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# High-Resolution Melt PCR Analysis for Genotyping of *Ureaplasma parvum* Isolates Directly from Clinical Samples

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***Ureaplasma* sp. infection in neonates and adults underlies a variety of disease pathologies. Of the two human *Ureaplasma* spp., *Ureaplasma parvum* is clinically the most common. We have developed a high-resolution melt (HRM) PCR assay for the differentiation of the four serovars of *U. parvum* in a single step. Currently *U. parvum* strains are separated into four serovars by sequencing the promoter and coding region of the multiple-banded antigen (MBA) gene. We designed primers to conserved sequences within this region for PCR amplification and HRM analysis to generate reproducible and distinct melt profiles that distinguish clonal representatives of serovars 1, 3, 6, and 14. Furthermore, our HRM PCR assay could classify DNA extracted from 74 known (MBA-sequenced) test strains with 100% accuracy. Importantly, HRM PCR was also able to identify *U. parvum* serovars directly from 16 clinical swabs. HRM PCR performed with DNA consisting of mixtures of combined known serovars yielded profiles that were easily distinguished from those for single-serovar controls. These profiles mirrored clinical samples that contained mixed serovars. Unfortunately, melt curve analysis software is not yet robust enough to identify the composition of mixed serovar samples, only that more than one serovar is present. HRM PCR provides a single-step, rapid, cost-effective means to differentiate the four serovars of *U. parvum* that did not amplify any of the known 10 serovars of *Ureaplasma urealyticum* tested in parallel. Choice of reaction reagents was found to be crucial to allow sufficient sensitivity to differentiate *U. parvum* serovars directly from clinical swabs rather than requiring cell enrichment using microbial culture techniques.***

*Ureaplasma* spp. are among the smallest known self-replicating organisms (700- to 1,000-kb genome), second only to *Mycoplasma genitalium*. Only two species are known to infect humans, *Ureaplasma parvum* and *Ureaplasma urealyticum*, both of which are most commonly associated with neonatal diseases (1) including bronchopulmonary dysplasia (BPD) (2), intraventricular hemorrhage (2), necrotizing enterocolitis (3), and pneumonia (4). In adults, *Ureaplasma* spp. have been reported to be one of the leading causes of nongonococcal urethritis in addition to *Chlamydia trachomatis*. *Ureaplasma* spp. are also causally associated with preterm birth, where they are the organism most commonly isolated from preterm pregnancy tissues, particularly in cases of histological chorioamnionitis (5).

Many studies have attempted to investigate whether *U. parvum* or *U. urealyticum* is more clinically relevant; however, this issue has not been accurately resolved. For instance, Heggie et al. (6) found no differences in association between infant colonization with *U. parvum* or *U. urealyticum* and the development of BPD, while Abele-Horn et al. (7) reported a significant association between *U. urealyticum* and BPD compared with *U. parvum*. Despite these differences, it is generally accepted that *U. parvum* is the more commonly isolated of the two species from clinical samples (1).

*U. parvum* and *U. urealyticum* are currently classified into 14 distinct serovars based upon a serotyping system developed in 1982 (8). Serovar 1 (SV1), SV3, SV6, and SV14 belong to *U. parvum*, and the remaining 10 belong to *U. urealyticum*. Similar to the species debate, there are numerous conflicting studies attempting to link a specific serovar with disease. This is further confounded through the use of flawed serotyping methodologies, many of which were reported shortly after the conception of the original serotyping scheme and showed multiple cross-reactions among individual serovars (9). Despite efforts to improve on this (10), disparities in serotyping methodologies have not been suitably resolved.

When used in combination with standard microbial culture protocols, molecular methods have become the mainstay of *Ureaplasma* sp. diagnostics; however, beyond species level discrimination there is a complete lack of one-step genotyping assays capable of accurate serovar discrimination. Teng et al. (11) described an endpoint PCR which was used to discriminate between *U. parvum* and *U. urealyticum* based upon amplicon size (403 bp versus 448 bp) when a section of the multiple-banded antigen (MBA) gene was targeted. This assay also allowed serovar detection for *U. parvum*, following amplicon sequencing; however, this approach to *U. parvum* detection and genotyping is both laborious and expensive. It is not possible to identify *U. urealyticum* serovars based upon the MBA gene due to high levels of homology within this gene between serovars. The most recent attempt at serovar detection of *U. parvum* and *U. urealyticum* was a set of real-time PCR assays described by Xiao et al. (12) which utilized a wide range of targets. However, a multiplex approach was not applied; instead a process of elimination was employed to identify the correct serovar. Numerous specificity problems, particularly with *U. urealyticum* serovars (13), were reported with this assay shortly after its

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publication, suggesting that horizontal gene transfer between *Ureaplasma* spp. may be a major confounding factor in the ability to use serovar characterization as a diagnostic method (14). Such gene transfer was particularly common in *U. urealyticum*, whereas in the vast majority of cases, *U. parvum* was accurately characterized based upon serovar status.

Although this research cast doubts over the validity of the current *Ureaplasma* sp. serotyping and genotyping classification schemes, at present these remain the gold standard for the subclassification of *U. parvum* and *U. urealyticum* serovars. It is highly likely that they will continue to remain in place until development of a validated, genotyping approach, such as multilocus sequence typing, similar to that used for *Staphylococcus* (15) and *Streptococcus* (16) spp. In order to accurately define *U. parvum* serovar distribution within clinical samples, there is the need for a rapid, cost-effective molecular test that may be implemented on a diagnostic level. The aim of this study was to develop and evaluate an HRM PCR assay for the single-step serovar classification of *U. parvum* directly from clinical samples. To the best of our knowledge, there are very few similar studies that exist.

## MATERIALS AND METHODS

***Ureaplasma* sp. isolates.** In total, 84 isolates of *U. parvum* and 10 isolates of *U. urealyticum* were examined. Reference strains of *U. parvum* (SV1 [ATCC 27813], SV3 [ATCC 27815], SV6 [ATCC 27818], and SV14 [ATCC 33697]) were obtained from the American Type Culture Collection (ATCC). Other control strains used included the sequenced prototype control strains DFK1 (SV1), HPA5 (SV3), HPA2 (SV6), and HPA32 (SV14), previously characterized for complement sensitivity (17).

Clinical *U. parvum* isolates were obtained from various sources in the United Kingdom. These included 13 isolates from preterm neonatal patients previously investigated for antibiotic sensitivity (18) as well as 52 isolates collected between 2008 and 2013 from preterm neonatal patients by the Health Protection Agency, England, Colindale, United Kingdom (provided by Victoria Chalker), the University Hospital of Wales, Cardiff, United Kingdom (provided by Cora Doherty and Jenny Calvert), and Derriford Hospital, Plymouth, United Kingdom (provided by Nicola Maxwell). Isolates from 11 sexual health patients from the Royal Glamorgan Hospital, Wales, United Kingdom (provided by Lucy Jones), were also included in the study.

All neonatal samples were obtained from either bronchoalveolar lavage (1 ml/kg of body weight) retrieved as described by Davies et al. (19) or as endotracheal secretions suctioned from the intubation catheter. All neonatal patients were preterm and had either respiratory distress syndrome or BPD. All sexual health patients were attending a clinic for urethritis and bacterial vaginosis investigations, and samples were collected as endocervical charcoal swabs.

Sequenced prototype control strains of *U. urealyticum* were included in HRM analyses to validate the specificity of the primers for *U. parvum*. The original serotyping control strains for *U. urealyticum* (SV2, SV4, SV5, and SV7 to SV13; provided by Janet Robertson, University of Calgary, Canada, and the Institute of Medical Microbiology, University of Aarhus, Denmark) were also investigated.

***Ureaplasma* sp. culture.** All isolates were subcultured from −80°C stocks using a 1:10 serial dilution in *Ureaplasma* sp. selective medium (USM) (Mycoplasma Experience Ltd., Surrey, United Kingdom) and incubated at 37°C for up to 48 h. Exponential-phase positive cultures were harvested from the highest dilution series with pH color change (*Ureaplasma* spp. grow as a nonturbid culture, and production of ammonium ions, altering the pH from 6.5 to >9, is used to identify growth) and frozen at −80°C for subsequent purification.

**Purification of isolates.** In order to ensure purity of sequenced prototype strains and ATCC strains, a triple-cloning process was employed. Thirty microliters of a 1:100 dilution of each strain was plated on prepoured *Ureaplasma* sp. selective agar plates (Mycoplasma Experience), and individual colonies were picked using a sterile 1-ml, 27-gauge insulin needle and syringe under ×20 magnification on an inverted tissue culture microscope. Colonies were resuspended in 180 μl of USM and grown overnight at 37°C. This process was repeated three times.

**Multiple-banded antigen sequencing to characterize *Ureaplasma* sp. isolate serovars.** Five hundred microliters of USM containing each strain was pelleted at 17,135 × *g*, 4°C, in a refrigerated benchtop centrifuge (PrismR, Appleton Woods, United Kingdom) for 20 min, and all supernatant was removed. The pellet was resuspended in 40 μl PCR grade water and heated to 95°C for 5 min to cause cell lysis. Standard *U. parvum*/*U. urealyticum* speciation PCR was subsequently performed as described by Teng et al. (11). PCR cycling conditions consisted of 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 40 s. Successful amplification was confirmed on a 1% agarose gel (Life Technologies, Glasgow, United Kingdom), and only samples containing a 403-bp amplicon (*U. parvum*) were used for sequence determination. Any samples with 448-bp (*U. urealyticum*) amplicons (except for *U. urealyticum* prototype control isolates) were not used in this study.

Amplicons were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and sequenced by Eurofins MWG Operon (Ebersberg, Germany). Serovar identity was determined by sequence homology to nucleotide databases using the NCBI nucleotide Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Clinical samples.** To determine the ability of the HRM assay to reliably detect/genotype *U. parvum* direct from clinical samples, vaginal swabs were collected from a small cohort of pregnant women in Perth, Western Australia, Australia. Ethics approval was granted by the Women and Newborn Health Service prior to sample collection (2056-EW). During a routine antenatal visit, 40 pregnant women (gestational age, 13 to 26 weeks; mean, 21 weeks) provided self-collected vaginal swabs using universal transport media (UTM) swab kits (Copan Diagnostics Inc., CA), designed to enhance the viability of *Ureaplasma* sp. cells postcollection. Samples were stored at 4°C immediately after collection and transferred to the laboratory for processing. Swabs resting in UTM within sample collection tubes were vortexed for 10 s and then rotated against the side of the tube to remove all liquid. Swabs were then discarded, and 250 μl of UTM liquid was used for DNA extraction from each sample.

**DNA extraction.** DNA was extracted using the Siemens (Munich, Germany) sample preparation kit 1.0 on an automated Kingfisher Duo extraction platform (Thermo Fisher Scientific Inc., MA) in accordance with the manufacturer's instructions. All extracts were eluted in a final volume of 100 μl of elution buffer (Siemens).

***Ureaplasma* sp. detection within clinical samples prior to HRM analysis.** In order to assess *Ureaplasma* sp. colonization status, vaginal swab DNA was screened using a real-time PCR assay targeting the urease genes of *U. parvum* and *U. urealyticum* as described by Yi et al. (20), adapted for use on a ViiA7 real-time PCR system (Life Technologies). Reaction mixtures (final concentration) consisted of 1× TaqMan Fast advanced master mix (Life Technologies), 0.9 μM primers UU1613F and UU1524R (Life Technologies), 0.25 μM probes UU-parvo (6-carboxyfluorescein [FAM]) and UU-T960 (VIC) (Life Technologies), 5 μl of template DNA, and nuclease-free water (Ambion, Life Technologies) to a final volume of 20 μl. PCR cycling conditions consisted of an initial denaturation/*Taq* activation at 95°C for 20 s, followed by 40 quantification cycles of 95°C for 1 s and 60°C for 20 s (data acquisition). Positive standards for both *U. parvum* and *U. urealyticum* were included in each run.

**High-resolution melt PCR analysis. (i) HRM primer design.** Primers were designed to anneal to conserved regions that were homologous to all four *U. parvum* serovars flanking the MBA gene. Specifically, whole-genome shotgun sequences of ATCC type strains representing each serovar were retrieved from GenBank (Table 1), and a multiple sequence alignment was performed using Clustal Omega (21). One region of the MBA gene was identified as suitable for HRM primer design. This was based on

**TABLE 1** GenBank accession numbers and single-nucleotide variations used for HRM primer and assay design

| Serovar | ATCC clone | GenBank accession no. | Nucleotide position[a] | Base change | SNP class[b] | HRM melt curve shift[b] (°C) |
|---|---|---|---|---|---|---|
| 1 | 27813 | NZ_ABES01000001 | 102 | C/T | 1 | >0.5 |
| | | | 114 | A/G | 1 | >0.5 |
| 3 | 27815 | NC_010503 | | | | |
| 6 | 27818 | NZ_AAZQ01000001 | 81 | G/A | 1 | >0.5 |
| | | | 122 | G/A | 1 | >0.5 |
| | | | 131 | G/A | 1 | >0.5 |
| | | | 162 | A/G | 1 | >0.5 |
| 14 | 33697 | NZ_ABER01000002 | 29 | G/T | 2 | 0.2–0.5 |

[a] Numbering is based on nucleotide position from the start codon.
[b] Single-nucleotide polymorphism (SNP) class and HRM melt curve shift are based on results by Venter et al. (22).

a GC content greater than 25% and adequate regions of sequence homology between all four serovars that incorporated sufficient nucleotide variants to differentiate each by HRM analysis (22). Serovar 3 was used as the "reference" strain to annotate such nucleotide variations (Table 1). Primers were designed using Primer3Plus (23) to amplify the shortest possible suitable PCR product (305 bp). The absence of potential secondary structures in both primers and amplicons was confirmed using DINAMelt (24). To increase the stringency and efficiency of primer annealing, the primers were designed to have a minimum GC content of 50% within the first four bases at the 3′ region. To ensure that the primers were species specific, potential primer sequence homology to other bacterial and mammalian species was assessed using Primer-BLAST (25). No potential homology to other species was detected, including homology to *U. urealyticum* or human DNA that may be copurified from clinical specimens.

**(ii) *In silico* HRM analysis.** The Web-based tool uMELT (26) was used to predict high-resolution fluorescent DNA melting curves and denaturation profiles of PCR products to ensure that distinct profiles were generated by the four serovars of *U. parvum*.

**(iii) DNA quantitation.** All *Ureaplasma* sp. isolate DNA was quantified using a double-stranded DNA (dsDNA) high-sensitivity quantitation kit (Molecular Probes, Life Technologies) on the Qubit 2.0 fluorometer (Life Technologies) in accordance with the manufacturer's instructions. Aliquots (50 μl) from DNA extracts were adjusted to 0.02 ng/μl and stored at 4°C, along with stock extracts, until completion of HRM analyses.

**(iv) HRM PCR analysis.** DNA extracts from pure *U. parvum* and *U. urealyticum* (control) isolates, combinations of DNA extracts from the four ATCC serovar *U. parvum* isolates, and vaginal swabs were screened in triplicate using HRM PCR on a ViiA7 real-time PCR system (Life Technologies). DNA from pure *U. parvum* control isolates representing each of the four serovars (one of each) was included as standards with each run. Due to the reagent-specific nature of HRM PCR, two types of commercially available reagent kits for this instrument were utilized in order to establish potential differences in HRM curve profiles between serovars, as well as to establish the sensitivity of each to detect/genotype *U. parvum* directly from clinical samples (vaginal swabs). Reaction mixtures used for each kit (final concentration) were MeltDoctor HRM master mix (Life Technologies), consisting of 1× MeltDoctor HRM master mix, 0.3 μM primers UPHRM-F (5′ TGCAATCTTTATATGTTTTCGTT 3′) and UPHRM-R (5′ GATCTTTAAAGTTTTCAATTTCGT 3′) (Life Technologies), 5 μl of template DNA, and nuclease-free water (Ambion, Life Technologies), to a final volume of 20 μl, and the MeltDoctor HRM reagent kit (Life Technologies), consisting of 1× AmpliTaq Gold 360 buffer, 1.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), 0.3 μM primers UPHRM-F and UPHRM-R (Life Technologies), MeltDoctor HRM dye (1×), AmpliTaq Gold 360 DNA polymerase (0.1 U/μl), and nuclease-free water (Ambion, Life Technologies), to a final volume of 20 μl.

PCR cycling conditions were identical for both reaction mixtures. These consisted of an initial denaturation/*Taq* activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (data acquisition). To provide data on *U. parvum* serovar status, amplicons were subsequently subjected to an HRM step where the temperature was raised to 95°C for 10 s and then lowered to 60°C for 1 min. The temperature was then raised to 95°C at a rate of 0.025°C/s (continuous data acquisition), held at 95°C for 15 s, and then lowered to 60°C for 15 s.

HRM profiles were analyzed using the ViiA7 real-time PCR system software, v1.2.1 (Life Technologies).

## RESULTS

**Multiple-banded antigen sequencing of clinical *Ureaplasma* sp. isolates.** Of the 76 tentatively positive *U. parvum* clinical isolates examined, 74 showed 100% identity to genomic sequences for *U. parvum*. These consisted of 23, 31, and 20 representations of SV1, SV3, and SV6, respectively. No clinical isolates of SV14 were identified. Two samples achieved only 94% identity to the closest match, and these were excluded from subsequent HRM analysis.

**Real-time PCR detection of *U. parvum*/*U. urealyticum* from vaginal swabs.** Of the 40 vaginal swabs subjected to DNA extraction, 18 tested positive for *Ureaplasma* spp. Of these, 14 were positive for *U. parvum*, 2 for *U. urealyticum*, and 2 for both *U. parvum* and *U. urealyticum*.

**HRM PCR analysis. (i) Control isolates of *U. parvum* SV1, SV3, SV6, and SV14.** All control (ATCC and prototype) isolates showed amplification with the HRM primer set and produced serovar-specific melt profiles, consisting of two defined peaks (Fig. 1i and ii). Combinations of SV1 and SV3, SV1 and SV6, SV1 and SV14, and SV3 and SV14 were separated from one another based upon the first peak only (Fig. 2i to iv). SV6 and SV14 were separated from one another based upon the second peak only (Fig. 2vi), and SV3 and SV6 could be separated based upon either of the two peaks (Fig. 2v). This was reproducible with serovar-specific peak melting temperatures ($T_m$) and minimal variance in $T_m$ between replicates (Table 2). SV6 recorded the highest peak $T_m$ total variances of 0.191°C and 0.187°C for peaks 1 and 2, respectively.

**(ii) Control isolates of *U. urealyticum*.** No amplification or melt curve was produced by any serovar of *U. urealyticum*.

**(iii) Single *Ureaplasma* sp. isolates.** In all cases, melt profiles produced by the 74 clinical isolates of *U. parvum* tested matched one of the four serovar control standards (SV1, SV3, SV6, and SV14) included with every run. Peak $T_m$ values between isolates for respective serovars showed only minimal variance, the two largest of which were recorded for SV3 at 0.537°C and 0.535°C total variance for peaks 1 and 2, respectively (variance representative of 93 replicates, 31 isolates in triplicate). In all cases, the *U. parvum* serovar as indicated by melt profile was correctly matched to serovar identity in accordance with the previously sequenced 403-bp serovar-specific amplicon.

Increased DNA levels in reactions (up to 0.62 ng/μl) resulted in amplification at earlier cycles of the PCR assay, yet had no effect on melt profiles, which remained as previously described for DNA levels normalized to 0.02 ng/μl.

**(iv) Combinations of DNA extracts from ATCC control isolates.** When DNA extracts containing multiple serovars were examined (all possible combinations were tested), serovar combination-specific melt profiles were not produced. However, all melt profiles produced by various combinations of serovar extracts were easily distinguished from those of single-serovar isolates. In all but one case, multiple serovar extracts produced >2 peaks

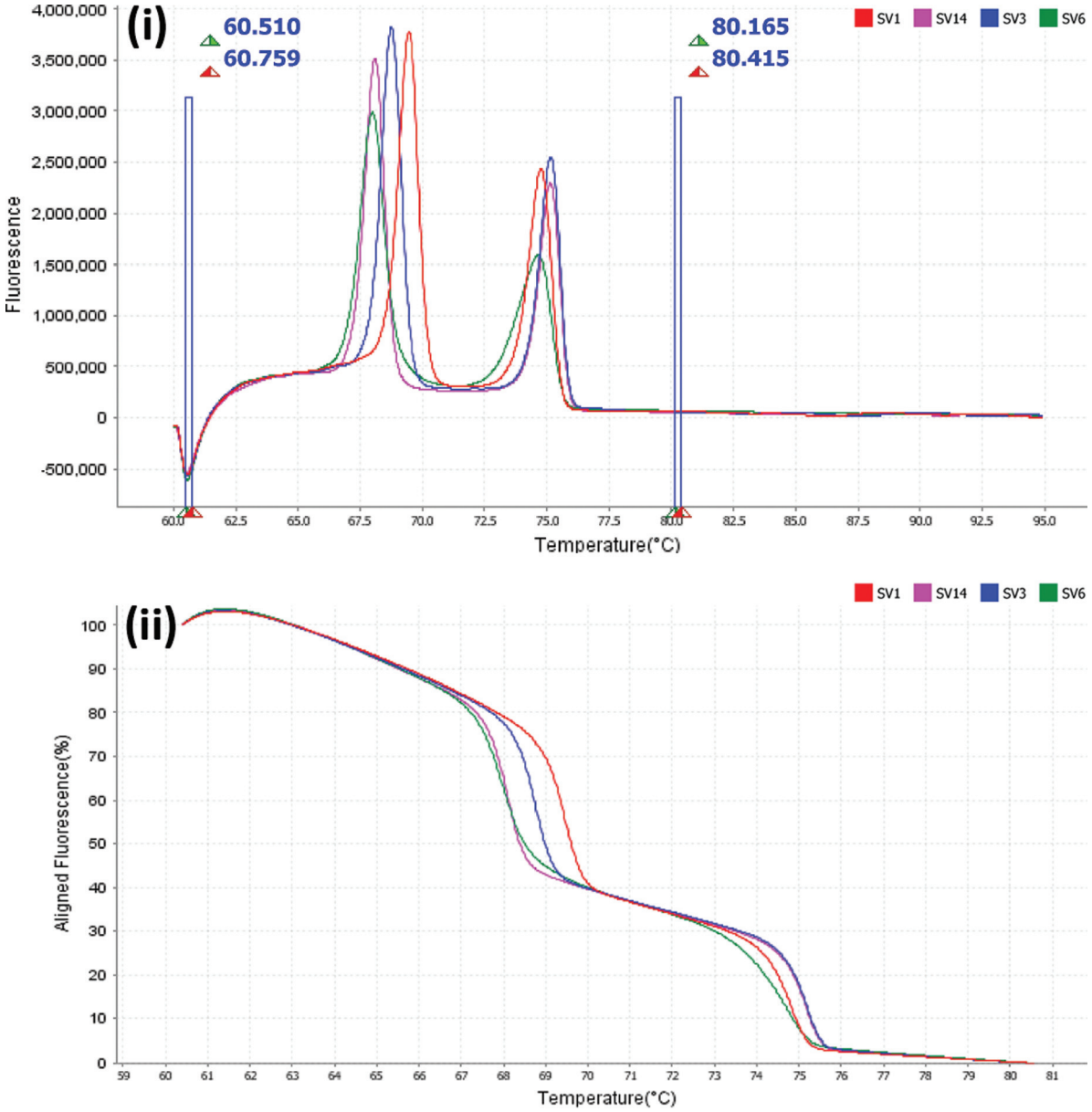


**FIG 1** (i) Derivative high-resolution melt curves comparing individual *U. parvum* ATCC serovars 1, 3, 6, and 14 using MeltDoctor premix reagents. (ii) Aligned high-resolution melt curves comparing individual *U. parvum* ATCC serovars 1, 3, 6, and 14 using MeltDoctor premix reagents.

(Fig. 3i to iv and vi to xi), and in the case (SV3 plus SV14) where two peaks were produced (Fig. 3v), the first peak was broad, undefined, and not similar to those in any of the four serovar-specific melt profiles.

**(v) Vaginal swabs.** Of the 18 vaginal swab DNA extracts positive for *Ureaplasma* spp., 12 showed positive amplification with the *U. parvum* HRM primer set. Eleven of these extracts produced melt profiles that enabled *U. parvum* genotyping, and 1 produced a melt profile with >2 peaks, indicative of multiple serovar colonization. In addition, despite producing no positive amplification, as indicated by the absence of SYTO-9 dye fluorescence during the PCR phase, weak melt profiles indicative of single serovars were still produced by a further four samples that showed positive amplification for *U. parvum* in the previous real-time PCR assay targeting the urease gene. Two samples showed no amplification or melt profiles, and these had been previously shown to be *U. parvum* negative and *U. urealyticum* positive by detection of the urease gene.

**(vi) Reagent-specific effects.** All four *U. parvum* serovars were reliably separated using both reagent types tested. However, there were large differences in fluorescence levels and peak melting temperatures for each serovar between kits. When the MeltDoctor HRM master mix (premix kit) was used, fluorescence levels for control isolate melt profiles were consistently between $1.5 \times 10^6$ and $4 \times 10^6$ units, depending on the serovar (Fig. 1). These values were substantially lower than those for the same isolate melt profiles run with the MeltDoctor HRM reagent kit, where the master mix was constructed separately. Fluorescence values when this kit

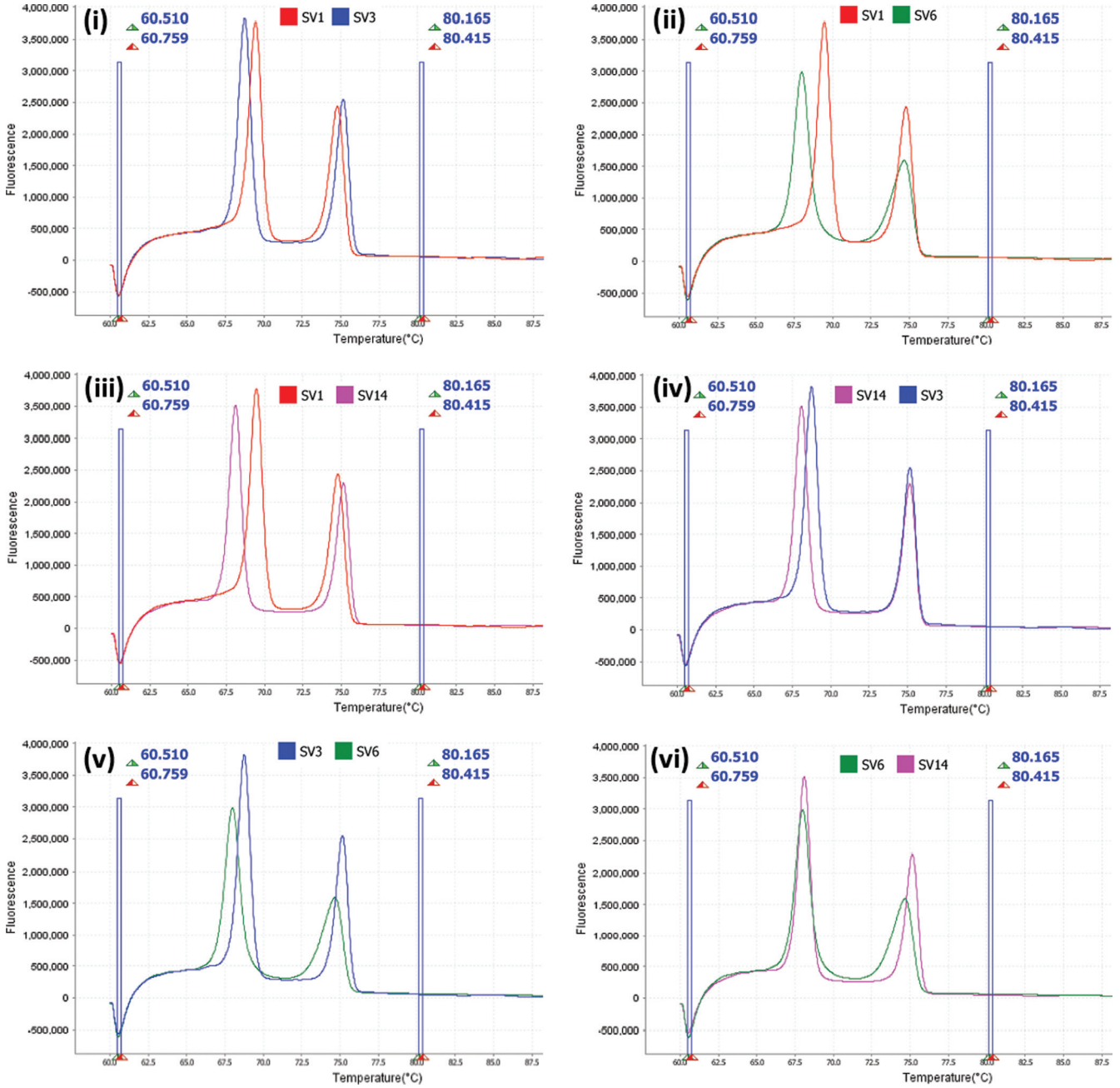


**FIG 2** Derivative high-resolution melt curves comparing individual *U. parvum* ATCC serovars 1 and 3 (i), 1 and 6 (ii), 1 and 14 (iii), 14 and 3 (iv), 3 and 6 (v), and 6 and 14 (vi).

**TABLE 2** Mean $T_m$, $T_m$ standard deviations, and $T_m$ variances[a] for ATCC *U. parvum* reference strains

| Serovar | Peak 1 | | | Peak 2 | | |
|---|---|---|---|---|---|---|
| | Mean $T_m$ (°C) | SD (°C) | Total variance (°C) | Mean $T_m$ (°C) | SD (°C) | Total variance (°C) |
| 1 | 69.343 | 0.052 | 0.128 | 74.675 | 0.046 | 0.110 |
| 3 | 68.675 | 0.056 | 0.159 | 75.100 | 0.046 | 0.132 |
| 6 | 67.904 | 0.064 | 0.191 | 74.600 | 0.061 | 0.187 |
| 14 | 68.077 | 0.069 | 0.189 | 75.139 | 0.073 | 0.182 |

[a] Values are representative of nine replicates (three triplicate runs).

was used were consistently between $10 \times 10^6$ and $23 \times 10^6$ units, depending on the serovar (Fig. 4). Mean peak melting temperatures recorded for each control serovar isolate followed a similar trend, with lower peak temperatures recorded when using the MeltDoctor HRM master mix kit (Table 3).

Despite these differences in fluorescence signal and peak melt temperatures, *U. parvum* serovars from pure isolate DNA were easily distinguished using either kit, as long as standards were included with each run.

For genotyping *U. parvum* from vaginal swabs, however, the MeltDoctor HRM master mix failed to detect amplification in any

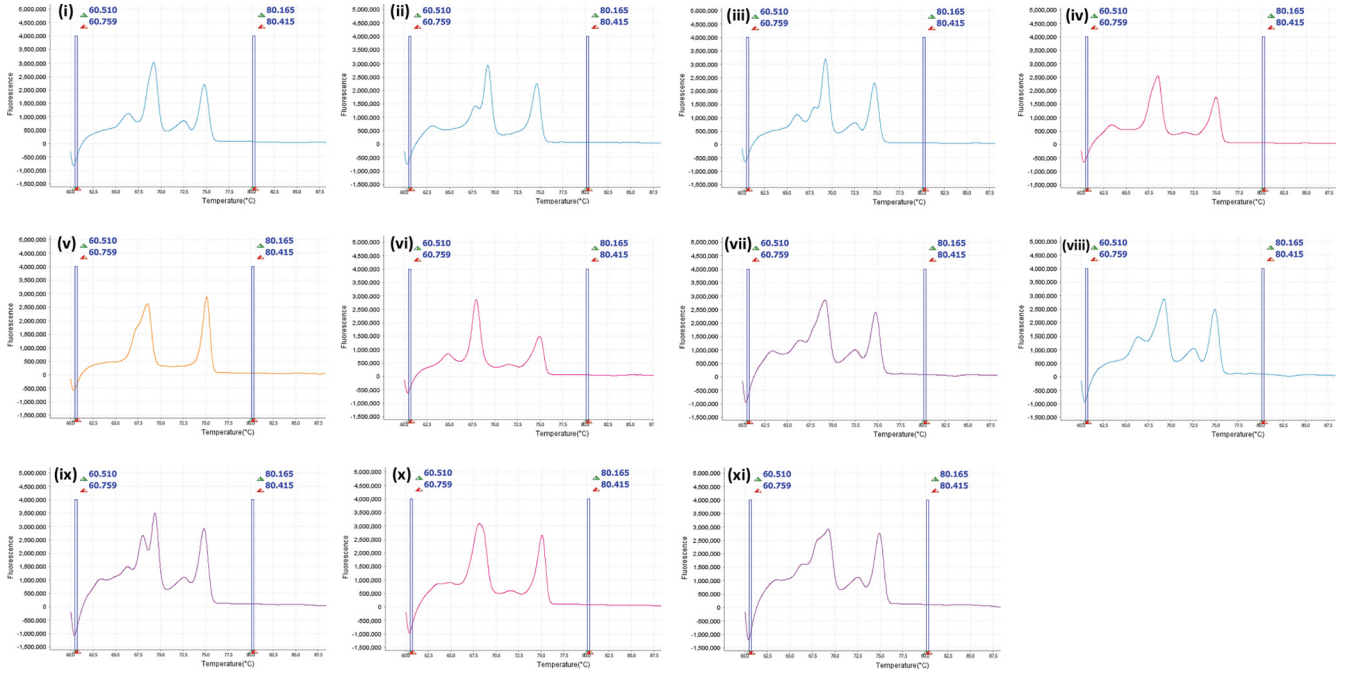


**FIG 3** Derivative high-resolution melt curves for combinations of *U. parvum* ATCC serovars 1 and 3 (i), 1 and 6 (ii), 1 and 14 (iii), 3 and 6 (iv), 3 and 14 (v), 6 and 14 (vi), 1, 3, and 6 (vii), 1, 3, and 14 (viii), 1, 6, and 14 (ix), 3, 6, and 14 (x), and 1, 3, 6, and 14 (xi).

of the 18 samples and produced only one melt profile (weak) from a single sample. The MeltDoctor HRM reagent kit reproduced the previously described urease gene results for vaginal swabs.

## DISCUSSION

Of the two *Ureaplasma* species known to infect humans, *U. parvum* is the most commonly isolated from clinical samples, typically representing 48 to 86% of human *Ureaplasma* spp. reported (1, 13, 27, 28). Since the inception of molecular assays that have allowed the genotyping of *U. parvum*, a small number of studies have described serovar distribution within different clinical samples. Sung et al. (28) reported that SV3 and SV6, either alone or in combination, accounted for 96% of *U. parvum* isolates obtained from endotracheal and/or nasopharyngeal aspirates from preterm infants at risk of BPD. However, they failed to find an association between the presence of any *U. parvum* or *U. urealyticum* serovar in patients and the development of moderate to severe BPD. Similarly, in a study that examined 1,061 *U. parvum*/*U. urealyticum* isolates from a range of sample/disease types, Xiao et al. (13) found that SV3 was the most common of all *U. parvum* serovars (65%) but could not define any consistent pattern between specific serovars and disease groups. In contrast, in a study of endo-

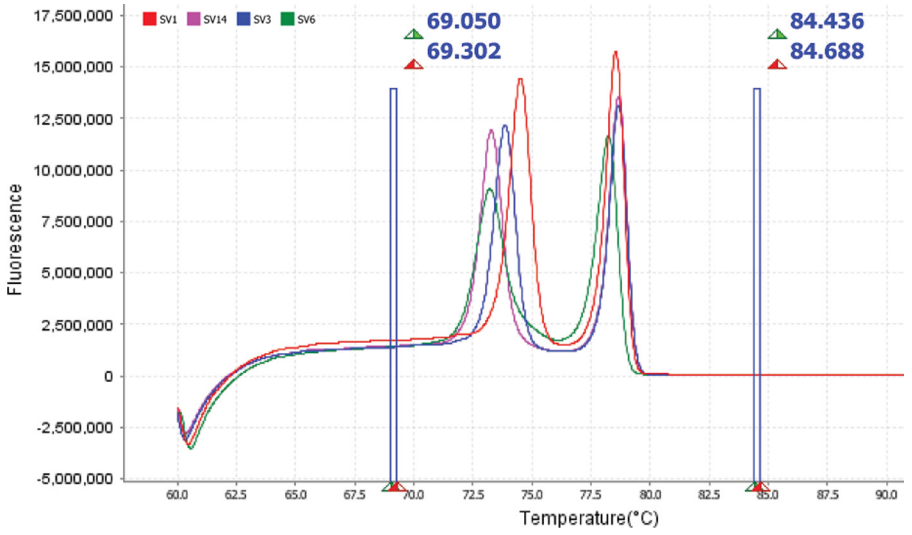


**FIG 4** Derivative high-resolution melt curves comparing individual *U. parvum* ATCC serovars 1, 3, 6, and 14 using the MeltDoctor HRM reagent kit.

**TABLE 3** Differences in ATCC *U. parvum* reference strain mean $T_m$ peaks between MeltDoctor HRM master mix (premix) and MeltDoctor HRM reagent kits

| Serovar | Kit | Mean $T_m$ (°C) for peak: | |
|---|---|---|---|
| | | 1 | 2 |
| 1 | Premix | 69.343 | 74.675 |
| 1 | Reagent | 78.518 | 74.429 |
| 3 | Premix | 68.077 | 75.139 |
| 3 | Reagent | 78.698 | 73.811 |
| 6 | Premix | 68.675 | 75.100 |
| 6 | Reagent | 78.224 | 73.096 |
| 14 | Premix | 67.904 | 74.600 |
| 14 | Reagent | 78.680 | 73.208 |

cervical, urethral, and vaginal swabs, De Francesco et al. (27) reported that SV1 and the combination of SV3 and SV14 (not separated beyond this description) were the most frequent isolates (37% and 39%, respectively), followed by SV6 (24%). Interestingly, this study found that *U. parvum* SV6 was significantly associated with a normal vaginal flora, in contrast to the SV3/SV14 combination, which was correlated with an absence of lactobacilli. This study also found correlations between *U. parvum* serovar type and the age of women.

Irrespective of discrepancies in their findings, previously described assays for serovar characterization of *U. parvum* have been both laborious and expensive, combining the use of endpoint PCR and sequencing (11, 29) or four separate single-plex (12) or two separate duplex (30) real-time PCR assays. Here, we have described the first assay for *U. parvum* serovar characterization using an HRM PCR approach. Due to the low GC nucleotide content, which is a feature of the *Ureaplasma* sp. genome, this represents a significant achievement, considering the need to identify a homologous amplicon with >25% GC content and suitable nucleotide variation within to allow discrimination between all four *U. parvum* serovars based on DNA melting dynamics. This assay was highly specific for *U. parvum*, with no amplification detected in *U. urealyticum* isolates, and, in cases of single-serovar colonization, allowed serovar characterization through the use of internal standards. Although it was not possible to identify individual *U. parvum* serovars when clinical samples indicated colonization by multiple serovars, cases where more than one serovar were present were easily distinguished from single-serovar colonization by the melt profile produced. We were able to validate our assay with only two isolates of SV14, one from the ATCC and another a prototype clinical isolate. However, the failure to identify any new SV14 strains from 76 new patient samples by either MBA sequencing or our HRM method reflects the rare distribution of this serovar in clinical samples. We are confident that the melt profiles produced by the two isolates used in this study are representative of *U. parvum* SV14; however, the only way this can be thoroughly validated is through collection of more clinical isolates of this serovar, which is likely to take many years.

Surprisingly, there were substantial differences in melt curve profiles between the two MeltDoctor HRM kits tested during this study. It is difficult to explain this, largely because the concentrations of reagents in the MeltDoctor HRM master mix (premix) are proprietary and not obtainable from the manufacturer (Life Technologies). This kit is provided as "optimized" for HRM assays. The ~66%-lower difference in fluorescence units detected in melt curves using the premix kit suggests that much lower levels of SYTO-9 dye are incorporated into this master mix. It is unknown whether an increased dye concentration contributes to a higher melting temperature for amplicons, although the major factor governing this is nucleotide composition, which should be identical in both assays. Regardless of these differences, when used for genotyping of *U. parvum* from clinical isolates, both HRM kits produced the same serovar characterization results so long as internal standards were included in each run. However, when used as a means of genotyping *U. parvum* directly from clinical samples, the premix kit is not suitable as it lacks the sensitivity of the reagent kit. It should be noted, however, that a limitation of our HRM assay for the genotyping of *U. parvum* directly from clinical samples is that, in cases of colonization by multiple serovars, we were unable to determine the serovar combinations present based upon melt curve analyses and instead could identify these only as "mixed" *U. parvum*. In such scenarios, one would need to employ microbiological culture and individual colony purification of samples to attempt to separate the multiple serovars. The effect of dominant/minority colonization on melt curves is also unknown. This is a potential limiting factor in the clinical application of this assay; however, previous studies have suggested that, unlike *U. urealyticum*, *U. parvum* may be more commonly found as one or two serovars within a clinical sample (28).

Although our HRM assay is highly effective at differentiating the serovars of *U. parvum* in cases of single-serovar colonization, it is important to note that it was not specifically designed as a detection tool for the initial screening of clinical samples. It is still very important to identify the presence of *U. urealyticum*, which may be of high clinical relevance to certain conditions, such as pelvic inflammatory disease/endometritis, nongonococcal urethritis, and BPD (13). As such, the HRM assay described here is designed to complement assays that initially detect *U. parvum* and *U. urealyticum*, as we have utilized it in this study with the urease gene assay described by Yi et al. (20). When used in combination with this, our HRM assay provides a much more rapid, cost-effective means of detecting single-serovar colonization by *U. parvum* than is currently available. When applied to large sample sets, it has the potential to provide valuable information relating to *U. parvum* serovar status and specific clinical conditions. Its suitability for use as a molecular diagnostic test will be confirmed through further testing on large numbers of *U. parvum* clinical isolates and different clinical sample types.

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