniae*, *S. salivarius*, *S. sanguinis*, *V. dispar*, *V. parvula* and *Xanthomonas sp*.

**Conclusion:** The DNA of many pathogenic oral bacteria can be identified in the amniotic cavity of healthy pregnant women at term when utilising culture-independent techniques. Given DNA is not always present in the vagina, the study findings fulfill one criterion necessary for oral haematogenous spread to the amniotic cavity.

**Keywords:** Amniotic fluid; Culture independent; Intrauterine infection; Oral bacteria; Periodontal disease

### Introduction

An estimated one million infants die each year from preterm birth (PTB), defined as birth prior to 37 weeks gestation [1]. PTB causes significant mortality and morbidity, including respiratory distress syndrome, cardiovascular defects, sleep apnoea, dermatological conditions, immune system defects and central nervous system impairment [2]. The annual global economic cost of PTB exceeds fifty billion dollars. This represents a combination of medical costs, special needs services, as well as loss of household and labour market productivity owing to the ongoing disability of individuals [1]. There are many causes of PTB, however intrauterine infection (IUI) is a frequent and important contributing factor [3].

IUI accounts for 25 - 45% of spontaneous preterm deliveries [4]. Until recently, studies utilising culture-based techniques concluded the amniotic cavity was sterile under normal circumstances, prior to the initiation of labour [5]. The 'gold standard' for microbiological analysis involved collecting samples of the amniotic fluid by amniocentesis and employing microbial cultures to identify the presence of

microorganisms [6]. Based on these techniques, less than 1% of women in labour at term had bacteria in their amniotic fluid, and for many years the isolation of organisms from amniotic fluid was deemed a pathological finding [6].

More recent studies have demonstrated that even in cases where there were clear indicators of IUI, cultures of swabs from the amniotic cavity were often negative. For instance, inflammatory markers Prostaglandin F2 and Interleukin 6 were found to be elevated in women with negative culture results [7,8]. Other studies documented histological evidence of inflammation and chorioamnionitis in the presence of negative cultures [9,10]. This led to questioning of the methodological approaches of earlier studies. Is the amniotic cavity really sterile?

Recently, it has been proposed that the incongruent findings might be attributed to traditional culturing methods failing to detect some microbial species [11-13]. With the advent of culture-independent techniques such as broad-range PCR and fast sequencing to detect the presence of microbial taxa in the amniotic cavity, it is estimated the prevalence of microbes is higher than previously detected by culture-based methods [6].

There are four main pathways for entry of organisms into the amniotic cavity. Bacteria may gain entry to the amniotic cavity through ascending migration from the vagina, haematogenous dissemination, retrograde access from the peritoneal cavity through the Fallopian tubes, or iatrogenic introduction during invasive procedures [6]. There is evidence suggesting that oral microflora may spread to the amniotic cavity and play a role in intrauterine infections leading to preterm birth [14].

The term periodontal disease describes a group of infections affecting the gingival tissues of the oral cavity. There are two main broad categories of periodontal disease - gingivitis and periodontitis. Gingivitis is an inflammation of the gingival tissues without the loss of supporting soft tissue or bone, and periodontitis is characterised by loss of bone and soft tissue attachment, tooth mobility and potential tooth loss [15].

In periodontal diseases, inflamed gingival tissues release significant amounts of pro-inflammatory cytokines that have the potential to cause systemic effects. The increased permeability of these inflamed tissues results in a release of bacteria that potentially could lead to 'seeding' of bacteria in the amniotic cavity, where they could cause IUI [16]. Oral bacteria have been isolated from the amniotic cavity in the setting of clinical chorioamnionitis [17].

It has previously been hypothesised that periodontal disease may increase adverse pregnancy outcomes [15]. Inflammatory markers such as prostaglandin E2 (PGE2), along with endotoxins produced by Gram-negative periodontal organisms, are present in periodontal inflammation, and also regulate the normal physiological process of parturition. Release of these inflammatory markers, particularly PGE2, has the potential to initiate labour and lead to PTB [16]. Gingivitis and periodontitis have a significant incidence in pregnant women, providing support for the involvement of periodontal pathogens in IUI [15].

The manner by which oral bacteria might seed the amniotic cavity is unclear. Two leading theories are that seeding could arise by ascending migration from the vagina or haematogenous dissemination.

The aim of the present study was to document whether oral bacteria are present in the amniotic space of healthy pregnant women at term. If so, the secondary aim was to explore whether the presence of such bacteria was universally associated with the presence of the same bacteria in the vagina, or whether bacteria could be present in the amniotic cavity without being present in the vagina in individual women. This latter finding would constitute one piece of evidence in support of haematogenous dissemination. Oral bacteria in the vagina and amniotic cavity of healthy women giving birth at term were assessed utilising culture-independent methods and ultrafast sequencing to identify bacteria at the taxon level.

## Methods

### Study population

The study population consisted of 43 healthy pregnant women, booked for an elective caesarean section at term at Auburn Hospital in metropolitan Sydney, Australia, who were able to understand English and could complete a short questionnaire.

### Recruitment of participants

Women were recruited from the Obstetric Services prior to their planned delivery. A qualified nurse recruited all participants. Prior to recruitment all women had undergone a physical and dental examination and any detected problems had been corrected.

A short questionnaire was used to collect baseline data on age, ethnicity, gestational age, past pregnancies including any complications encountered, reasons for an elective caesarean delivery and complications during the current pregnancy such as infection, fever, vaginal discharge or pain, as well as past medical and surgical history. The information obtained through the questionnaires was de-identified.

### Consent process

To obtain consent, pregnant women were approached and requested to participate in the study, and were given verbal background information about the research. In addition, participants were provided with a plain English information sheet along with a consent form outlining the aims of the research project, contact details of the researchers involved, confidentiality information, any potential risks to the participants, as well as the right to refuse participation or withdraw their consent at any time, with no effect on their medical care.

Institutional ethics approval was granted by The University of Notre Dame Australia, The Western Sydney Local Health District and the Auburn Hospital in Sydney.

### Samples for analysis

Each participant agreed to have four swabs taken under sterile field conditions prior to or during their caesarean section. They were:

1. A vaginal swab collected from the posterior fossae of the upper vagina. This sample was obtained immediately prior to the caesarean section, just before insertion of the indwelling catheter;
2. An amniotic fluid sample collected immediately prior to delivery of the baby by the attending obstetrician;
3. A swab collected from the left axilla of the newborn immediately after delivery from the uterus by the attending obstetrician;
4. A swab collected from the maternal surface of the placenta immediately after its removal from the uterus by the attending obstetrician.

The samples were collected using plastic swabs with Dacron tips and were allocated a de-identified code for confidentiality and to avoid bias. Samples collected from the amniotic fluid, left axilla of the baby and placenta were pooled as amniotic cavity samples. All amniotic cavity samples were collected using aseptic techniques within a sterile operating field.

### DNA extraction, purification and sequencing

The swabs were stored at 4°C and sent to the University of Notre Dame Australia in Sydney for DNA extraction and purification. It was performed using QIAamp DNA Mini Kits (Qiagen; Chadstone Centre, VIC, Australia) according to the manufacturer's instructions. The concentration and quality of DNA was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies; Wilmington, DE, USA). The composition of the microbial communities in the amniotic cavity was determined by high-throughput sequencing of the 16S rDNA gene utilising a Roche 454 FLX instrument with Titanium

reagents. Tag-encoded amplicon pyrosequencing analyses were performed at the Research and Testing Laboratory (Lubbock, TX, USA) based upon established and validated protocols. These techniques have been used to analyse a broad range of environmental and health related microbiomes. The raw sequence data derived from the high-throughput sequencing process were analysed employing a pipeline developed at the same laboratory.

### Data analyses

Data were entered into a Minitab and password protected. The analyses of the sequence data yielded the number of reads (abundance) and identification of bacterial DNA (diversity) for each participant. It was from the detection of the bacterial DNA, that we inferred the presence of the bacterial species. This information was sent to the School of Medicine, Sydney where further analyses were conducted to characterise the microflora in the samples and assess the relative abundance of various phylotypes against a predefined list of oral bacteria.

### Bacteria targeted in the study

A literature search was performed for bacterial genera found in the oral cavity. A list was then compiled for bacteria isolated from various sites of the oral cavity such as the buccal mucosa, tongue, dentition, supra- and subgingival plaque [18-24]. The sequencing data were analysed searching for the presence of these predefined bacteria.

### Results

Out of 45 women approached, consent and final data were available from 43 women. Table 1 summarises the demographic data of participants.

Mean age	31.5 years
<b>Racial background</b>	
Caucasian	9 (20.9 %)
Asian	14 (32.6%)
Middle Eastern	8 (18.6%)
Indian	7 (16.3%)
Pacific Islander	5 (11.6%)
Mean gestational age	38.7 weeks
Median parity	1 (IQR 1-2)
<b>Complications in pregnancy</b>	
Diabetes	6 (14.0%)
Breech presentation	4 (9.3%)
Hypertension	2 (4.75)
Small for gestational age baby	2 (4.75%)
No complications	29 (67.4%)

**Table 1:** Demographic and pregnancy data of participants.

The mean gestational age at sampling was 38.7 weeks. All samples were collected at the time of caesarean section. During the pregnancy, none of the women had a symptoms of disease with an infectious aetiology. The minority of women were Caucasian, consistent with the racial background of women delivering at Auburn Hospital, which has a high proportion of women from Asian, Middle Eastern, Indian and Pacific Islander origin. Most women were parous, and the present pregnancy was uncomplicated. The most common indication for delivery was repeat caesarean section.

A summary of the taxa found in the oral cavity that were detected in both the vaginal and amniotic cavity are summarized in Table 2. Taxa isolated from the amniotic cavity but not the vagina included *A. xylosoxidans*, *A. tumefaciens*, *B. subtilis*, *Bartonella sp*, *Bergeyella sp*, *C. concisus*, *C. curvus*, *C. durum*, *D. microaerophilus*, *G. haemolysans*, *G. morbillorum*, *G. adiacens*, *G. elegans*, *K. pneumoniae*, *L. casei*, *L. paracasei*, *L. fermentum*, *P. aeruginosa*, *P. fluorescens*, *P. pseudoalcaligenes*, *P. stutzeri*, *R. microluginosa*, *S. maltophilia*, *S. pneumoniae*, *S. salivarius*, *S. sanguinis*, *V. dispar*, *V. parvula* and *Xanthomonas spp*.

*Streptococcus. mitis* was found in the amniotic cavity alone in 10/46 women. It was detected in the vagina sample of only one woman.

Genus	Species
<i>Achromobacter</i>	<i>A. xylosoxidans</i>
<i>Acinetobacter</i>	<i>A. baumannii</i>
<i>Atopobium</i>	<i>A. Parvulum</i> , <i>A. rimae</i> , <i>A. vaginae</i>
<i>Finexgoldia</i>	<i>F. Magna</i>
<i>Fusobacterium</i>	<i>F. nucleatum</i> , <i>F. periodonticum</i>
<i>Lactobacillus</i>	<i>L. acidophilus</i> , <i>L. crispatus</i> , <i>L. gasseri</i> , <i>L. iners</i> , <i>L. jensenii</i> , <i>L. johnsonii</i> , <i>L. reuteri</i> , <i>L. rhamnosus</i> , <i>L. salivarius</i> , <i>L. vaginalis</i>
<i>Peptostreptococcus</i>	<i>P. anaerobius</i>
<i>Prevotella</i>	<i>P. bivia</i> , <i>P. buccalis</i>
<i>Propionibacterium</i>	<i>P. acnes</i>
<i>Pseudomonas</i>	<i>P. fluorescens</i>
<i>Staphylococcus</i>	<i>S. aureus</i> , <i>S. caprae</i> , <i>S. epidermidis</i> , <i>S. warneri</i>
<i>Streptococcus</i>	<i>S. agalactiae</i> , <i>S. anginosus</i> , <i>S. intermedius</i> , <i>S. mitis</i>
<i>Veillonella</i>	<i>Veillonella atypica</i>

**Table 2:** Oral bacteria identified in the genital tract of pregnant women.

The bacterial species most frequently isolated from the amniotic cavity alone were *C. curvus* (12 participants), *P. aeruginosa* (7 participants), *S. salivarius* (6 participants), *C. concisus* (5 participants), *L. casei* (4 participants), *L. paracasei* (4 participants), *Xanthomonas* (4 participants), *K. pneumoniae* (3 participants), *A. xylosoxidans* (3 participants), *P. fluorescens* (3 participants) and *P. pseudoalcaligenes* (3 participants). The remaining taxa were isolated less frequently, but were also detected in the amniotic cavity only.

No differences were detected in the taxa present in the amniotic cavity of women with an uncomplicated pregnancy compared to those diagnosed with a pregnancy complication. However, the study lacked

power to identify if specific subtypes of pregnancy complications might be associated with changes in taxa.

## Discussion

Our first novel finding is that utilising culture independent techniques we have identified that the intra-amniotic space is colonised by many bacteria previously identified as dental pathogens. The oral cavity is a large reservoir for microorganisms comprising of over 700 bacterial species [18]. It appears the intra-amniotic space is also colonised by many bacterial species.

Our second key finding was that many, but not all, bacteria identified from the intra-amniotic space were detected in the vagina. This supports an argument for multiple methods of transmission between dental and intra-amniotic sites, specifically both ascending and via haematogenous routes.

The biological plausibility for oral microorganisms reaching the intra-amniotic space through a haematogenous pathway has been described in the literature. Wu et al described the human gingiva as an oestrogen dependent tissue, postulating that the rise in sex hormones in pregnancy plays a role in altering the topography and permeability of the gingival tissues [25]. This change can lead to an increased risk for bacterial spread from organisms routinely implicated in periodontal disease and translocation to the intrauterine space [16].

The Oral Conditions and Pregnancy (OCAP, 2004) study noted that the incidence of preterm birth was significantly higher in women with periodontal disease (28.6%) than those with a healthy periodontium (11.2%) [26]. Other studies have also reported an association between periodontal disease and risk of preterm birth and low birth weight [27, 28]. Clinical trials investigating maintenance of periodontal health in these women, suggest oral prophylaxis and periodontal treatment can lead to a 50% reduction in preterm births and a 57% reduction in preterm low birth weight [15].

The notion that oral bacteria have the potential for distant site infection is not new. Gendron et al. provided the earliest descriptions of the systemic significance of oral infections [19]. Along with major body systems such as the cardiac, respiratory, gastrointestinal and skeletal systems, spontaneous preterm birth resulting from amniotic fluid infection was one of the areas where the oral-systemic link was postulated [19]. Several studies have explored the oral-haematogenous route of infection and the concept that opportunistic infections from the oral cavity have a role in intra amniotic infection [16,17,19,26,28].

Microbial invasion of the amniotic cavity by oral bacteria can occur via the gastrointestinal tract, the vagina, and ascending into the uterus through the cervix. Detecting oral taxa in the intra-amniotic space that were also present in the vagina provides support for the ascending route of infection. This route is considered the most common route for female genital tract infection [6].

As outlined in Table 2, a number of oral bacteria were detected in the genital tract of pregnant women. Of these, 29 bacterial species were detected exclusively in the amniotic fluid, and not in the vagina. *Campylobacter curvus* was the most commonly identified oral bacteria detected in the amniotic fluid of 12/46 participants. The two *Campylobacter* spp. found only in the amniotic space are both opportunistic pathogens associated with gingivitis and periodontal disease, and also are emerging infections in the gastrointestinal tract [29].

*Pseudomonas aeruginosa* was isolated in amniotic cavity alone of 7/43 women. This species has been associated with periodontal disease in a previous study [30].

*Lactobacillus* species have been known to play a role in progression of dental caries, with high counts of certain *Lactobacilli* being associated with a higher caries risk [31]. *L. casei* was isolated in amniotic fluid but not vaginal samples in 4/43 of the pregnant women; the species has been associated with acid production in development of dental caries [24].

It has been established that oral-colonising bacteria could be present in the amniotic cavity of healthy pregnant women at term. The differences between vaginal and intra-amniotic bacterial populations suggest that some bacteria in the amniotic cavity may have originated from colonisation sites outside the female reproductive tract. The results for bacteria found in the intra-amniotic cavity but not the vagina of the same woman fulfil a criterion for evidence of haematogenous spread. Firm conclusions that the bacteria originated from the oral cavity cannot yet be drawn since some of these bacteria also can be found in other body sites [19], and haematogenous spread from those sites could also have taken place.

Despite these limitations, we have shown that a proportion of oral genera were not detected in the vagina of the participants, thus fulfilling one criterion necessary to support haematogenous spread of bacteria to the amniotic cavity. In future studies, the periodontal status of women undergoing an elective caesarean section could be assessed to investigate any correlations between their periodontal and genital microbial compositions. This study investigated the presence of oral bacteria in the amniotic cavity of healthy pregnant women at term. It would be interesting to move forward and conduct similar studies in women at risk of preterm birth, with the utilization of culture independent techniques.

## Acknowledgement

We are grateful to Dr. Hadia Mukhtar, Ms Susie Nanayakkara and the Maternity Unit Team for organizing the participants and collection of samples at Auburn Hospital.

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