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Isolation of separate *Ureaplasma* species from endotracheal secretions of twin patients.

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Short title: *Ureaplasma* isolated from the lungs of dizygotic twins

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Abbreviations:

ePTB: Early preterm birth

NICU: Neonatal Intensive Care Unit

CPAP: Continuous positive airway pressure

NEC: Necrotising enterocolitis

ETA: Endotracheal aspirates

USM: Ureaplasma selective media

CCU: Colour Changing Unit

MBA: Multiple-banded antigen

Contributors' statements:

Michael L. Beeton: Collated the data and wrote the manuscript

Nicola C. Maxwell: Consented parents, collected samples and analysed clinical data

Victoria J. Chalker: Developed and validated the qPCR assay for clinical patient samples and oversaw molecular quantification of *U. parvum*

Rebecca J. Brown: Extracted clinical samples and performed the qPCR analysis on samples

Ali F. Aboklaish: Quantified bacteria by culture techniques and performed immunoblot analysis.

O. Brad Spiller: Designed the experiments, coordinated all data collection and data analysis

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Abstract:

Isolation of *Ureaplasma* spp. from preterm neonates and the association with development of bronchopulmonary dysplasia has been previously investigated. However, few studies have contrasted the nature of infection in twins. Here we report that dizygotic twins (1 female, 1 male) born at 24 weeks gestation both yielded culturable *Ureaplasma* from endotracheal secretions. The samples were part of a serial blind collection cohort of ventilated premature neonates, and analysis of repeat cultures showed stable separate infections over a period of 17 and 21 days respectively. Immunoblot and probe-specific qPCR analysis determined that Twin1 was solely infected with *Ureaplasma parvum* (specifically serovar 6 by gene sequencing), while Twin2 was solely infected with *Ureaplasma urealyticum* (specifically genotype A- serovars 2, 5, and 8 by gene sequencing). Immunoblot analysis found that the major surface antigen (multiple-banded antigen; MBA) altered relative mass for both strains during the course of infection. Quantitative PCR analysis of extracted endotracheal aspirates confirmed no evidence of mixed infection for either twin. Failure of sentinel ventilated preterm infants on the same ward to acquire *Ureaplasma* infection after the first week of birth suggest no cot-to-cot transfer of *Ureaplasma* infection occurred. This study demonstrated not only a contrasting clinical outcome for a set of twins infected with two separate species of *Ureaplasma*, but also the first real-time demonstration of MBA alteration and evolution of *Ureaplasma* over the course of a clinical infection.

Introduction:

The Office for National Statistics has reported a steady rate of preterm birth (<37 weeks gestation) of 7.1-7.3% since 2009 in the UK. Those born <28 weeks gestation (early preterm; ePTB) are of greatest clinical significance; 66% of which are associated with chorioamnionitis and *Ureaplasma* spp. are the most common microorganisms isolated from cases of ePTB.^{1, 2} Furthermore, respiratory *Ureaplasma* spp. infection is found to be concentrated (up to 65%) in ventilated ePTB postnatally.³ However, the mechanism by which *Ureaplasma* gain entry to the amniotic sac as well as the differential pathogenicity of the two species is still under debate.

Only two previous twin studies on intrauterine infection or chorioamnionitis have examined *Ureaplasma* spp, but preferential infection of the presenting twin has been reported.^{4, 5} Here we present a unique case study examining repeated endotracheal samples where each twin was solely infected with a separate species of *Ureaplasma*, with different clinical outcomes as well as evidence of bacterial evolution during infection.

Methods:

Patient details and ethical approval

The mother was fit and well, her first pregnancy, a non-smoker during pregnancy with no abnormal antenatal serology. She presented with spontaneous onset of labour at 24 +4 weeks gestation followed by vaginal delivery of twins (Twin1 cephalic – female and Twin2 breech - male).

Twin1 was transferred from birth hospital to nearest tertiary neonatal intensive care unit (NICU) on day 1. She was extubated on day 20 to continuous positive airways pressure (CPAP) after commencing diuretics, but as a result of increasing apnoeas, bradycardias and desaturations required re-intubation on day 23. Re-intubation was accompanied by administration of antibiotics (teicoplanin and gentamicin). The twin was extubated on day 31.

Due to high ventilation requirements and recurrent pulmonary haemorrhages Twin1 was too unstable to transfer on day 1 and arrived at the NICU on day 5. This infant remained on ventilation until his death on day 32. Primary cause of death was determined as necrotising enterocolitis (NEC) with secondary causes listed as prematurity, chronic lung disease of prematurity and intracranial haemorrhage. No data on chorioamnionitis was available.

Ethical consent was received and approved by local ethics board

Sample transport and culture methods.

Endotracheal aspirates (ETA) samples were taken as part of the Ureatrack study and were transported to University Hospital of Wales, Cardiff, for analysis. Infectious *Ureaplasma* spp. titre was determined by culture in *Ureaplasma* selective media (USM) (Mycoplasma Experience, UK) in triplicate upon arrival, while aliquots were frozen (-80°C) for later DNA extraction and quantitative PCR determination of *U. parvum*, *U. urealyticum* and *M. hominis* genomes at Public Health England by published methods.^{6, 7}

Analysis of *Ureaplasma* isolates

Positive cultures from each sample were subjected to *Ureaplasma* spp. PCR genotyping analysis and results were confirmed by sequencing the purified DNA amplicons (Eurofins MWG, Germany).⁸ Cultured *Ureaplasma* isolates from each positive patient sample were also analysed by previously published immunoblot analysis, using a panel of characterised monoclonal antibodies (Virostat, Portland, ME) that recognise the serovar-determining multiple-banded antigen (MBA) [submitted manuscript] that can differentiate between *Ureaplasma parvum* and *Urealyticum urealyticum* as well as some serovars.⁹ MBA protein mass was determined relative to PageRuler™ protein mass standards (ThermoFisher, UK).

Results:

Culture and qPCR data graphs

Bacterial loads were quantified by both culture and multiplex real-time qPCR. Values for bacterial load over the course of infection followed a similar pattern by each method. ETA samples from Twin1 were negative for *U. parvum* on day 1 (Figure 1), but progressively increased in titre with a maximum load of 10^4 CCU on day 10. Titres then dropped by day 24 with a final negative result. The first sample for Twin2 on day 12 was strongly positive for *Ureaplasma* with 10^4 CCU, but became increasingly positive with high titres of 10^7 CCU (2.8×10^5 copies/ μ l *U. urealyticum*) until the patient died.

Species determination of Ply130 and Ply131

Analysis methods (primer-probe pairs from qPCR, amplification by speciation PCR primers, species-specific monoclonal antibodies by immunoblot analysis), demonstrated that all culture positive samples from Twin1 contained only *U. parvum* (designated strain Ply130) while all culture positive samples from Twin2 contained only *U. urealyticum* (designated strain Ply131). There was no contamination of any sample by the alternate *Ureaplasma* species or *Mycoplasma hominis* as confirmed by qPCR.

Analysis of the MBA surface antigen over the course of infection

Immunoblot analysis using monoclonal antibodies that detect both *U. urealyticum* and *U. parvum* (clone 6522; Figure 2A) or specifically *U. parvum* (clones 6523; Figure 2B) found that the surface MBA protein changed considerably over the course of

infection. A single discrete band of approximately 80 kDa was initially noted for strain Ply130 on day four post-birth which was accompanied by a second larger band of 100 kDa by day six. At day ten a further two bands were apparent (36 kDa and 50 kDa) and were the predominant MBA species until day 23. For isolate Ply131 a single MBA species of 60 kDa was seen on days 12 and 18 post birth, but this was replaced by two bands of 50 kDa and 130 kDa. The larger 130 kDa band was then present until the final sample prior to patient death.

Discussion:

We present a case study of dizygotic twins both with *Ureaplasma* positive endotracheal aspirate samples, but interestingly harbouring two separate species. Of particular interest is the observed alteration to the bacterial major surface antigen mass over the course of infection.

For Twin1 the titres of *U. parvum* (Ply130) obtained from samples rapidly increased from the first sample, but a sample taken on day 24 failed to grow *Ureaplasma* by culture, despite detection of residual bacterial genomic DNA by the sensitive PCR methods. Although this culture-negative result coincided with the administration of teicoplanin and gentamicin this will not have an impact on the *Ureaplasma* colonisation status due to the pathogen's intrinsic resistance to these antibiotics.¹⁰

The first sample for Twin2 was not taken until 10 days after birth as poor patient clinical status precluded transport to the research hospital. While *U. urealyticum* was not detected in the initial sample, high levels of *U. urealyticum* were present in a sample taken three days later and all subsequent samples tested until the death of the patient 21 days later as a result of NEC. *Ureaplasma* spp. infection have been reported to contribute to the development of NEC. *Ureaplasma* has been detected in gastric aspirate samples from preterm human neonates, and experimental intrauterine *Ureaplasma* infection in pregnant sheep was found to impair development of the fetal ovine gut in an IL-1-dependent manner.^{11, 12} Further, Okogbule-Wonodi *et al.*, have reported a 2-fold increased NEC prevalence in *Ureaplasma*-infected neonates born <33 weeks and a 3.3-fold increase those born <28 weeks with *Ureaplasma* infection.¹³

The greater pathogenic potential of *U. urealyticum* relative to *U. parvum* has been reported among urethritis patients as well as individuals presenting with miscarriage which is consistent with the worse clinical outcome for Twin2 compared to Twin1.¹⁴

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The repeated sampling nature of this study gave a unique opportunity to track the evolution of antigen variability of each *Ureaplasma* strain throughout the course of the infection. Altered mass of the MBA, caused by insertion or deletion of repeat units in the external portion of the protein, is likely in response to host immune response especially since immunoglobulin and complement have been shown important to *Ureaplasma*-cidal activity of human sera.^{16,12} This is the first real-time demonstration of MBA alteration in a clinical infection: The *U. parvum* MBA from the strain infecting Twin1 decreased in mass, while the *U. urealyticum* from Twin2 increased. An increase in molecular weight of the major surface antigen has been shown to be important to evading complement-mediated killing by serum in the closely related species *Mycoplasma pulmonis*.¹⁷ Future studies will collect serum samples from mothers and neonates, in parallel to ETS samples, to determine if MBA alteration coincides with altered serum killing of matched *Ureaplasma* isolates.

Previous reports have shown discordance in microbial infection between twins. In 1990, Romero and colleagues performed amniocentesis on women carrying twins with intact membranes, presenting with preterm labour.⁴ Of the five pairs with evidence of microbial invasion of the amniotic cavity that subsequently delivered preterm, *Ureaplasma spp.* was cultured from three of these pairs and invariably only from the presenting (never from the non-presenting) twin. Unfortunately this study

predated the differentiation of *Ureaplasma* species. In a second study by Mazor *et al.*, examining microbial invasion of preterm twins, only the presenting twin was infected in five out of nine cases, and microbial titre was always higher for the presenting twin in the remaining cases where both twins were infected.¹⁸ *Ureaplasma* spp. were the most commonly observed pathogen in this study. These data suggest that microbial invasion occurs via ascending infection, and a retrospective study on chorioamnionitis and funisitis examining 1156 twins found that dichorionic placentas conferred significant protection against the spread of chorioamnionitis from the presenting to the non-presenting gestational sac.⁵

In conclusion we report the first known case of twins independently infected with different *Ureaplasma* species, where different clinical outcomes were observed and both bacterial species showed antigenic evolution across serial samples.

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References

1. Lahra MM, Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. *American journal of obstetrics and gynecology* 2004; **190**: 147-51.
2. Goldenberg RL, Andrews WW, Goepfert AR et al. The Alabama Preterm Birth Study: umbilical cord blood *Ureaplasma urealyticum* and *Mycoplasma hominis* cultures in very preterm newborn infants. *American journal of obstetrics and gynecology* 2008; **198**: 43 e1-5.
3. Sung TJ, Xiao L, Duffy L et al. Frequency of ureaplasma serovars in respiratory secretions of preterm infants at risk for bronchopulmonary dysplasia. *The Pediatric infectious disease journal* 2011; **30**: 379-83.
4. Romero R, Shamma F, Avila C et al. Infection and labor. VI. Prevalence, microbiology, and clinical significance of intraamniotic infection in twin gestations with preterm labor. *American journal of obstetrics and gynecology* 1990; **163**: 757-61.
5. Phung DT, Blickstein I, Goldman RD et al. The Northwestern Twin Chorionicity Study: I. Discordant inflammatory findings that are related to chorionicity in presenting versus nonpresenting twins. *American journal of obstetrics and gynecology* 2002; **186**: 1041-5.
6. Beeton ML, Chalker VJ, Maxwell NC et al. Concurrent titration and determination of antibiotic resistance in ureaplasma species with identification of novel point mutations in genes associated with resistance. *Antimicrobial agents and chemotherapy* 2009; **53**: 2020-7.
7. Payne MS, Goss KC, Connett GJ et al. A quantitative analysis of *Ureaplasma urealyticum* and *Ureaplasma parvum* compared with host immune response in preterm neonates at risk of developing bronchopulmonary dysplasia. *Journal of clinical microbiology* 2012; **50**: 909-14.
8. Teng LJ, Zheng X, Glass JI et al. *Ureaplasma urealyticum* biovar specificity and diversity are encoded in multiple-banded antigen gene. *Journal of clinical microbiology* 1994; **32**: 1464-9.
9. Beeton ML, Daha MR, El-Shanawany T et al. Serum killing of *Ureaplasma parvum* shows serovar-determined susceptibility for normal individuals and common variable immuno-deficiency patients. *Immunobiology* 2012; **217**: 187-94.
10. Beeton ML, Chalker VJ, Jones LC et al. Antibiotic resistance among clinical *Ureaplasma* isolates recovered from neonates in England and Wales between 2007 to 2013. *Antimicrobial agents and chemotherapy* 2015.
11. Beeton ML, Maxwell NC, Davies PL et al. Role of pulmonary infection in the development of chronic lung disease of prematurity. *The European respiratory journal* 2011; **37**: 1424-30.
12. Wolfs TG, Kallapur SG, Knox CL et al. Antenatal ureaplasma infection impairs development of the fetal ovine gut in an IL-1-dependent manner. *Mucosal immunology* 2013; **6**: 547-56.
13. Okogbule-Wonodi AC, Gross GW, Sun CC et al. Necrotizing enterocolitis is associated with ureaplasma colonization in preterm infants. *Pediatric research* 2011; **69**: 442-7.
14. Zhang N, Wang R, Li X et al. Are *Ureaplasma* spp. a cause of nongonococcal urethritis? A systematic review and meta-analysis. *PLoS One* 2014; **9**: e113771.

15. Abele-Horn M, Wolff C, Dressel P et al. Association of *Ureaplasma urealyticum* biovars with clinical outcome for neonates, obstetric patients, and gynecological patients with pelvic inflammatory disease. *Journal of clinical microbiology* 1997; **35**: 1199-202.
16. Zheng X, Teng LJ, Watson HL et al. Small repeating units within the *Ureaplasma urealyticum* MB antigen gene encode serovar specificity and are associated with antigen size variation. *Infection and immunity* 1995; **63**: 891-8.
17. Simmons WL, Dybvig K. The Vsa proteins modulate susceptibility of *Mycoplasma pulmonis* to complement killing, hemadsorption, and adherence to polystyrene. *Infection and immunity* 2003; **71**: 5733-8.
18. Mazor M, HersHKovitz R, Ghezzi F et al. Intraamniotic infection in patients with preterm labor and twin pregnancies. *Acta obstetrica et gynecologica Scandinavica* 1996; **75**: 624-7.

Figure 1. Quantification of *Ureaplasma* spp. from endotracheal aspirated during the course of infection. *Ureaplasma* spp. were quantified by both real-time q-PCR (solid line) and routine colour changing units (CCU) (dashed line). *U. parvum* (square) was isolated from Twin1 and *U. urealyticum* (triangle) was isolated from Twin2.

Figure 2. Immunoblot analysis of the multiple-banded antigen of *Ureaplasma* isolated from endotracheal secretions from Twin1 and Twin2. The multiple-banded antigen was detected with monoclonal antibody 6522 (recognises both *U. parvum* and *U. urealyticum*) (A). Monoclonal antibody 6523 (*U. parvum* specific) detected the isolates from Twin1, but not Twin2 (B).

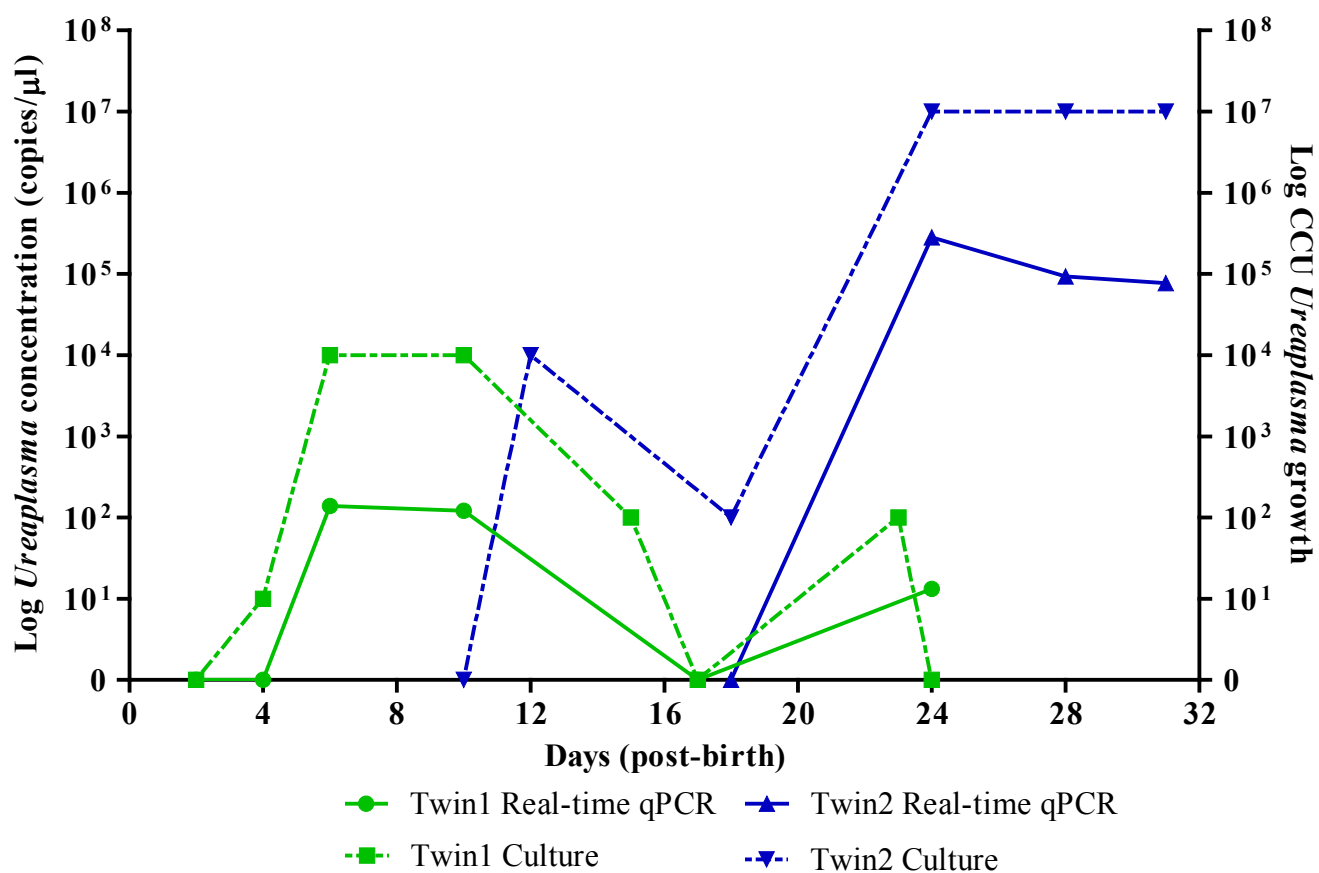


Figure 1. Quantification of *Ureaplasma* spp. from endotracheal aspirated during the course of infection. *Ureaplasma* spp. load was quantified by both real-time q-PCR (dashed line) and routine colour changing units (solid line). Final sample for Twin 1 represents point of extubation whereas final sample for Twin 2 represents end of life. *U. parvum* (square) was isolated from Twin1 and *U. urealyticum* (triangle) was isolated from Twin2. CCU = colour changing units (bacterial growth measurement).

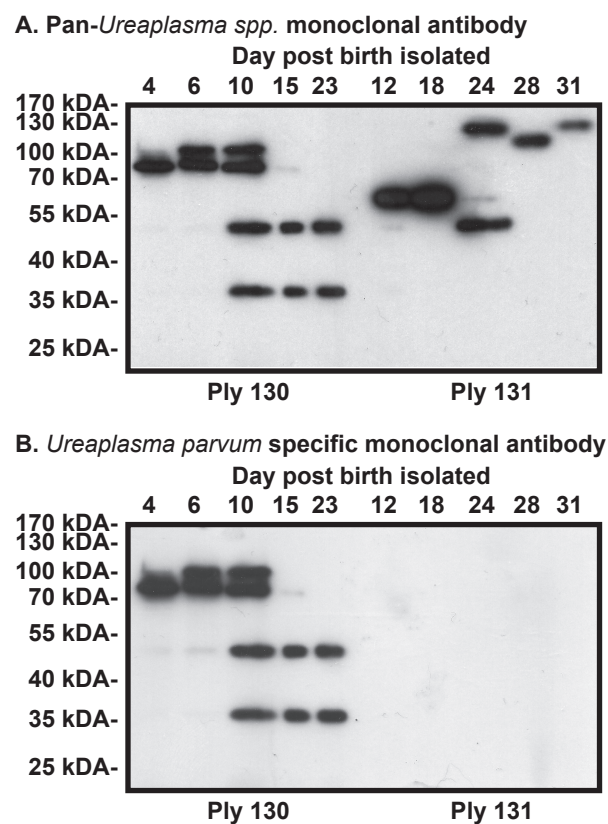


Figure 2. Immunoblot analysis of the multiple-banded antigen of *Ureaplasma* strains isolated from endotracheal secretions from Twin1 and Twin2. The major surface antigen (multiple-banded antigen) detected by incubation with monoclonal antibody 6522 that recognises a core epitope conserved in both *U. parvum* and *U. urealyticum* (A). Stripping and re-probing the blot with a monoclonal antibody that only recognises the multiple banded antigen for *U. parvum* (6523) detected the isolates from Twin1, but not Twin2 (B).