One-step simultaneous detection of Ureaplasma parvum and genotypes SV1, SV3 and SV6 from clinical samples using PlexPCR technology

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Abstract

Ureaplasma spp. are associated with preterm birth. In recent times, it has become apparent that Ureaplasma parvum, but not Ureaplasma urealyticum, is of most relevance. We recently demonstrated this in Australian pregnant women and using high-resolution melt (HRM) PCR, further showed that U. parvum genotype SV6 was of particular significance. However, our assay was unable to identify multiple genotypes in the same sample, required a separate species-level qPCR for low titre samples and was not ideal for diagnostic laboratories due to the nature of HRM PCR result interpretation. Consequently, our current study developed a novel, one-step PlexPCR assay capable of detecting U. parvum and genotypes SV1, SV3 and SV6 in a single reaction directly from clinical samples. We then validated this using vaginal swab DNA from our Australian cohort of pregnant women. The PlexPCR was highly sensitive, detecting all targets to between 0.4 × 10⁻⁹ ng DNA (SV3) and 0.4 × 10⁻⁶ ng DNA (U. parvum, SV1 and SV6). Compared to our HRM PCR, the PlexPCR defined genotype distribution in all seven cases previously reported as ‘mixed’, and detected another eight cases where multiple genotypes (two) were present in samples previously reported as single genotypes using HRM PCR.

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from culture to various molecular assays. In addition, the vast majority of previous research examining the role of Ureaplasma spp., in negative pregnancy outcomes has failed to provide data of diagnostic value simply by not detecting beyond the genus level (Lamont et al. 1987; Carey et al. 1991; Horowitz et al. 1995a, b; Abele-Horn et al. 1997, 2000; Kafetzis et al. 2004; Harada et al. 2008; Rezeberga et al. 2008; Goyender et al. 2009; Kacerovsky et al. 2009; Breugelmans et al. 2010; Choi et al. 2012).

We recently showed that vaginal colonization by Ureaplasma parvum only, and not Ureaplasma urealyticum, is significantly associated with preterm birth (Payne et al. 2016). These data clarified that of earlier studies by Mitsunari et al. (Mitsunari et al. 2005) and Kataoka et al. (Kataoka et al. 2006), both whom reported significant associations between vaginal U. parvum and preterm birth, but not for U. urealyticum. Of further potential diagnostic value is our previous finding that vaginal colonization by U. parvum genotype serovar 6 (SV6) was significantly associated with preterm birth compared to genotypes SV1 and SV3 and was present in four of five cases of preterm birth <34 weeks GA (Payne et al. 2016). This was a novel finding that is currently being validated in a larger cohort study, however, the data are supported in part by that of Xiao et al. (2011), who reported a significant association between the presence of U. parvum genotype SV6 in placental tissue and histologic chorioamnionitis, a hallmark of in utero infection and associated with preterm birth (Goldenberg et al. 2000).

Due to the highly fastidious nature of Ureaplasma spp., molecular detection is generally preferred over culture. A number of PCR assays have been published over the past three decades, with the first capable of genus-level detection published in 1993 (Robertson et al. 1993). Although some of these PCR assays are able to identify Ureaplasma spp. at the genotype level (Kong et al. 2000; Cao et al. 2007; Xiao et al. 2010; Payne et al. 2013), to the best of our knowledge, none exist that are capable of concurrent genus and multiple genotype detection in a single reaction directly from clinical samples. Three years ago we developed a high-resolution melt (HRM) PCR assay that was capable of detecting U. parvum and differentiating single genotype colonizations directly from clinical samples (Payne et al. 2013). We further validated this assay in our most recent study, where it was used to provide novel data on vaginal U. parvum genotype distribution and spontaneous preterm birth (Payne et al. 2016). However, this assay had some inherent weaknesses. These were its inability to resolve multiple genotype colonizations; the fact that HRM curves can show slight variance, making use in a diagnostic lab less than ideal; and that in order to maintain a high sensitivity, it was best used in a two-step screening process, combined with the species-level detection assay of Yi et al. (2005). There is a current need for a specific, sensitive, single-step real-time PCR assay for the detection of U. parvum and its clinically relevant genotypes (SV1, 3 and 6) for use in women at a high risk of preterm birth. Such an assay would likely be of great use in other human disease phenotypes where there is an association with U. parvum.

The aim of the current study was to design a multiplex real-time PCR assay to concurrently detect U. parvum and genotypes SV1, 3 and 6, and subsequently validate its clinical utility by comparing with results from our previous HRM assay.

Results and discussion

PlexPCR has previously been shown to have highly favourable multiplexing characteristics (Mokany et al. 2013) and most recently was employed in an assay to simultaneously detect Mycoplasma genitalium and macrolide resistance (Tabrizi et al. 2016). Our current study compared the new PlexPCR UP assay with results from our previously developed U. parvum HRM assay (Payne et al. 2013), as used in our recent study that investigated vaginal colonization by a range of microbes and association with preterm birth (Payne et al. 2016).

One of the inherit weaknesses of our HRM PCR assay was its inability to distinguish between multiple genotypes within the same sample. However, we suggested that although this was a potential limiting factor in terms of clinical applications, previous studies at the time had reported that unlike U. urealyticum, U. parvum typically occurred as either one or two genotypes within the same sample (Sung et al. 2011; Payne et al. 2013). Our subsequent study, utilizing the HRM assay, appeared to qualify this with only seven cases (from three participants) identified as having multiple genotypes based on the HRM curve profiles generated (Payne et al. 2016).

Our current study, however, has shown that cases of multiple colonizations, although still low, are more common than previously thought. At the species level, the PlexPCR assay showed 100% concordance with the results of our previous study (188 U. parvum-positive samples). However, in terms of genotype discrimination, the PlexPCR was far superior to the HRM assay, uncovering the number and identity of the genotypes in samples previously classified as ‘mixed’ (Payne et al. 2016), and also identifying numerous cases of dual genotype colonization where the HRM assay had indicated only one was present (Table 1). In nearly all of these cases, the HRM assay detected the genotype identified as present in higher levels (dominant), suggesting that low-level colonization events were missed as they did not influence melt curve profiles.
Although unexpected, this result is not surprising, as we previously commented that we did not know what the effect of dominant/minority colonization events on melt curves would be (Payne et al. 2013). The clinical significance of cocolonization by dual U. parvum genotypes, however, is completely unknown. A retrospective analysis of our 2016 study shows that, in terms of negative pregnancy outcome, for all identified cases (12 samples, 7 participants), preterm birth only occurred in one instance (Payne et al. 2016). The effect of multiple U. parvum genotype vaginal colonization on pregnancy outcome is currently being examined in a large prospective cohort study of pregnant women. The effects of multiple genotype colonization in other clinical disease phenotypes warrants further research, particularly for Bronchopulmonary Dysplasia in preterm infants, a condition known to be associated with the presence of Ureaplasma spp. (Waites et al. 2011).

In addition to detecting multiple genotypes of U. parvum within the same clinical sample, we also found that the PlexPCR assay was highly sensitive, detecting U. parvum DNA of each genotype in single-template triplicate reactions (template from only one genotype added—mastermix still containing all four probes and primer pairs) down to at least 0.4 × 10⁻⁵ ng DNA (Table 2). For the generic U. parvum target (UreC gene), detection was observed down to 0.4 × 10⁻⁶ ng DNA (Table 3). These high levels of sensitivity were also maintained in multi-template format (Table 4) and are far superior to the previous combined duplex real-time PCR/HRM assay, which was limited in sensitivity by the HRM component. This is evident in the three cases (all 28-week samples) of no/weak HRM PCR amplification from our recent study which resulted in no identification of U. parvum genotypes, despite the initial real-time PCR indicating that U. parvum was present (Table 1). In all of these cases, the PlexPCR assay confirmed U. parvum as being present, and identified the genotype. Furthermore, the genotype identified was consistent with the genotype identified in the sample from the earlier time point (BL) (Table 1), consistent with the results seen in our previous study (Payne et al. 2016).

The PlexPCR assay was also highly specific for U. parvum, with no amplification detected for any of the closely

Table 1 Differences in Ureaplasma parvum genotype detection between the HRM and PlexPCR assays

| STUDY ID | BL HRM PCR | BL PlexPCR | 28-week HRM PCR | 28-week PlexPCR | 36-week HRM PCR | 36-week PlexPCR |
|----------|------------|------------|-----------------|-----------------|-----------------|-----------------|
| UPCAN1   | SV3        | SV3 dom/SV6| N/A             | N/A             | N/A             | N/A             |
| UPCAN12  | SV6        | SV6 dom/SV3| N/A             | N/A             | SV6             | SV6             |
| UPCAN18  | SV3        |            | NO AMP          | SV3             | N/A             | N/A             |
| UPCAN23  | MIXED      | SV3/SV6 equal| N/A         | N/A             | MIXED SV3/SV6 equal| N/A             |
| UPCAN57  | SV6        | SV6 dom/SV3| SV6             | SV6             | SV6 dom/SV3     | SV6 dom/SV3     |
| UPCAN93  | SV6        | SV6 dom/SV3| N/A             | N/A             | N/A             | N/A             |
| UPCAN94  | SV6        | SV6 dom/SV3| MIXED           | MIXED           | SV6 dom/SV3     | SV6 dom/SV3     |
| UPCAN187 | SV3        |            | WEAK AMP        | SV3             | SV3             | SV3             |
| UPCAN190 | SV1        |            | WEAK AMP        | SV1             | SV1             | SV1             |
| UPCAN199 | MIXED      | SV6        | MIXED           | SV6             | SV6             | SV6             |

dom, dominant; NO AMP, no amplification; MIXED, multiple U. parvum genotypes detected, but identification not possible; WEAK AMP, weak amplification, no genotype identification; bold text indicates differences.

Table 2 Limits of detection for all Ureaplasma parvum genotypes in single-template reactions

| Probe target | Reproducible limit of detection (mean Ct [SD]; ng of DNA) | Maximum limit of detection (mean Ct [SD]; ng of DNA; # of replicates/3) |
|--------------|----------------------------------------------------------|------------------------------------------------------------------------|
| Ureaplasma parvum SV1 | 29 [0.5]; 0.4 × 10⁻⁶ | 29 [0.5]; 0.4 × 10⁻⁶; 3 |
| Ureaplasma parvum SV3 | 28 [0.3]; 0.4 × 10⁻⁵ | 31 [1.7]; 0.4 × 10⁻⁶; 2 |
| Ureaplasma parvum SV6 | 33 [2.2]; 0.4 × 10⁻⁶ | 33 [2.2]; 0.4 × 10⁻⁶; 3 |

Table 3 Limits of detection for the U. parvum generic target for all Ureaplasma parvum genotypes in single-template reactions

| Probe target | Reproducible limit of detection (mean Ct [SD]; ng of DNA) | Maximum limit of detection (mean Ct [SD]; ng of DNA; # of replicates/3) |
|--------------|----------------------------------------------------------|------------------------------------------------------------------------|
| Ureaplasma parvum SV1 | 25 [0.5]; 0.4 × 10⁻⁶ | 28 [0.7]; 0.4 × 10⁻⁷; 2 |
| Ureaplasma parvum SV3 | 26 [0.6]; 0.4 × 10⁻⁶ | 26 [0.6]; 0.4 × 10⁻⁶; 3 |
| Ureaplasma parvum SV6 | 26 [0.2]; 0.4 × 10⁻⁶ | 27 [N/A]; 0.4 × 10⁻⁷; 1 |
| Ureaplasma parvum SV14 | 26 [0.6]; 0.4 × 10⁻⁶ | 26 [0.6]; 0.4 × 10⁻⁶; 3 |
Table 4 Limits of detection for all Ureaplasma parvum genotypes and the U. parvum generic target in multitemplate reactions

| Probe target            | Reproducible limit of detection (mean Ct [SD]; ng of DNA) | Maximum limit of detection (mean Ct [SD]; ng of DNA; # of replicates/3) |
|-------------------------|----------------------------------------------------------|------------------------------------------------------------------------|
| Ureaplasma parvum SV1   | 29 [0.3]; 0.4 × 10⁻⁶                                      | 31 [0.2]; 0.4 × 10⁻⁷; 2                                               |
| Ureaplasma parvum SV3   | 28 [0.2]; 0.4 × 10⁻⁵                                      | 33 [2.5]; 0.4 × 10⁻⁶; 2                                               |
| Ureaplasma parvum SV6   | 32 [1.6]; 0.4 × 10⁻⁶                                      | 32 [1.6]; 0.4 × 10⁻⁶; 3                                               |
| Ureaplasma parvum (UreC)| 24 [0.3]; 0.4 × 10⁻⁶                                      | 27 [N/A]; 0.4 × 10⁻⁸; 1                                               |

Table 5 Specificity testing of the PlexPCR assay

| DNA template              | Reproducible limit of detection (probe target; mean Ct [SD]; ng of DNA) | Maximum limit of detection (probe target; mean Ct [SD]; ng of DNA; # of replicates/3) |
|---------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| Ureaplasma parvum SV14    | SV6; 39 [0.5]; 0.4 × 10⁻⁶                                             | SV6; 39 [0.5]; 0.4 × 10⁻¹                                                             |
| Ureaplasma urealyticum    | No amplification                                                      | No amplification                                                                      |
| Mycoplasma hominis        | No amplification                                                      | No amplification                                                                      |
| Mycoplasma genitalium     | No amplification                                                      | No amplification                                                                      |

related species tested (Table 5), nor for DNA from any of the 40 swabs that were identified as positive only for U. urealyticum in the previous study (Payne et al. 2016). Although some very minor cross-reactivity was observed between U. parvum SV14 DNA template and the U. parvum SV6 probe (Table 5), we believe this is of little clinical concern. This is due to the fact that it only occurred with the highest titre DNA levels (0.04 ng) and even with these, Ct values were almost beyond the limit of detection (Ct 39; 40 cycle reactions). In addition, the PlexPCR assay deliberately excluded SV14 as a target as detection of this genotype in clinical samples is extremely rare (Xiao et al. 2011). We did not identify any SV14 isolates in our previous study of over 500 samples (Payne et al. 2016) and were only able to access two different SV14 isolates from international sample collections in our original HRM study (Payne et al. 2013). Exclusion of this genotype also allowed us to include the generic UreC U. parvum target in the assay, which in the rare case of a single SV14 colonization, would show as detection of UreC only and none of the SV targets. In such instances this would allow any interested parties to pursue further downstream analyses to identify the genotype, best achieved through the use of our HRM PCR assay (Payne et al. 2013), which has been shown to identify single colonizations by SV14. However, multiple genotype colonizations that include SV14 would remain undetected within both our current assay and our HRM assay.

The PlexPCR assay developed in this study provides a rapid, specific and sensitive means of detecting U. parvum and its clinically relevant genotypes directly from clinical samples in a single reaction. It is superior in both sensitivity and clinical application to our previous HRM PCR assay and will be a useful tool for further investigations into the role of U. parvum in human disease, in particular the role of multiple genotype colonizations. The assay may be of specific appeal to clinicians who wish to pursue antenatal U. parvum investigations, especially in pregnant women who already have existing risk factors for preterm birth.

Materials and methods

PCR assay design

The multiplex assay was developed using PlexPCR technology which employs novel PlexPrimers (Tan et al. 2017) coupled with PlexZyme (formerly known as MNAzyme) (Mokany et al. 2013) detection in qPCR. PlexPrimers selectively amplify target sequences and PlexZymes enable high-level multiplexing of similar genotypes in a single well. The PlexPCR UP assay (SpeeDx Pty Ltd, Sydney, NSW, Australia) was developed as a multiplex qPCR assay reporting across four channels: detection of U. parvum via the Urease C (UreC) gene, and detection of U. parvum genotypes SV1, SV3 and SV6 via the multiple-banded antigen (MBA) gene.

The PlexPCR UP assay was performed as per manufacturer’s instructions (SpeeDx). Briefly, DNA was mixed with Plex Mastermix to a final volume of 20 µl. All testing was performed in 96-well plates on the ViiA 7 real-time PCR system (Life Technologies, Carlsbad, CA, USA) using the following cycling program: initial denaturation at 95°C for 2 min; 10 cycles of 95°C for 5 s, 61°C for 30 s (−0.5°C cycle); and 40 cycles of 95°C for 5 s, 52°C for 40 s (with data acquisition). All data were analysed using QuantStudio Real-Time PCR Software ver. 1.3 (Life Technologies).

Sensitivity testing

DNA extracts from pure cultures of U. parvum genotypes SV1, 3 and 6 were quantified using a dsDNA high-sensitivity kit (Life Technologies) on a Qubit fluorometer (Life Technologies) and normalized to 0.02 ng µl⁻¹ of DNA in nuclease-free water (Ambion, Life Technologies, Carlsbad, CA, USA). A 10-fold dilution series (to a maximum of
10⁻⁷) of each of these DNA extracts were established and these were used in both single-template (2 μl) and multi-template (genotypes SV1, 3 and 6) (2 μl of each, 6 μl in total) formats to assess sensitivity levels. All samples were run in triplicate.

Specificity testing

To assess assay specificity, the PlexPCR UP assay was tested in triplicate with pure DNA extracts from all U. parvum genotypes (SV1, 3, 6 and 14) and the closely related species, Ureaplasma urealyticum, Mycoplasma hominis and Mycoplasma genitalium.

Validation

A retrospective study was conducted that accessed DNA extracted from vaginal swabs collected from pregnant women at three gestational time points during their pregnancies (18–26 (baseline), 28 and 36 weeks); the original study was known as the UPCAN study (Payne et al. 2016). DNA extracts stored at −20°C were accessed from all swabs that had previously been shown to be positive for U. parvum by real-time PCR (Payne et al. 2016) and subsequently genotyped using a HRM PCR assay (Payne et al. 2013). This consisted of 72 baseline (BL) samples, sixty-three 28-week samples and fifty-three 36 week samples; 188 in total. In addition, 40 swabs (BL, 28 and 36 weeks) that had been identified as positive only for U. urealyticum were also analysed to ensure primer/probe specificity was maintained within clinical sample analyses. In total, DNA extracts from 228 vaginal swabs were validated using the PlexPCR UP assay. The study was approved by the Human Research Ethics Committee of the Western Australian Department of Health, Women and Newborn Health Service (2056/EW).

Acknowledgements

LLF was supported by an Australian Government Research Training Program Scholarship and a Professor Gordon King Postgraduate Scholarship provided by the Women’s and Infants’ Research Foundation (WIRF). Funding for this research was provided by WIRF in the form of a Capacity Building Grant held by MSP.

Conflict of Interest

Authors RT, LYT and EM are employees of SpeeDx Pty Ltd, an Australian molecular diagnostics company. SpeeDx is the owner of PlexPCR and will distribute the U. parvum assay when it becomes available to commercial laboratories. RT, LYT and EM’s involvement in the study consisted of expertise in PCR assay design and use of PlexPCR technology. They were not involved in any data analysis or validation of the PlexPCR assay on human clinical samples; this was all performed by MSP and LLF.

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