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Development of a Rapid and High-Throughput Multiplex Real-Time PCR Assay for *Mycoplasma hominis* and *Ureaplasma* Species

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Bacterial commensals of the human genitourinary tract, *Mycoplasma hominis* and *Ureaplasma* species (*parvum* and *urealyticum*) can be sexually transmitted, and may cause nongonococcal urethritis, pelvic inflammatory disease, and infertility. *Mycoplasma hominis* and *Ureaplasma* species may also cause severe invasive infections in immunocompromised patients. Current culture-based methods for *Mycoplasma/Ureaplasma* identification are costly and laborious, with a turnaround time between 1 and 2 weeks. We developed a high-throughput, real-time multiplex PCR assay for the rapid detection of *M. hominis* and *Ureaplasma* species in urine, genital swab, body fluid, and tissue. In total, 282 specimens were tested by PCR and compared with historic culture results; a molecular reference method was used to moderate discrepancies. Overall result agreement was 99% for *M. hominis* (97% positive percentage agreement and 100% negative percentage agreement) and 96% for *Ureaplasma* species (96% positive percentage agreement and 97% negative percentage agreement). Specimen stability was validated for up to 7 days at room temperature. This multiplex molecular assay was designed for implementation in a high-complexity clinical microbiology laboratory. With this method, >90 samples can be tested in one run, with a turnaround time of 4 to 5 hours from specimen extraction to reporting of results. This PCR test is also more labor effective and cheaper than the conventional culture-based test, thus improving laboratory efficiency and alleviating labor shortages. *(J Mol Diagn 2023, 25: 838–848; https://doi.org/10.1016/j.jmoldx.2023.07.004)*

Part of the Mollicutes class, *Mycoplasma hominis* (renamed as *Metamycoplasma hominis*) and *Ureaplasma* species (*parvum* and *urealyticum*) are known commensals of the human genitourinary (GU) tract. However, these organisms can also be sexually transmitted, with the potential to contribute to or cause nongonococcal urethritis,[1–3] pelvic inflammatory disease,[4,5] postpartum fever,[6,7] and infertility.[8,9] Moreover, these pathogens can also be transmitted to neonates, with the capability to cause pneumonia,[10,11] bacteremia,[12,13] and meningitis.[14,15] *Mycoplasma hominis* and *Ureaplasma* species can also cause severe invasive infections in immunocompromised patients, including pneumonia, septic shock, and infections of the pleural space.[16] Because of their ability to deplete arginine and disrupt the host urea cycle (*M. hominis*)[17,18] and their high efficiency at converting urea to ammonia via urease production (*Ureaplasma* species),[19–21] these organisms have further been implicated in the development of hyperammonemia syndrome in immunosuppressed patients (eg, solid organ[16,22,23] or stem cell transplant recipients[24] and those with hematologic malignant cancers[25,26]). This is a serious condition in which serum ammonia levels accumulate to dangerously high levels, resulting in neurologic abnormalities, cerebral edema, and death.

Identification of *Mycoplasma* and *Ureaplasma* species by standard diagnostic methods is challenging because of unique features: lack of a rigid cell wall precluding observation by staining techniques, requirements for complex growth media and growth conditions, extreme sensitivity to

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desiccation, and small bacterial colony size.[27] Current culture-based methods are costly and laborious, with typical turnaround time between 1 and 2 weeks. Screening of culture plates by microscopy is required for colony observation and organism identification and requires highly trained technologists, limiting method scalability. Overgrowth of specimens with non-*Mycoplasma*/*Ureaplasma* bacterial species, as well as significant growth sensitivity of the Mollicutes to inhibitory factors, may also interfere with *M. hominis* and *Ureaplasma* species detection by culture.[27] Therefore, a diagnostic method for rapid identification of *M. hominis* and *Ureaplasma* species is warranted. We describe development and validation of a high-throughput, multiplex real-time PCR assay for the rapid detection of *M. hominis* and *Ureaplasma* species (*parvum*/*urealyticum*) for clinical diagnosis.

Materials and Methods

Primer/Probe Design

All primer/probe sets were designed to amplify a gene segment of <150 bp and to have similar GC content and melting temperature. The Oligo Calc: Oligonucleotide Properties Calculator tool (*http://biotools.nubic.northwestern.edu/OligoCalc.html*, last accessed December 4, 2022) was used to assess and adjust each parameter accordingly. Each primer/probe set was synthesized by IDT (San Diego, CA) as a custom PrimeTime real-time quantitative PCR (qPCR) probe assay.

The *M. hominis* primer/probe set anneals to the *tuf* gene, which encodes the elongation factor thermal unstable protein. The *tuf* target corresponds to 63,613 to 63,696 of GenBank accession number CP055150 (*https://www.ncbi.nlm.nih.gov/nuccore/CP055150*), amplifying an 84-bp product. The *M. hominis* probe is labeled with Cy5. The intended *U. parvum* primer/probe set anneals to a gene encoding the caseinolytic peptidase B homolog (ClpB) chaperone protein, a member of the AAA + superfamily of ATPases. The ClpB target corresponds to 275,679 to 275,818 of GenBank accession number AP018561 (*https://www.ncbi.nlm.nih.gov/nuccore/AP018561*), amplifying a 140-bp product. The *U. parvum* probe is labeled with FAM. The intended *U. urealyticum* primer/probe set anneals to a gene encoding an ATP-binding cassette (ABC) transporter permease, corresponding to 13,186 to 13,285 of GenBank accession number CP039963 (*https://www.ncbi.nlm.nih.gov/nuccore/CP039963*). This target amplifies a 100-bp product, and the *U. urealyticum* probe is labeled with TET (equivalent to JOE).

The internal control (IC) primer/probe set targets a human housekeeping gene, RNase P, which encodes an essential endonuclease in tRNA maturation. The RNase P target corresponds to 21 to 104 of GenBank accession number NM_006413 (*https://www.ncbi.nlm.nih.gov/nuccore/NM_006413*), amplifying an 84-bp product. The IC probe is labeled with SUN (equivalent to VIC) (Table 1).

Nucleic Acid Extraction of Specimens

The Applied Biosystems MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA; reference A48383) was used to extract nucleic acid from specimens with the Thermo Scientific KingFisher Flex Purification System. The manufacturer's protocol for isolation of nucleic acid from biofluids and transport media was followed. Three separate wash plates were prepared with the addition of 1000 μL wash buffer, 1000 μL 80% ethanol, and 500 μL 80% ethanol in each sample well to respective plates. The elution plate contained 100 μL Elution Solution in each sample well. The sample plate was prepared by adding 10 μL of Proteinase K to each sample well. Preparation of all KingFisher extraction plates occurred at a clean reagent workstation with laminar flow capability in a separate pre-amplification room.

Binding Bead Mix was prepared according to the following sample well calculation: 530 μL Binding Solution, 20 μL Total Nucleic Acid Magnetic Beads, and 20 μL of human serum as IC for monitoring RNase P detection. For multiple reactions, an overage of 10% to 20% was prepared. The specimens were vortexed, and 400 μL was added to respective wells of the sample plate. After gentle inversion, 570 μL of the Binding Bead Mix was added to each well of the sample plate. The MVP_Flex program was selected on the instrument, and the prepared plates were loaded for extraction while wearing appropriate personal protective equipment. A negative extraction control, composed of sterile universal viral transport (UVT; Becton Dickinson, Franklin Lakes, NJ; reference 220220), and a positive extraction control, composed of UVT seeded with *M. hominis* (23114), *U. parvum* (27815), and *U. urealyticum* (27618), were extracted in parallel along with all clinical and contrived specimens (Figure 1).

Multiplex Real-Time PCR

Real-time PCR was performed on an Applied Biosystems 7500 Fast Dx (Thermo Fisher Scientific). Each PCR contained 2.5 μL PrimeTime Gene Expression Master Mix (2×; IDT; reference 1055771) with low concentration reference dye, 0.25 μL *M. hominis* qPCR Assay (20×), 0.25 μL *U. parvum* qPCR Assay (20×), 0.25 μL *U. urealyticum* qPCR Assay (20×), 0.25 μL RNase P qPCR Assay (20×), 0.5 μL PCR-grade water, and 1 μL of extracted specimen nucleic acid as template. Each PCR contained a 500 nmol/L concentration of all forward and reverse primers and a 250 nmol/L concentration of all probes. Optimal PCR conditions for PrimeTime Gene Expression Master Mix were based on IDT guidance for fast cycling: i) polymerase activation at 95°C for 3 minutes, ii) denaturation at 95°C for 5 seconds, and iii) annealing/extension at 60°C for 30 seconds. The

Table 1 *Mycoplasma hominis* and *Ureaplasma* Species Multiplex PCR Primer and Probe Sequences

Species	Target gene	Primers/probes	Primer sequence	5′ Probe reporter dye; 3′ quencher
M. hominis	*tuf*	Forward	5′-CGCTGGATTATTGCTACGTG-3′	
		Reverse	5′-TGAGGAACGATTGTCTTAGG-3′	
		Probe	5′-AGCGAAGTTGAACGTGGACAAG-3′	Cy5 (668); TAO/Iowa Black RQ
Ureaplasma parvum	ClpB ATPase	Forward	5′-CCATTATCGGTCGAGATGAAGAA-3′	
		Reverse	5′-ATGAACAATTCGGTATGCAAGC-3′	
		Probe	5′-TGTCTTAATTGGTGAACCTGGTG-3′	FAM (520); ZEN/Iowa Black FQ
Ureaplasma urealyticum	ABC transporter permease	Forward	5′-GGAATTGCAATGGGTTTGATCTC-3′	
		Reverse	5′-CTCCTAAGTTATCGGCTGATTGT-3′	
		Probe	5′-CCGTTAGCAACAATTCCTGTGTC-3′	TET* (539); ZEN/Iowa Black FQ
Human RNase P (internal control)	RNase P	Forward	5′-TGTTTGCAGATTTGGACCTGC-3′	
		Reverse	5′-AATAGCCAAGGTGAGCGGCT-3′	
		Probe	5′-AAGGCTCTGCGCGGACTTGTGGA-3′	SUN* (554); ZEN/Iowa Black FQ

*Integrated DNA Technologies TET and SUN reporter dyes are equivalent to JOE and VIC reporter dyes, respectively, on the Applied Biosystems 7500 instrument.

ABC, ATP-binding cassette; ClpB, caseinolytic peptidase B homolog.

denaturation and annealing/extension steps were performed for 40 cycles. For each PCR run, the negative extraction control and positive extraction control nucleic acid templates were run in parallel with all clinical and contrived specimens (Figure 1).

Preparation of LOD Material and Contrived Specimens

Quantified material for limit of detection (LOD) studies was prepared in-house with three ATCC (Manassas, VA) strains: *M. hominis* 23114, *U. parvum* 27815, and *U. urealyticum* 27618. Each strain was cultured in Remel 10 B Arginine Broth (Thermo Fisher Scientific; reference R20305) at 35°C with 9% CO_2. *Mycoplasma hominis* and *U. parvum* were cultured for 18 to 19 hours without rocking. As *U. urealyticum* is especially sensitive to urea depletion and elevated pH, this strain was cultured for only 3.5 hours with rocking. After broth amplification, each culture was 10-fold serially diluted to 10^{-6} in UVT, with each dilution plated (100 μL) to an A-8 selective agar plate (Thermo Fisher Scientific; reference R20204) and incubated at 35°C with 9% CO_2 in an enclosed, humidified container for at least 1 week. Bacterial colonies were counted from the A-8 plates with an inverted microscope, using a grid format, by two independent readers (J.P.Y. and E.G.C.) (Figure 1). The counts were averaged, and bacterial colony-forming units (CFUs)/mL were estimated through back-calculation.

Contrived negative and positive specimens to be tested by the assay were generated by pooling of known negative clinical matrix and by seeding known concentrations of bacteria (*Mycoplasma* and/or *Ureaplasma* species) into either sterile holding media (UVT) or into confirmed negative clinical matrix in UVT [genital swab, urine, body fluid, lower respiratory fluid, or ESwab (Becton Dickinson)]. Contrived specimen sets were generated by a single individual (J.L.C.) and were seeded at low to high organism concentration for each target: *M. hominis* (6.3×10^1 to 7.0×10^4 CFUs/mL), *U. parvum* (3.2×10^2 to 3.5×10^4 CFUs/mL), and *U. urealyticum* (6.6×10^1 to 7.3×10^3 CFUs/mL). Separate individuals (J.P.Y. and E.G.C.) were blinded to contrived specimen identity and performed specimen processing and assay performance.

Precision

For intra-assay precision, one negative extraction control and one positive extraction control were tested in triplicate in the same assay run. For interassay precision, two technologists blinded to 14 contrived specimens performed testing on two separate assay runs. A separate technologist generated the specimens with negative pooled vaginal/cervical genital swab clinical matrix in UVT seeded with low to high *M. hominis* and/or *Ureaplasma* species concentrations, as described in the previous paragraph.

Analytical Specificity

Several bacterial species that may be encountered in validated specimen types were tested to determine analytical specificity: *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Lactobacillus*, *Moraxella catarrhalis*, *Neisseria gonorrhoeae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, and *Streptococcus viridans*. Fresh growth of each organism was harvested from a blood agar plate (5% sheep blood), normalized to 0.5 McFarland in saline, and diluted 1:100 to yield approximately 1.5×10^6 CFUs/mL in UVT. Each organism was tested by the assay at this concentration. Other *Mycoplasma* and *Ureaplasma* species were also tested: *M. fermentans*, *M. genitalium*, *M. pneumoniae*, *U. diversum*, and *U. pacifica*. Because of the complex growth conditions required for these species,

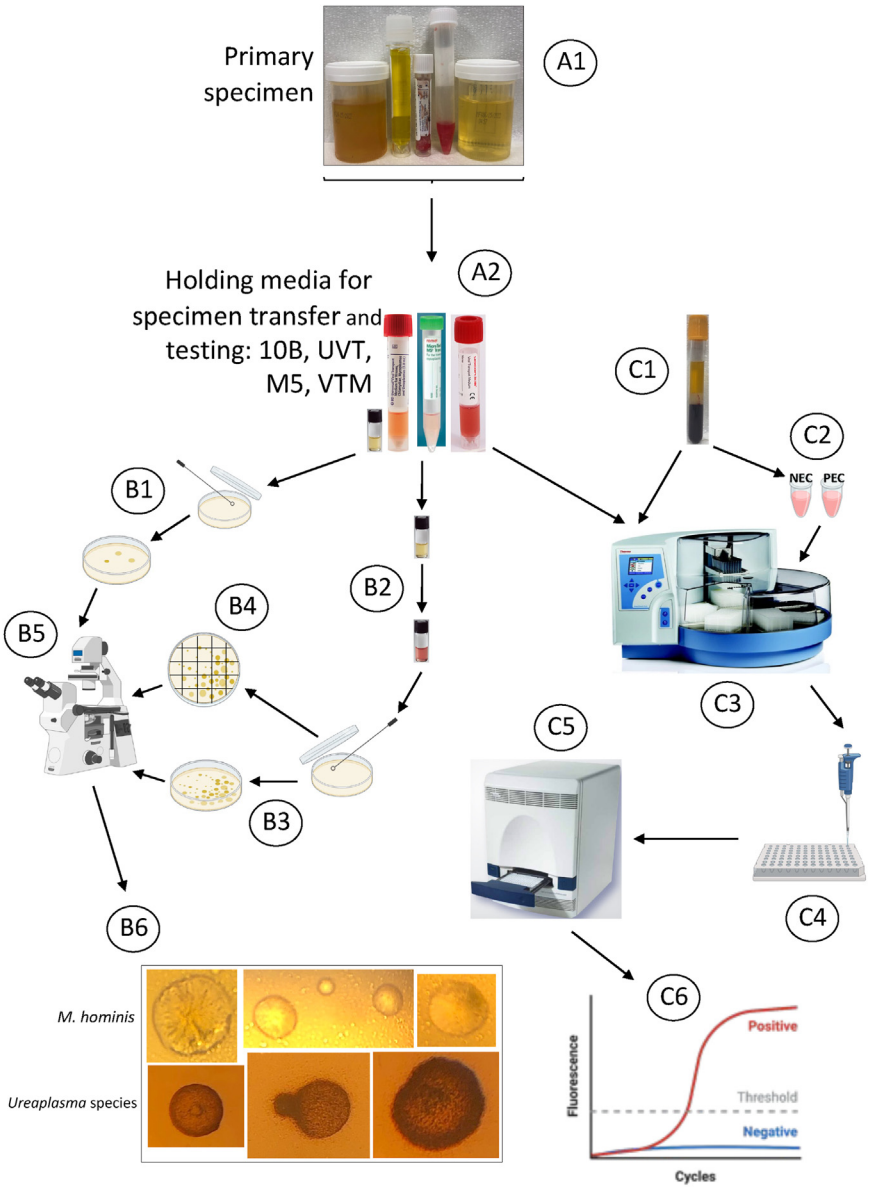

Figure 1 *Mycoplasma/Ureaplasma* culture-based testing versus molecular testing workflows. **A1:** Primary specimens: urine, lower respiratory fluid, body fluid, and ESwab. **A2:** Holding media into which primary specimens have been collected directly (ie, genital swabs) or have been transferred to for transport and storage. **B1:** Direct plating of specimen and culture on A-8 agar. **B2:** Amplification of specimen in 10 B Arginine Broth (color change to pink indicates growth). **B3:** Plating and culture of broth-amplified specimen on A-8 agar. **B4:** Application of grid for colony counting to prepare limit-of-detection material. **B5:** Use of inverted microscope for agar plate screening. **B6:** Characteristic *Mycoplasma/Ureaplasma* colony morphologies. **C1:** Each specimen is spiked with human serum as internal control for monitoring RNase P detection. **C2:** Negative extraction control (NEC) and positive extraction control (PEC) samples are run in parallel with each test batch. **C3:** Specimen nucleic acid extraction with KingFisher Flex Purification System. **C4:** PCR plate setup. **C5:** PCR assay performance with Applied Biosystems 7500 Fast Dx PCR System. **C6:** Depiction of PCR amplification curves. Some icons were sourced from BioRender.com (Toronto, ON, Canada). *M. hominis*, *Mycoplasma hominis*; UVT, universal viral transport; VTM, viral transport medium.

frozen stocks obtained from ATCC were diluted 1:2 directly into UVT and tested by the assay.

Specimen Stability

Pooled female vaginal/cervical genital swab–negative clinical matrix in UVT was seeded with *M. hominis* 23114, *U. parvum* 27815, and *U. urealyticum* 27618 at the established LOD for each strain. Aliquots of this contrived specimen were held at either 4°C or room temperature (approximately 22°C), and each condition was sampled at days 0, 3, 5, and 7. Specimen collections at each time point were extracted and tested by the *M. hominis* and *Ureaplasma* species multiplex PCR assay. Organism detection was evaluated by the generation of cycle threshold values for each assay target (*M. hominis*, *U. parvum*, and *U. urealyticum*).

Reference Methods

Routine *Mycoplasma/Ureaplasma* culture used both direct specimen plating and specimen broth amplification. Primary specimens are collected in or transferred to appropriate holding media and plated to A-8 agar and inoculated to 10 B Arginine Broth within 24 hours of collection. With incubation at 35°C (9% CO_2), A-8 plates are monitored for up to 7 days for the presence of *Mycoplasma* and *Ureaplasma* bacterial colonies, and 10 B broths are monitored for up to 7 days for a change in pH. Broths that indicate a pH change (color change to pink) are subcultured to A-8 plates, which

are further incubated and monitored for *Mycoplasma/Ureaplasma* colony development (Figure 1).

For discrepancy reconciliation, specimens were sent to an outside reference laboratory able to perform molecular testing for *Mycoplasma* and *Ureaplasma* species. This PCR reference method could detect *M. hominis* and *Ureaplasma* species (*parvum/urealyticum*) by PCR.

Method Comparison Calculations and Statistical Analysis

Method comparison calculations (percentage agreement and 95% CI) were performed using the MedCalc: Diagnostic Test Evaluation Calculator tool (*https://wwwmedcalcorg/calc/diagnostic_testphp*, last accessed June 2, 2023). Statistics to generate κ coefficients for each target were performed using the GraphPad by Dotmatics Quantify Agreement with Kappa tool (GraphPad Software, Boston, MA).

Results

Primer/Probe Design and PCR Optimization

To select the best-performing primers and probes, multiple sets were designed for each bacterial species. Early assessment of the *M. hominis tuf* housekeeping gene among 20 clinical isolates from different individuals (GU, oral, and synovial fluid sources) had found low levels of intraspecies sequence variability and a low frequency of recombination occurring in this gene.[28] Consequently, based on the work of Cunningham et al,[29] two primer/probe sets were designed to target the *M. hominis tuf* gene with Cy5 as the reporter: one set was inside of the published primers, and the other set targeted another unique region of the gene.

For *U. parvum*, primer/probe sets were designed to target different putative housekeeping or essential genes with *in silico* conservation among type strains of all four *U. parvum* serovars (1, 3, 6, and 14)[30]: ClpB ATPase gene and ABC transporter permease gene. The *U. parvum* probes were labeled with FAM as the reporter. For *U. urealyticum*, primer/probe sets were designed to target different genes with TET as the reporter. On the basis of the work of Xiao et al,[30] a primer/probe set was designed inside of the published *U. urealyticum* Giant *Ureaplasma* Membrane-Anchored Protein gene primers. Two other primer/probe sets were designed to target predicted *U. urealyticum* essential genes displaying *in silico* conservation to most type *U. urealyticum* strain serovars (2, 4, 8, and 10 to 13)[30]: UvrD-helicase gene and ABC transporter permease gene.

All primer/probe sets were tested in single-plex reactions with appropriate control DNA to assess overall performance in a 5-μL total volume PCR. On the basis of robust PCR amplification with intended targets, along with limited cross-reactivity, the following primer/probes were selected for further evaluation: i) *M. hominis* set targeting the unique region of the *tuf* gene, ii) *U. parvum* set targeting the ClpB ATPase gene, and iii) *U. urealyticum* sets targeting the Giant *Ureaplasma* Membrane-Anchored Protein gene and the ABC transporter permease gene. The selected primer/probes were further tested in multiplex PCR combination reactions with appropriate control DNA and clinical specimens to assess overall performance. Optimal multiplex assay performance was achieved with targeting of the *U. urealyticum* ABC transporter permease gene, and thus, the final *Mycoplasma/Ureaplasma* species primer/probe set combinations were selected (Table 1).

In silico analysis of the *M. hominis* primer/probe set showed collective mapping to the *tuf* gene in 12 of 18 publicly available complete reference genomes with 100% coverage and 100% identity, whereas only 86% to 95% coverage (100% identity) was observed for the remaining 6 genomes. In addition to better performance in the multiplex assay, this *M. hominis* set was also chosen because of its better specificity defined *in silico*. *In silico* analysis of the *U. parvum* primer/probe set showed mapping to the ClpB ATPase gene in 11 of 11 publicly available complete reference genomes, with 100% coverage and 100% identity. This target was also conserved among all *U. parvum* serotypes (1, 3, 6, and 14). *In silico* analysis of the *U. urealyticum* primer/probe set showed mapping to the ABC transporter permease gene in five of five publicly available complete reference genomes, with 100% coverage and 100% identity. Excluding serovar 7, this set was confirmed to map to type strains of all *U. urealyticum* serovars (2, 4, 5, and 8 to 13). The reference genome of the serovar 7 type strain (ATCC 27819) is of an incomplete genome assembly. This likely explains why primer/probe mapping to the type strain could not be established.

Assay Performance and Method Comparison

A method comparison study was performed, with a clinically validated culture-based method for *Mycoplasma/Ureaplasma* detection assigned as the reference standard (Figure 1). Urine, genital swab, and miscellaneous specimens held in various holding media were tested by the *M. hominis* and *Ureaplasma* species multiplex PCR assay. For clinical specimens, *Mycoplasma/Ureaplasma* culture results had been previously generated as part of the laboratory diagnostic testing workflow. For contrived specimens, various negative clinical matrices were seeded with known concentrations and combinations of *M. hominis*, *U. parvum*, and *U. urealyticum* from the previously prepared LOD material. Various contrived specimens were also held in different temperature conditions to assess specimen stability. A total of 285 specimens were tested by the PCR assay. Three of these specimens had no comparable culture result: in one specimen, the culture test had been cancelled; and in two specimens, a culture result could not be generated because of microbial overgrowth. Of the 282 specimens that could be compared with culture, 221 were clinical

Table 2 Method Comparison Summary Table for the *Mycoplasma hominis* and *Ureaplasma* Species Multiplex PCR Assay

Sample types and characteristics	No. of samples	
Total specimens tested with comparative data, *N*	282	
Total positive specimens, *n*	113	
Total negative specimens, *n*	169	
Holding media, *n* (%)*		
Remel 10B Arginine Broth	130 (46)	
Becton Dickinson universal viral transport	131 (46)	
Remel MicroTest M5 transport media	14 (5)	
YOCON viral transport medium	7 (2)	
Specimens, *n* (%)		
Urine	124 (44)	
Genital swab	124 (44)	
Lower respiratory tract (tracheal aspirate and BAL fluid)	5 (2)	
Body fluid (pleural)	6 (2)	
Wound or surgical swab (finger and shoulder)	4 (1)	
Other†	19 (7)	
Result agreement analysis	**Initial assessment**	**Discrepancy reconciliation**
M. hominis result agreement with culture method, *n*/total (%)	272/282 (96)	279/282 (99)
Positive percentage agreement, *n*/total (%) [95% CI]	58/67 (87) [76–94]	65/67 (97) [90–100]
Negative percentage agreement, *n*/total (%) [95% CI]	214/215 (100) [97–100]	214/215 (100) [97–100]
κ Coefficient	0.898	0.97
Ureaplasma species result agreement with culture method, *n*/total (%)	257/282 (91)	272/282 (96)
Positive percentage agreement, *n*/total (%) [95% CI]	82/90 (91) [83–96]	86/90 (96) [89–99]
Negative percentage agreement, *n*/total (%) [95% CI]	175/192 (91) [86–95]	186/192 (97) [93–99]
κ Coefficient	0.801	0.919
Invalid rate, *n*/total (%)‡	1/169 (0.6)	

*Media types in which primary specimens are held and from which testing is performed.
†Includes contrived specimens used for analytical specificity experiments. Bacterial isolates were spiked into sterile universal viral transport medium only.
‡On repeated testing, this single invalid result repeated as negative.
BAL, bronchoalveolar lavage.

specimens and 61 were contrived specimens. Overall, a total of 113 positive specimens (*M. hominis* and/or *Ureaplasma* species positive by culture or seeding) and 169 negative specimens (neither *M. hominis* nor *Ureaplasma* species recovered by culture or seeding) were tested. Result concordance between the two methods was assessed overall and by individual assay target: *M. hominis* and *Ureaplasma* species (Tables 2 and 3).

On initial assessment, PCR detection for *M. hominis* displayed 87% positive percentage agreement (PPA; 95% CI, 76%–94%) (58/67) and 100% negative percentage agreement (NPA; 95% CI, 97%–100%) (214/215); the κ coefficient was 0.898. PCR detection for *Ureaplasma* species displayed 91% PPA (95% CI, 83%–96%) (82/90) and 91% NPA (95% CI, 86%–95%) (175/192); the κ coefficient was 0.801. Overall, 30 of the 282 specimens yielded discordant results between culture and PCR. Of these, 29 specimens had sufficient remaining volume to be sent to a reference laboratory for *M. hominis*/*Ureaplasma* species qualitative PCR testing. Testing by the outside molecular reference method reconciled 19 of the 30 discordant results (Supplemental Table S1). After discrepancy reconciliation, PCR detection for *M. hominis* displayed 97% PPA (95% CI, 90%–100%) (65/67) and 100% NPA (95% CI, 97%–100%) (214/215); the κ coefficient was 0.970. PCR detection for *Ureaplasma* species displayed 96% PPA (95% CI, 89%–99%) (86/90) and 97% NPA (95% CI, 93%–99%) (186/192); the κ coefficient was 0.919 (Table 2).

Precision

For intra-assay precision, triplicate testing of the negative extraction control and positive extraction control yielded a 100% RNase P internal control detection rate and a 100% detection rate of each *Mycoplasma*/*Ureaplasma* assay target, respectively. For interassay precision, negative specimens had 100% result concordance for RNase P IC detection, and positive specimens had 100% result concordance for *M. hominis* and *Ureaplasma* species assay targets. Compared C_T values had SDs $\leq$ 3 and percentage CV $\leq$ 25%.

Analytical Sensitivity and Specificity

For analytical sensitivity, titered strains (*M. hominis* 23114, *U. parvum* 27815, and *U. urealyticum* 27618) were

Table 3 Description of Tested Clinical and Contrived Specimens

Sample types and characteristics	No. of samples
Total specimens tested with comparative data, *N*	282
Clinical specimens, *n*	221
Male	54 (24)
Female	167 (76)
Positive specimens by culture, *n*	80
Urine	42 (53)
Genital swab	37 (46)
Body fluid (pleural)	1 (1)
Negative specimens by culture, *n*	141
Urine	78 (55)
Genital swab	61 (43)
Body fluid (pleural)	1 (1)
Lower respiratory tract (tracheal aspirate)	1 (1)
Contrived specimens, *n**	61
Positive specimens by seeding, *n*	33
Genital swab	23 (70)
Urine	4 (12)
Body fluid (pleural)	2 (6)
Lower respiratory tract (BAL fluid)	2 (6)
Wound or surgical swab (finger and shoulder)	2 (6)
Negative specimens (no paired culture data), *n*	28
Genital swab	3 (11)
Body fluid (pleural)	2 (7)
Lower respiratory tract (BAL fluid)	2 (7)
Wound or surgical swab (finger and shoulder)	2 (7)
Various bacterial species seeded in UVT alone†	19 (68)

Data are given as number (percentage) unless otherwise indicated.

*Single or pooled clinical specimens and analytical specificity samples seeded with various *Mycoplasma hominis/Ureaplasma* species concentrations.

†Includes contrived specimens used for analytical specificity experiments. Bacterial isolates were spiked into sterile UVT only.

BAL, bronchoalveolar lavage; UVT, universal viral transport.

individually 10-fold serially diluted (10^{-1} to 10^{-6}) in UVT. Three replicates of each strain at each dilution were then extracted and tested by the multiplex PCR. Assessment of overall assay performance, from specimen extraction to PCR amplification, demonstrated acceptable linearity of the assay targets based on standard curve evaluation (Figure 2). The LOD for each species was defined as the highest dilution and corresponding concentration at which three of three replicates still achieved detectable fluorescence signal: *M. hominis*, 77 CFUs/mL; *U. parvum*, 355 CFUs/mL; and *U. urealyticum*, 80 CFUs/mL. The organism dilutions (10^{-4}) at the described LOD were further validated by testing 20 replicates of the corresponding extracted nucleic acid in the same PCR assay run. All targets achieved a ≥95% detection rate.

LOD performance was additionally assessed in pooled lower respiratory tract specimens, including bronchoalveolar lavage and pooled body fluid (joint, pleural, and peritoneal) in UVT seeded at the following concentrations: *M. hominis*, 154 CFUs/mL; *U. parvum*, 710 CFUs/mL; and *U. urealyticum*, 480 CFUs/mL. For each matrix, 20 replicates were tested. For bronchoalveolar lavage and body fluid, all assay targets achieved a ≥95% detection rate.

For analytical specificity, primers and probes designed for each assay target were first evaluated *in silico*. Conservation of intended gene targets was confirmed among respective completed genomes (*M. hominis*, *U. parvum*, and *U. urealyticum*) deposited in National Center for Biotechnology Information at the time of this writing. In addition, among 19 non—*M. hominis* and non—*U. parvum/urealyticum* bacterial species tested by the assay, no cross-reactivity was observed. All species tested, which included common pathogens and commensal organisms, were not detected by the *M. hominis* and *Ureaplasma* species multiplex PCR. Cross-reactivity was observed between *U. parvum* and *U. urealyticum* targets. The *U. parvum* strain was detected by the *urealyticum* target at 10% (2/20), and the *U. urealyticum* strain was detected by the *parvum* target at 90% (18/20). Because of this cross-reactivity, the assay does not differentiate between *U. parvum* and *U. urealyticum*. Assay detection of both or either *Ureaplasma* target is reported as positive for *Ureaplasma* species.

An invalid result was defined as a failed RNase P IC signal without detection of *M. hominis* and/or *Ureaplasma* species. Little interference was observed with the assay, as was shown by a low invalid rate of 0.6% (1/169) among negative specimens (Table 2). On specimen re-extraction, this singe invalid result repeated as negative.

Specimen Stability

Whether specimens were held continuously at 4°C or at room temperature (approximately 22°C), *M. hominis*, *U. parvum*, and *U. urealyticum* targets were detectable in specimens seeded at the LOD up to 7 days after specimen collection. Clinical specimens held in UVT will be acceptable for PCR-based testing up to 7 days after collection at the stated conditions.

PCR Cutoff Values

PCR amplification plots of all tested samples were collectively assessed to determine C_T cutoff values for each assay target. Cutoff values for *M. hominis* and *U. parvum* detection were set at C_T of 40. Because high background was observed with the *U. urealyticum* target TET (JOE equivalent) reporter dye, a C_T cutoff of 35 was set for detection. For the RNase P IC, because of its robust amplification and to maintain consistency with the lowest target cutoff (*U. urealyticum*), a C_T cutoff of 35 was set for detection.

Discussion

We developed a rapid, high-throughput multiplex real-time PCR assay for *M. hominis* and *Ureaplasma* species detection from diverse specimens. The assay was highly accurate, with a result concordance of 99% for *M. hominis*

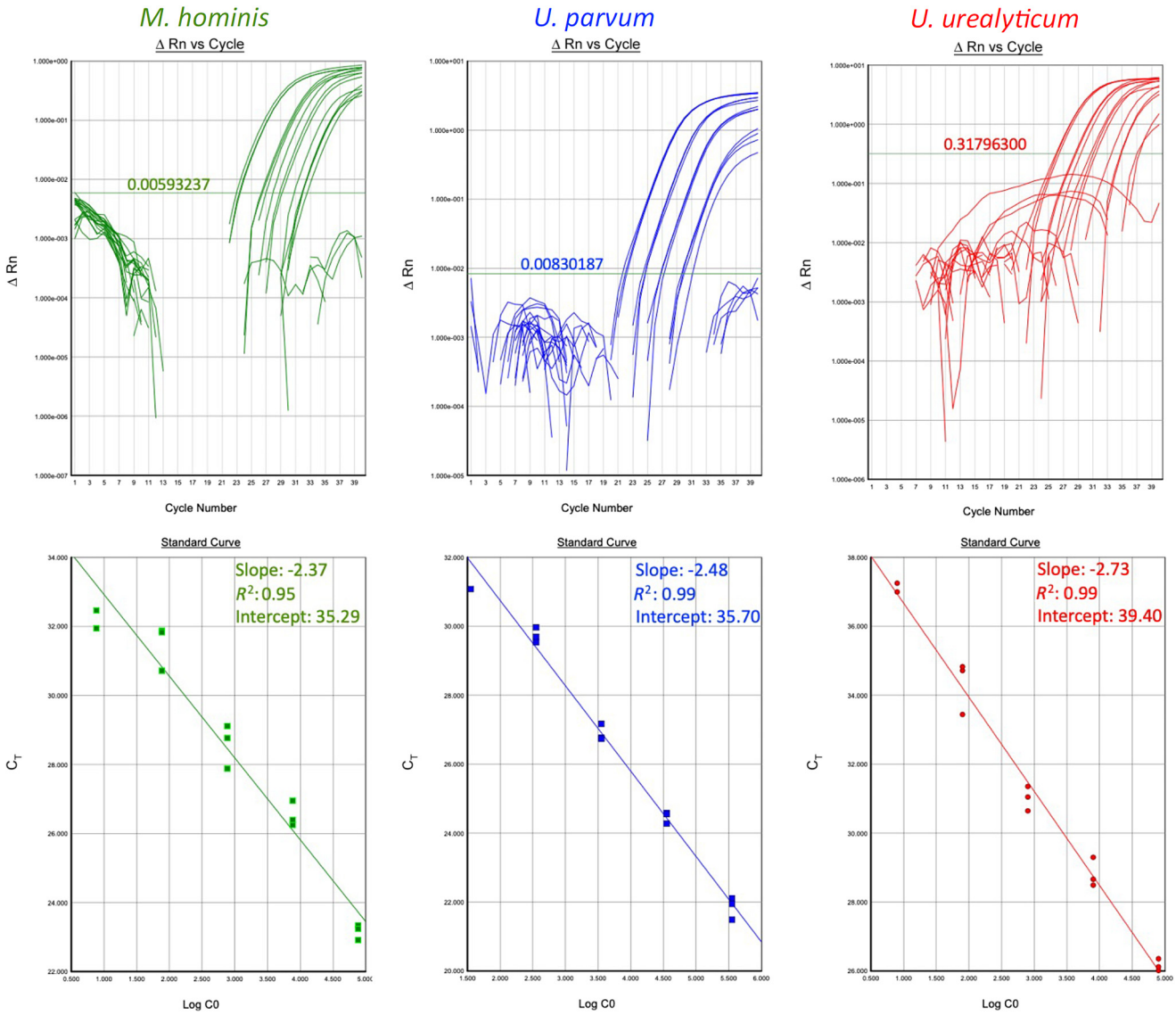

Figure 2 Multiplex PCR performance assessment and assay specifications. Titered strains (*Mycoplasma hominis* 23114, *Ureaplasma parvum* 27815, and *Ureaplasma urealyticum* 27618) were individually 10-fold serially diluted in universal viral transport. Three replicates of each strain dilution were then extracted and tested by the multiplex PCR. **Top panels:** Amplification plots of individual targets. **Bottom panels:** Standard curves of individual targets. C_T, cycle threshold; Log C0, estimated quantity (colony-forming units/mL); R^2, regression coefficient value; Rn, normalized reporter value.

(97% PPA and 100% NPA) and 97% for *Ureaplasma* species (96% PPA and 97% NPA). Some discordance from reference methods was expected because of inherent differences in overall method sensitivity (ie, culture versus molecular), the imprecise nature of colony screening by microscopy, and the potential loss of organism viability before culture.

The analytical sensitivity for each target reached a low LOD (<500 bacterial CFUs/mL). For analytical specificity, *in silico* analysis confirmed target gene conservation among only the intended *Mycoplasma*/*Ureaplasma* species. In addition, no false positives were observed when 19 other bacterial species were tested by the assay. For specimen stability, *M. hominis* and *Ureaplasma* species were reliably detected up to 7 days after specimen collection, stored at either 4°C or room temperature. Performance of this assay also allowed us to overcome microbial overgrowth observed in 2 of 285 specimens that impeded initial culture assessment: 1 vaginal swab was confirmed as negative, and 1 cervical/vaginal swab was confirmed as positive for *Ureaplasma* species.

The aim of the current study was to develop a molecular assay to effectively replace routine *Mycoplasma*/*Ureaplasma* culture being performed in the laboratory, freeing up valuable technician time for other essential clinical testing. Performance validation on clinical specimens held in appropriate transport media (ie, UVT) also allows for maintenance of *Mycoplasma*/*Ureaplasma* organism

viability in case phenotypic drug susceptibility testing must be pursued.[16,31,32] Considering that GU specimens are the highest submissions for this labor-intensive culture and that *M. hominis* and *Ureaplasma* species have been associated with urogenital infections and pregnancy complications,[11] many GU specimens (>200) were included in the validation.

Mycoplasma hominis and/or *Ureaplasma* species can also be significant pathogens affecting solid organ transplant recipients, associated with infections of the pleural space, mediastinum, peritoneum, and surgical sites, among others.[16,33,34] Our institution performed >700 solid organ transplants in 2021. Although rare, the development of hyperammonemia syndrome in lung transplant recipients (incidence up to 4.1% with 69% mortality)[35] and in other highly immunocompromised individuals is also of significant concern. A subset of these cases has been attributed to opportunistic disseminated infection with *M. hominis* or *Ureaplasma* species.[35] Pathogenic transmission of *U. parvum* from donor lungs to the recipient, resulting in infectious sequelae, has also been documented.[36] As the trajectory of this condition is accelerated—hyperammonemia noted as early as 5 days, with peak ammonia levels approximately 15 days after transplant[35]—rapid detection of *M. hominis*/*Ureaplasma* species to assist in diagnosis is essential. Thusly, bronchoalveolar lavage, tracheal aspirate, pleural fluid, and wound and surgical tissue specimens were further validated to aid in the detection of disseminated *M. hominis*/*Ureaplasma* species infection (Tables 2 and 3). The diversity of specimen types validated for this assay will better serve our transplant recipient population.

Earlier publications describing molecular detection methods for both *M. hominis* and *Ureaplasma* species relied on the performance of two separate real-time PCR assays for the detection of each species[29] or involved the performance of a nine-plex real-time PCR assay with melt curve analysis for differentiation of several sexually transmitted pathogens.[37] Using a four-plex design (including IC), our assay allows for the simultaneous detection of *M. hominis* and *Ureaplasma* species in a single PCR using a TaqMan-based single-probe system reliant on extension phase detection, whereas methods described by Cunningham et al[29] and Xiao et al[30] had used fluorescence resonance energy transfer–based dual-probe systems reliant on annealing phase detection. Although earlier molecular methods had prioritized the differentiation of *U. parvum* and *U. urealyticum* species,[29,30,38,39] which may provide useful information for overall *Ureaplasma* infection epidemiology, this capability does not impact clinical practice. Thus, although rare (10%) *U. parvum* cross-reaction with the intended *U. urealyticum* target was observed, we proceeded with assay validation for generic *Ureaplasma* species detection.

On the basis of work by Cunningham et al[29] demonstrating 91% sensitivity and 99% specificity with culture and outside 16 S ribosomal DNA testing of discordant samples, this study similarly targeted the *M. hominis tuf* gene for species detection, which yielded comparable results (97% PPA and 100% NPA) (Table 2). For *Ureaplasma* species detection, prior PCR assays had targeted the urease gene.[38] Yet, cross-reactivity with *S. aureus* occurred because of homology with a gene encoding a urease accessory protein requiring further primer modification.[39] The assay herein targets *Ureaplasma* species AAA family ATPase and ABC transporter permease genes, ensuring high sensitivity and specificity based on *in silico* and *in vitro* testing.

Limitations of the assay include the inability to differentiate between *U. parvum* and *U. urealyticum*. Currently, there is no known clinical utility for differentiation of *Ureaplasma* species.[40] Targeting both *parvum* and *urealyticum* species individually has provided higher confidence in the overall sensitivity of *Ureaplasma* species detection. Another limitation is the reliance on seed and recovery studies for validation of nongenital and nonurine specimens. Because of the infrequent recovery of *M. hominis* and *Ureaplasma* species from non-GU sources from clinical specimens, spike-in studies were necessary to perform the validation.

In summary, we developed and validated a multiplex real-time PCR assay that can be implemented in a high-complexity clinical microbiology laboratory for high-volume testing. With this method, >90 samples can be tested in one run, with a turnaround time of 4 to 5 hours from specimen extraction to reporting of results. Furthermore, this test is more cost-effective in terms of both labor and reagents (approximately $10/test). Reagent cost savings are realized as this assay was optimized to use a 5 μL PCR, which saved significant amount of PCR master mix, the most expensive component of the test. Stresses on microbiology laboratories continue to mount as critical clinical testing obligations increase, while significant staffing shortages persist. The *M. hominis* and *Ureaplasma* species multiplex PCR assay provides high accuracy and improves labor efficiency, helping to alleviate some of this strain. This molecular assay will replace *Mycoplasma*/*Ureaplasma* culture in our laboratory, becoming the method of choice for patient diagnostic testing. Continued advances in molecular diagnostics, along with the help of automation platforms, are part of the solution to the laboratorian workforce conundrum.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2023.07.004*.

References

1. Cox C, McKenna JP, Watt AP, Coyle PV: Ureaplasma parvum and Mycoplasma genitalium are found to be significantly associated with microscopy-confirmed urethritis in a routine genitourinary medicine setting. Int J STD AIDS 2016, 27:861−867
2. Couldwell DL, Gidding HF, Freedman EV, McKechnie ML, Biggs K, Sintchenko V, Gilbert GL: Ureaplasma urealyticum is significantly associated with non-gonococcal urethritis in heterosexual Sydney men. Int J STD AIDS 2010, 21:337−341
3. Deguchi T, Shimada Y, Horie K, Mizutani K, Seike K, Tsuchiya T, Yokoi S, Yasuda M, Ito S: Bacterial loads of Ureaplasma parvum contribute to the development of inflammatory responses in the male urethra. Int J STD AIDS 2015, 26:1035−1039
4. Taylor-Robinson D: Infections due to species of Mycoplasma and Ureaplasma: an update. Clin Infect Dis 1996, 23:671−682. quiz 683-684
5. Mardh PA, Westrom L: Tubal and cervical cultures in acute salpingitis with special reference to Mycoplasma hominis and T-strain mycoplasmas. Br J Vener Dis 1970, 46:179−186
6. Harwick HJ, Purcell RH, Iuppa JB, Fekety FR Jr: Mycoplasma hominis and abortion. J Infect Dis 1970, 121:260−268
7. Eschenbach DA: Ureaplasma urealyticum as a cause of postpartum fever. Pediatr Infect Dis 1986, 5:S258−261
8. Sleha R, Bostikova V, Hampl R, Salavec M, Halada P, Stepan M, Novotna S, Kukla R, Slehova E, Kacerovsky M, Bostik P: Prevalence of Mycoplasma hominis and Ureaplasma urealyticum in women undergoing an initial infertility evaluation. Epidemiol Mikrobiol Imunol 2016, 65:232−237
9. Huang C, Zhu HL, Xu KR, Wang SY, Fan LQ, Zhu WB: Mycoplasma and Ureaplasma infection and male infertility: a systematic review and meta-analysis. Andrology 2015, 3:809−816
10. Cassell GH, Waites KB, Watson HL, Crouse DT, Harasawa R: Ureaplasma urealyticum intrauterine infection: role in prematurity and disease in newborns. Clin Microbiol Rev 1993, 6:69−87
11. Waites KB, Katz B, Schelonka RL: Mycoplasmas and ureaplasmas as neonatal pathogens. Clin Microbiol Rev 2005, 18:757−789
12. Goldenberg RL, Andrews WW, Goepfert AR, Faye-Petersen O, Cliver SP, Carlo WA, Hauth JC: The Alabama Preterm Birth Study: umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. Am J Obstet Gynecol 2008, 198:43.e1−43.e5
13. Cassell GH, Waites KB, Crouse DT, Rudd PT, Canupp KC, Stagno S, Cutter GR: Association of Ureaplasma urealyticum infection of the lower respiratory tract with chronic lung disease and death in very-low-birth-weight infants. Lancet 1988, 2:240−245
14. Waites KB, Rudd PT, Crouse DT, Canupp KC, Nelson KG, Ramsey C, Cassell GH: Chronic Ureaplasma urealyticum and Mycoplasma hominis infections of central nervous system in preterm infants. Lancet 1988, 1:17−21
15. Viscardi RM, Hashmi N, Gross GW, Sun CC, Rodriguez A, Fairchild KD: Incidence of invasive ureaplasma in VLBW infants: relationship to severe intraventricular hemorrhage. J Perinatol 2008, 28:759−765
16. Chang SY, Price TK, Beaird OE, Gaynor PT, Schaenman JM, Carlson ME, Kubak BM, Yang S, Multani A: Mycoplasma hominis infections in solid organ transplant recipients: clinical characteristics, treatment outcomes, and comparison of phenotypic and genotypic susceptibility profiles. Transpl Infect Dis 2022, 24: e13822
17. Rouse HC, Bonifas VH, Schlesinger RW: Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. Virology 1963, 20:357−365
18. Schimke RT, Barile MF: Arginine metabolism in pleuropneumonia-like organisms isolated from mammalian cell culture. J Bacteriol 1963, 86:195−206
19. Romano N, La Licata R, Russo Alesi D: Energy production in Ureaplasma urealyticum. Pediatr Infect Dis 1986, 5:S308−S312
20. Smith DG, Russell WC, Ingledew WJ, Thirkell D: Hydrolysis of urea by Ureaplasma urealyticum generates a transmembrane potential with resultant ATP synthesis. J Bacteriol 1993, 175:3253−3258
21. Fleming D, Cunningham SA, Patel R: Contribution of uremia to Ureaplasma-induced hyperammonemia. Microbiol Spectr 2022, 10: e0194221
22. Nowbakht C, Edwards AR, Rodriguez-Buritica DF, Luce AM, Doshi PB, De Golovine A, Bynon JS, Nigo M: Two cases of fatal hyperammonemia syndrome due to Mycoplasma hominis and Ureaplasma urealyticum in immunocompromised patients outside lung transplant recipients. Open Forum Infect Dis 2019, 6:ofz033.
23. Leger RF, Silverman MS, Hauck ES, Guvakova KD: Hyperammonemia post lung transplantation: a review. Clin Med Insights Circ Respir Pulm Med 2020, 14:1179548420966234
24. Graetz R, Meyer R, Shehab K, Katsanis E: Successful resolution of hyperammonemia following hematopoietic cell transplantation with directed treatment of Ureaplasma parvum infection. Transpl Infect Dis 2018, 20:e12839
25. Smith M, Crews JD, Cheek N, Srivastava R, Appachi E: Hyperammonemic encephalopathy due to Ureaplasma parvum infection in an immunocompromised child. Pediatrics 2019, 144:e20190601
26. Placone N, Kao RL, Kempert P, Ruiz ME, Casillas JN, Okada M, Gibson JB, Maggi C, O'Brien K, Nattiv R, Gallant NM, Abrams SH: Hyperammonemia from Ureaplasma infection in an immunocompromised child. J Pediatr Hematol Oncol 2020, 42:e114−e116
27. Hutto C: Congenital and perinatal infections a concise guide to diagnosis. Infectious Disease. Totowa, NJ, Humana Press, 2006. pp. 271−288
28. Sogaard IZ, Boesen T, Mygind T, Melkova R, Birkelund S, Christiansen G, Schierup MH: Recombination in Mycoplasma hominis. Infect Genet Evol 2002, 1:277−285
29. Cunningham SA, Mandrekar JN, Rosenblatt JE, Patel R: Rapid PCR detection of Mycoplasma hominis, Ureaplasma urealyticum, and Ureaplasma parvum. Int J Bacteriol 2013, 2013:168742
30. Xiao L, Glass JI, Paralanov V, Yooseph S, Cassell GH, Duffy LB, Waites KB: Detection and characterization of human Ureaplasma species and serovars by real-time PCR. J Clin Microbiol 2010, 48: 2715−2723
31. Krausse R, Schubert S: In-vitro activities of tetracyclines, macrolides, fluoroquinolones and clindamycin against Mycoplasma hominis and Ureaplasma ssp. Isolated in Germany over 20 years. Clin Microbiol Infect 2010, 16:1649−1655
32. Beeton ML, Spiller OB: Antibiotic resistance among Ureaplasma spp. Isolates: cause for concern? J Antimicrob Chemother 2017, 72: 330−337
33. Steffenson DO, Dummer JS, Granick MS, Pasculle AW, Griffith BP, Cassell GH: Sternotomy infections with Mycoplasma hominis. Ann Intern Med 1987, 106:204−208
34. Haller M, Forst H, Ruckdeschel G, Denecke H, Peter K: Peritonitis due to Mycoplasma hominis and Ureaplasma urealyticum in a liver transplant recipient. Eur J Clin Microbiol Infect Dis 1991, 10:172
35. Krutsinger D, Pezzulo A, Blevins AE, Reed RM, Voigt MD, Eberlein M: Idiopathic hyperammonemia after solid organ transplantation: primarily a lung problem? a single-center experience and systematic review. Clin Transplant 2017, 31:e12957
36. Fernandez R, Ratliff A, Crabb D, Waites KB, Bharat A: Ureaplasma transmitted from donor lungs is pathogenic after lung transplantation. Ann Thorac Surg 2017, 103:670−671
37. Hu XM, Xu JX, Jiang LX, Deng LR, Gu ZM, Xie XY, Ji HC, Wang WH, Li LM, Tian CN, Song FL, Huang S, Zheng L, Zhong TY: Design and evaluation of a novel multiplex real-time PCR melting curve assay for the simultaneous detection of nine sexually transmitted disease pathogens in genitourinary secretions. Front Cell Infect Microbiol 2019, 9:382

38. Cao X, Wang Y, Hu X, Qing H, Wang H: Real-time TaqMan polymerase chain reaction assays for quantitative detection and differentiation of Ureaplasma urealyticum and Ureaplasma parvum. Diagn Microbiol Infect Dis 2007, 57:373–378
39. Vancutsem E, Soetens O, Breugelmans M, Foulon W, Naessens A: Modified real-time PCR for detecting, differentiating, and quantifying Ureaplasma urealyticum and Ureaplasma parvum. J Mol Diagn 2011, 13:206–212
40. Xiao L, Paralanov V, Glass JI, Duffy LB, Robertson JA, Cassell GH, Chen Y, Waites KB: Extensive horizontal gene transfer in ureaplasmas from humans questions the utility of serotyping for diagnostic purposes. J Clin Microbiol 2011, 49:2818–2826