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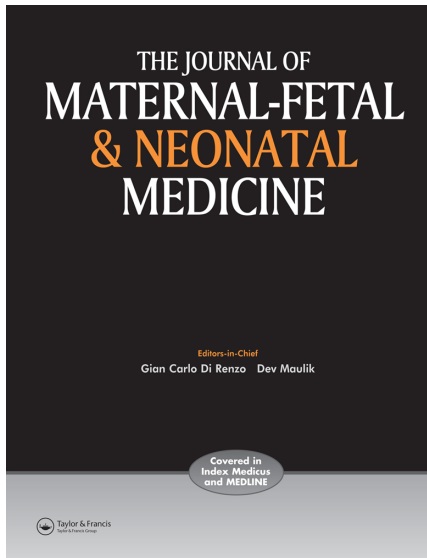
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The common vaginal commensal bacterium *Ureaplasma parvum* is associated with chorioamnionitis in extreme preterm labour

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Abstract

Objective: To assess the association of vaginal commensal and low grade pathogenic bacteria including *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, Group B *Streptococcus* (GBS), and *Gardnerella vaginalis*, in women who delivered preterm at less than 37 weeks gestation in the presence or absence of inflammation of the chorioamnionitic membranes.

Methods: A case control study involving women who delivered before 37 weeks gestation with and without inflammation of chorioamnionitic membranes.

A total of 57 placental samples were histologically examined for polymorphonuclear leukocyte infiltration of placental tissue for evidence of chorioamnionitis, and by type-specific nucleic acid amplification for evidence of infection with one or more of the target bacteria. Demographic data was collected for each mother.

Results: Amongst the 57 placental samples, 42.1% had chorioamnionitis and 24.6% delivered in the second trimester of pregnancy; *U. parvum*, *U. urealyticum*, *G. vaginalis* and GBS were all detected in the study with respective prevalence of 19.3%, 3.5%, 17.5% and 15.8%; *M. genitalium* and *M. hominis* were not detected. *U. parvum* was significantly associated with chorioamnionitis (p value = 0.02; OR 5.0; (95% CI 1.2-21.5) and was more common in women who delivered in the second (35.7%) compared to the third trimester of pregnancy (13.9%). None of the other bacteria were associated with chorioamnionitis or earlier delivery and all *G. vaginalis* positive women delivered in the third trimester of pregnancy (p value 0.04).

Conclusions: The detection of *U. parvum* in placental tissue was significantly associated with acute chorioamnionitis in women presenting in extreme preterm labour.

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Running title: *Ureaplasma parvum* and preterm labour

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Conclusions

The detection of *U. parvum* in placental tissue was significantly associated with acute chorioamnionitis in women presenting in extreme preterm labour.

Keywords

Pregnancy, placenta, trimester, intra-amniotic, *Ureaplasma*, prematurity, PCR, bacteria

Introduction

Preterm delivery is increasing and affects one baby in every 13 born (World Health Organisation, 2013). Preterm delivery, where a baby is born alive before completion of 37 gestational weeks, accounts for 75% of perinatal mortalities and a significant number of long term neonatal morbidities including chronic lung disease [1,2]. Intra-amniotic infection is currently a common cause of preterm delivery and is frequently caused by ascending genital tract infection [3,4]. Following ascent, microbes are capable of eliciting an inflammatory response in the chorioamniotic membrane by initiating an innate immune response resulting in the release of pro-inflammatory cytokines which are thought to trigger early labour [5,6]. Inflammation occurring across the chorioamniotic membrane is termed chorioamnionitis and has long been associated with preterm delivery [5,7-9].

Alteration of the vaginal microbiota and pH facilitates the ascent of microbes to the normally sterile amniotic cavity. The altered pH reflects a reduction in the protective lactobacilli species and an overgrowth of anaerobic commensals, including *Mycoplasma* and *Ureaplasma* species, a mechanism thought to facilitate ascending infection and adverse sequelae [4]. *Ureaplasma* and *Mycoplasma* are Gram negative facultative anaerobic bacteria which frequently colonise the genitourinary tract [10]. While *Ureaplasma urealyticum*, *Ureaplasma parvum* and *Mycoplasma hominis* can be regarded as commensal bacteria of the vaginal micro flora, there is supporting evidence they may act as low grade pathogens in pregnancy [11]. *Mycoplasma genitalium* is recognised as a low-grade urogenital pathogen that has been associated with cervicitis and complications during pregnancy [12-15]. *Gardnerella vaginalis* is a common vaginal commensal that is significantly altered in bacterial vaginosis, a microbial disturbance that is known to be linked to preterm labour [16]. *Streptococcus agalactiae* more commonly known as Group B Streptococcus [GBS], occurs as an intermittent vaginal coloniser present in up to 30% of pregnant women [17]. GBS is associated with early onset neonatal sepsis which has a significantly higher prevalence in preterm pregnancies [17, 18].

The present study aimed to assess if the detection of any of the 4 genital Mollicutes, *G. vaginalis* and/or GBS in placental tissue was associated with the presence of chorionic plate and/or membrane inflammation (chorioamnionitis) in a cohort of women who delivered less than 37 weeks gestation and if their presence was linked with earlier preterm delivery.

Methods

Inclusion and Exclusion Criteria

Placental specimens from women presenting in labour at the Royal Jubilee Maternity Hospital at <37 weeks gestation were eligible for enrollment and term pregnancies were excluded. In any pregnancy where a shared placenta was present, i.e in monochorionic twins or dichorionic triplets only one placental sample was included. Where twins or triplets did not share a placenta, independent samples for each baby were taken for analysis.

Demographic Data

Forty-nine pregnant women were enrolled into the study. There were 48 white European women and 1 Asian woman recruited. The median age of enrolled women was 29 years (range 18-42 years) with a median gestational age of 29 completed weeks (range 27-31 weeks). There were: 39/49 (79.6%) single pregnancies; 6/49 (12.2%) twin pregnancies and 4/49 (8.2%) triplet pregnancies. Six babies (sample numbers 2, 11, 37, 54, 55 and 64) were excluded from analysis due to sharing a placenta, leaving a total of 57 independent placental samples included in the study. A total of 33 of the 57 (57.9%) were delivered by Caesarean section, with a median birth weight of 1168g (range 890.75g –1598.75g).

Specimen Collection

Placental tissue samples were collected and sampled for later molecular analysis before being sent for histology to the Paediatric Pathology department of the Belfast HSC Trust. Deep placental tissue as opposed to superficial sampling at the mucosal surface was collected to decrease the risk of accidental bacterial contamination. Tissue samples were collected from the sub-chorionic plate area on the fetal side of the placenta. After lifting off the amnion membrane layer, a section of underlying tissue 1cm³ was dissected out aseptically and stored in sterile 25ml universal tubes at -70°C prior to nucleic acid extraction.

Histopathology

Each placental sample was subjected to a standard histopathological examination by a Consultant Paediatric Pathologist and categorised for the presence or absence of chorioamnionitis. The Pathologist was blinded to clinical outcomes and acute chorioamnionitis was defined if there were stereotypical patterns of inflammatory changes in chorionic plate or membranes (19).

Molecular testing

Nucleic acid extraction

Nucleic acid extracts were prepared using tissue protocol reagents from Qiagen QIAamp kits (Crawley, UK). Individual 20mg tissue samples were cut from stored specimens using sterile scalpel blades. They were homogenised in Buffer ATL (180µl) by bead-beating for 30 seconds in a 2ml micro-centrifuge tube using sterile 3mm tungsten carbide beads. A 20µl volume of Proteinase K was added and the homogenised samples were incubated at 56°C for 3 hours to completely dissolve the tissue. The resulting lysates were extracted using a standard QIAamp spin column protocol. Total nucleic acid from each specimen was eluted in Buffer AE (50µl).

Real-time quantitative PCR

Nucleic acid extracts were subjected to 6 real time TaqMan[®] qPCRs for the separate detection of: *U. urealyticum* and *U. parvum* urease genes (20); *M. hominis* 16s rRNA gene (21); *M. genitalium* MgpB gene (22); GBS sip gene (23); *G. vaginalis* 16s rRNA (24). Validation confirming the performance characteristics of each qPCR assay was undertaken separately before their application to the samples collected. Logarithmic serial dilutions of gene-specific plasmid standards with a known DNA concentration measured with a Nanodrop 2000 Spectrophotometer (Thermoscientific, Epsom, UK), were used to demonstrate a limit of detection between 10⁰-10¹ gene copies per reaction for each assay. No cross reactivity was observed for any of the qPCR assays when tested against a panel of nucleic acid extracts of 40 different species.

All assays were carried out using 1X Platinum[®] Quantitative PCR SuperMix-UDG (Life Technologies, Paisley, UK). Final working concentrations of reagents in the qPCR singular assays were as follows: 0.4 µM forward and reverse primer, 0.1 µM TaqMan[®] probe, 0.2 µg ml⁻¹ BSA (Thermoscientific, Epsom, UK) and 4mM MgCl₂ (Life Technologies, Paisley, UK). Final reaction volumes of 10µl made up of 2µl nucleic acid extract and 8µl mastermix were used. All real-time PCR reactions were performed using a LightCycler[®] 480 thermal cycler (Roche Diagnostics, Mannheim, Germany) with the following cycling conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 15 seconds and 59°C for 45 seconds. MS2 internal control was included in each real-time PCR run to monitor for inhibition, positive and negative controls specific to each qPCR assay were included in each run to monitor performance. Results were analysed using LightCycler[®] 480 software, version 1.5 (Roche Diagnostics, Mannheim, Germany) and recorded as cycle threshold (C_T) values; reactions which failed to produce a C_T value after 45 cycles were recorded as negative.

Statistical Analysis

Statistical analyses were performed using IBM SPSS 19.0 (SPSS Science, Chicago, IL, USA) to combine clinical and molecular data; p values of <0.05 (2 tailed) were regarded as significant. Placental tissue samples from women in the study were dichotomously categorised as (a) with or without chorioamnionitis (b) delivery in second (13-26 weeks gestation) or third (> 26 weeks gestation) trimesters of pregnancy. Fisher's Exact Test was used to compare the prevalence of the 6 organisms between the groups.

Results

Clinical characteristics

Amongst the 57 placentas, 24 (42.1%) and 33 (57.9%) respectively had or did not have histologically confirmed chorioamnionitis. A total of 14 (24.6%) and 43 (75.4%) women delivered respectively in the second and third trimester of pregnancy. Details from histopathology reports for each patient are outlined in table S1.

Molecular Data

Of the 57 placental samples tested, 26 (45.6%) were positive for at least one of the 6 target organisms. *U. parvum* was detected in 11 (19.3%) samples; *G. vaginalis* in 10 (17.5%) samples; GBS in 9 (15.8%) samples and *U. urealyticum* in 2 (3.5%) samples. *M. hominis* and *M. genitalium* were not detected in any samples. Placental samples positive for each bacterial target are outlined in table S2.

Molecular and Clinical Comparisons

The prevalence of *U. parvum* and marginally *U. urealyticum* and GBS were higher in samples with evidence of inflammation however only *U. parvum* reached statistical significance ($p=0.02$). Where chorioamnionitis was present or absent *U. parvum* was detected in 8 (33.3%) and 3 (9.1%) samples, GBS was detected in 4 (16.6%) and 5 (15.2%) samples and *U. urealyticum* was detected in 1 sample from each group (4.2% and 3% respectively). *G. vaginalis* was more commonly detected in samples without evidence of inflammation but this was not statistically significant ($p=0.12$) Where chorioamnionitis was present or absent, *G. vaginalis* was respectively detected in 2 (8.3%) and 8 (24.2%) samples. The comparison of organism prevalence between women with and without confirmed chorioamnionitis are summarised in Table 1.

The prevalence of organisms respectively detected in the second ($n=14$) and third ($n=43$) trimester of pregnancy were for *U. parvum* – 5 (35.7%) and 6 (14%); for *U. urealyticum* – 1 (7.1%) and 1 (2.3%) and for GBS – 3 (21.4%) and 6 (14%). All 10 *G. vaginalis* positive

samples were detected in the third trimester of pregnancy (23.3%). The comparisons between both trimesters of pregnancy are outlined in Table 2. The higher prevalence of *U. parvum* in women delivering in 2nd trimester compared to 3rd trimester approached statistical significance ($p=0.07$). The opposite association was seen for *G. vaginalis* where a higher prevalence in women delivering in the third trimester compared to the second trimester reached statistical significance ($p=0.04$). While GBS and *U. urealyticum* had higher prevalences in women delivering in the second trimester these did not approach statistical significance ($p=0.50$ and $p=0.40$ respectively).

Discussion

While intrauterine infection is regarded as an important cause of preterm delivery [3,4] the mechanism involved is less clear. The vaginal microbiota is thought to be protective against preterm labour and its disruption, as in bacterial vaginosis, is felt to be a risk factor allowing low pathogenic vaginal bacteria to ascend the genitourinary tract [3,4]. The focus of this study was to determine if common vaginal bacteria such as *Ureaplasma* and *Mycoplasma* species, GBS and *G. vaginalis* were associated with inflammatory changes in the placenta of women delivering at <37 weeks gestation. Highly sensitive qPCR assays, including ones that could discriminate between *U. urealyticum* and *U. parvum*, were chosen to overcome technical deficiencies of previous studies that have produced conflicting findings [25-30]. In the current study 57 placental tissue samples from 49 women were tested. *G. vaginalis*, GBS, *U. urealyticum* and *U. parvum* were detected while *M. genitalium* and *M. hominis* were not detected. *U. parvum* had the highest prevalence (19.3%), followed respectively by *G. vaginalis* (17.5%), GBS (15.8%) and *U. urealyticum* (3.5%).

Infiltration of the chorioamnion by polymorphonuclear leukocytes on histopathological examination was used as a biomarker of inflammation and was confirmed in 24 (42.1%) placentas in the study, suggesting that just over 40% of the preterm births were potentially linked to infection. While detection of *U. urealyticum* and GBS were more common in placental tissue with inflammation compared to tissue without, the proportions did not reach statistical significance. However, the detection rate of *U. parvum* in tissue with chorioamnionitis was significantly higher ($p=0.02$) than the rate detected in tissue without chorioamnionitis suggesting it could have an association with inflammation leading to preterm labour. A larger study should address this association and whether the association was linked to treatable dysbiotic disruption of the vaginal microbiota.

Traditionally *U. urealyticum* biovars 1 and 2 were regarded as a single species but recently they have been separated into two distinct species, namely *U. urealyticum* (formerly biovar 2) and *U. parvum* (formerly biovar 1). This has confounded results in publications prior to the separation [31]. Although previous studies associated *U. urealyticum* with stillbirth,

spontaneous abortion, chorioamnionitis and preterm labour [32], the majority failed to discriminate between both species and used insensitive detection methods [25-28]. Therefore clarity is lacking on the respective roles of *U. urealyticum* and *U. parvum* in pregnancy[33]. Although genital *Mycoplasma*, *Ureaplasma* and other pathogens have been associated with preterm delivery [34, 35], a high proportion of preterm births have no identifiable cause confirmed [36]. At present, the knowledge base regarding intrauterine infection is inconclusive, in particular the ability of *Mycoplasma* and *Ureaplasma* species to induce inflammatory responses in the uterine cavity [27]. Assessing the association of *Ureaplasma* species with chorioamnionitis in women delivering preterm is important to help assess their role as pathogens in intrauterine infection and preterm delivery [32,37]. The separate detection of *U. urealyticum* and *U. parvum* in clinical specimens from women with and without chorioamnionitis is a prerequisite for this type of study.

Previous studies have reported that inflammatory response induced by *U. parvum* is frequently associated with preterm delivery [38,39]. While *G. vaginalis* and *U. parvum* have similar colonisation prevalence rates of >50% in the vaginal microbiota of pregnant and non-pregnant women [40,41], their recovery in the current study in relation to chorioamnionitis and the gestational age at delivery were inverse. *U. parvum* showed a strong association with placenta inflammatory infiltration and with delivery in second trimester supporting an association with preterm labour. *G. vaginalis* showed no association with placenta inflammatory infiltration and was more prevalent at later gestational ages suggesting a non-pathogenic role in preterm labour.

Findings from this study coincide with those reporting a strong association between intrauterine infection and delivery at <30 weeks gestation [36]. Furthermore, a recent study reported a lower preterm delivery rate in pregnant women treated for *M. hominis* and *Ureaplasma* species [42]. Those findings and the results of the current study support the need for further research into the pathogenesis of *U. parvum* associated preterm labour and the potential for its prevention. Interestingly GBS showed no association with inflammatory infiltration of the placenta, and so did not appear to be linked to preterm labour through urogenital inflammation. However like *U. parvum* it was more common with earlier gestational delivery, suggesting GBS might have a non-inflammatory role in preterm labour. One possible explanation for this might be linked to the recent observation in gravid primates that in-utero infection with GBS can result in the down regulation of multiple cytokeratins and other cytoskeletal genes critical for maintenance of cellular membrane integrity and tensile strength, potentially leading to preterm labour [43]. These changes took place in the absence of inflammation. Additionally GBS has been shown to down-modulate the innate response to

infection in the chorioamnion which could again explain the apparent lack of inflammation in the placenta samples tested [44].

Although relatively small in overall numbers, we believe a real strength of the study was the recruitment of a number of women with extreme preterm pregnancies. From a technical perspective culture-based detection methods often used in the diagnosis of intrauterine infection in women with adverse pregnancy outcomes lack precision and are suboptimal in the identification of the highly fastidious species of the *Ureaplasma* and *Mycoplasma* genera. Thus another main strength of this study was the utilisation of qPCR assays with high analytical sensitivity and specificity to target bacteria that have been previously associated with pregnancy complications and with some that are also common in the vaginal microbiota. The application of these assays to placental tissue with and without histological evidence of chorioamnionitis, allowed a link to be established with a potential trigger of preterm labour. A perceived weakness in the study design could be exclusion of term pregnancies however since the question addressed was the association with inflammation in preterm delivery and not whether the infections triggered it, the rational controls had to be preterm deliveries lacking histological inflammation of the chorioamniotic membranes. The link seen with *U. parvum* and earlier preterm delivery suggests the association would be absent at term and it is probably causal.

The results suggest an association between *U. parvum* and inflammation of the chorioamniotic membrane in preterm labour; the increased number of positive women with delivery in the second trimester would also suggest an association with preterm delivery. The mechanism for *U. parvum* gaining access to the chorioamnion was not addressed. While a potential association for GBS in preterm delivery was also suggested by the findings, the data did not support a role in placental inflammation. The findings suggest the need for further investigation of the role of *U. parvum* as a trigger of preterm labour and the findings would support an extension of the present study to include term pregnancies.

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None

Disclosure of Interests: None

Details of ethics approval: All specimens were collected with informed consent under ethics approval from Office for Research Ethics Committees Northern Ireland (ORECNI) REC reference number: 07/NIR02/144. Date of approval 22nd February 2008.

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Table 1 Comparison of organism prevalence between women with or without chorioamnionitis and/or funisitis

	Chorioamnionitis		P value [#]	Odds ratio	95% confidence intervals	
	Present n=24	Absent n=33			lower	upper
<i>U.parvum</i>	33.3% (8)	9.1% (3)	0.02	5.0	1.2	21.5
<i>U.urealyticum</i>	4.2% (1)	3.0% (1)	0.82	1.4	0.08	23.4
<i>G.vaginalis</i>	8.3% (2)	24.2% (8)	0.12	0.3	0.05	1.4
GBS	16.6% (4)	15.2% (5)	0.88	1.1	0.27	4.7

[#]Fisher's Exact test.

Table 2 Comparison of organism prevalence between women delivering in second and third trimesters of pregnancy

	qPCR positives		P value [#]	Odds ratio	95% confidence intervals	
	second trimester delivery (13-26 weeks)	third trimester delivery (after 26 weeks)			Lower	upper
Total no. of samples	14	43	-	-	-	-
<i>U.parvum</i>	35.7% (5)	14% (6)	0.07	3.4	0.9	13.7
<i>U.urealyticum</i>	7.1% (1)	2.3% (1)	0.40	3.2-	0.2	55.3
<i>G.vaginalis</i>	0	23.3% (10)	0.04	-	-	-
GBS	21.4% (3)	14% (6)	0.50	1.6	0.4	7.9

[#]Fisher's Exact test.