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Evaluation of the MYCOPLASMA IST3 urogenital mycoplasma assay in an international multicentre trial

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Objectives: To evaluate the accuracy, susceptibility and specificity of MYCOPLASMA IST3, the next generation of the most popular culture-based *in vitro* diagnostic device designed to detect, identify and test the susceptibility of urogenital mycoplasma infections.

Methods: MYCOPLASMA IST3 was evaluated against culture- and molecular-based gold standard methodologies to detect, identify, enumerate and determine antimicrobial resistance for *Mycoplasma hominis and Ureaplasma* species in 516 clinical samples collected across France, Serbia and the UK. Sample types included vulvovaginal/endocervical or urethral swabs (dry swab or eSwab®), semen and urine samples, which included blinded analysis following addition of a panel of 80 characterized control strains.

Results: Overall species identification was excellent for both *Ureaplasma* spp. (98.4% sensitivity, 99.7% specificity) and *M. hominis* (95.7% sensitivity, 100% specificity) relative to combined colony morphology on agar and quantitative PCR standards. Non-dilution-based bacterial load estimation by the assay was accurate between 83.7% (*M. hominis*) and 86.3% (*Ureaplasma* spp.) of the time (increased to 94.2% and 100%, respectively, if ±10-fold variance was allowed) relative to colonies counted on agar. Resistance accuracy for *Ureaplasma* spp. varied from gold standards for only 11/605 of individual tests (major error rate = 1.8%) and for 14/917 individual tests for *M. hominis* (major error rate = 1.5%).

Conclusions: The redesigned MYCOPLASMA IST3 assay eliminated previous shortcomings by providing independent accurate resistance screening of *M. hominis and Ureaplasma* species, even in mixed infections, with CLSI-compliant thresholds. Specificity, sensitivity and enumeration estimates correlated closely with the confirmatory methods.

Introduction

Human urogenital mycoplasma infections were recognized as early as 1898 for *Mycoplasma hominis*[1] and 1954 for *Ureaplasma* spp.[2] By 1966, *in vitro* antimicrobial susceptibility studies were already being performed on these bacteria, with evidence of antimicrobial resistance.[3] Despite the controversy underlying the role of these organisms as causative agents in urogenital[4,5] and fertility[6] pathogenesis, reports of antimicrobial resistance continue to increase annually. Some studies have examined population urogenital mycoplasma epidemiology on a scale of 10 000–37 000 samples,[7–9] and because of the need for technical simplicity have relied on the use of culture-based, colour-changing assays that identify the species of mycoplasma infection and determine 'antimicrobial resistance' (e.g. growth of that species in the presence of a range of antibiotics set at threshold concentrations). All *Mycoplasma and Ureaplasma* species have evolved to have a reduced-size genome that necessitates a parasitic existence within the host to facilitate replication (due to breakages in several synthetic pathways). Thereby, the effective classes of antimicrobial agents are only those that disrupt DNA gyrase activity (fluoroquinolones) and those inhibiting prokaryotic protein synthesis

(tetracyclines and members of the macrolide/lincosamide/streptogramin/ketolide or MLSK class).

Many of the currently commercially available identification and antimicrobial resistance-determining devices for urogenital mycoplasmas were designed prior to the international standardization of antimicrobial thresholds being set by CLSI in 2011.[10,11] Additional design limitations in these devices (outlined in detail in the review by Beeton and Spiller),[12] aside from inappropriate choice or concentration of antimicrobials tested, include obfuscation of resistance results in a mixed infection containing *M. hominis* (inherently resistant to macrolides)[13] and *Ureaplasma* spp. (inherently resistant to lincosamides).[14] They also lack the ability to determine whether bacterial load, internationally set as between 10^4 and 10^5 colour-changing units (CCUs), is compliant with the resistance threshold concentrations that have been validated by CLSI.

The MYCOPLASMA IST2 *in vitro* medical device represents the most commonly used assay for determining resistance in culturable urogenital mycoplasmas. We have evaluated the third generation of this device (MYCOPLASMA IST3), which has the significant improvement of separating growth of *M. hominis* from *Ureaplasma* spp. for independent evaluation of CLSI-compliant concentrations of appropriate antimicrobials (choice and concentration differs between the two species), against gold standard broth microdilution and PCR methodologies. Validation was performed on a range of samples including swabs (vulvovaginal, endocervical and urethral), semen samples and urine collected in multiple clinical centres in the UK, France and Serbia. Additionally, we evaluated the accuracy of a novel enumeration read-out added to the assay (proprietary technology that did not require serial sample dilution), relative to the number of colonies that grew from serially diluted samples inoculated on A7 agar to assess sample bacterial load. Given the infrequency of sufficient numbers of antimicrobial-resistant isolates being collected, a set of 80 characterized archived resistant and susceptible *M. hominis and Ureaplasma* spp. strains were also evaluated following evaluator-blinded addition to sterile samples known to be free of endogenous mycoplasmas. For rigorous determination of antimicrobial resistance accuracy reporting, WGS analysis was used to identify the underlying mechanisms of resistance.

Materials and methods

Clinical samples

Clinical samples were from patients attending urology, fertility or sexually transmitted infection (STI) clinics and were collected through regular screening or for confirmation of a diagnosis of urogenital mycoplasmas. Participants from the UK were recruited on attendance at an independent sexual healthcare provider, HealthFirst Consulting (ethics granted by Cardiff University School of Medicine Research Ethics Committee). Following informed consent, anonymized leftover urine samples and endocervical swab samples were utilized for this research. Semen, male first-catch urine, urethral swabs and cervical or vaginal swab samples from anonymized participants in France were left over from clinical samples sent to CERBAlliance, Rhone Alpes-Centre Est, Lyon; they were regarded as 'waste' and therefore exempt from participant consent requirement (law 2012-300 of 12 March 2012 on research involving humans). Urethral, vulvovaginal and endocervical swab samples were provided by participants in Serbia attending Pancevo Health Institute, Pancevo (ethical approval 01-615/4-2018 granted by the Public Health Institute of Pancevo).

Reference isolates

A panel of 80 isolated strains (15 *Ureaplasma* spp. and 65 *M. hominis*) from previously published clinical cohorts,[15–17] as well as strains from the UROGEN study (IRAS 251053; 500 Welsh Sexual Health patients; O. B. Spiller and L. C. Jones, unpublished results), with known antimicrobial susceptibility or characterized resistance, were used as controls. These quality controls were coded (species and susceptibility unknown to the recipient) and aliquots containing 10^4 CCUs of viable bacteria were sent on dry ice to test facilities in France and the UK. These strains were kept at −80°C until required, then they were thawed and added immediately to 1 mL aliquots of clinical samples prior to processing for the MYCOPLASMA IST3 (samples also run without 'spiking' in parallel to examine endogenous infection). Spiked and unspiked samples were processed as per the study design protocol.

Study protocol

All samples were provided with a sequential anonymized code based on the country of origin (C001 for France, S001 for Serbia, U001 for Wales etc.). Data for all countries were recorded directly into the Medidata Rave web-based data capture platform. Initial data collected included sample type (semen, urine, vulvovaginal/endocervical swab, urethral swab), participant gender, date sample collected, swab type (dry swab, eSwab or not applicable) and the spike date (if applicable); further details are in the Supplementary Materials and methods, available as Supplementary data at *JAC* Online.

Following sample collection, contents from dry swabs were released by agitation into the R1 transport broth included in the assay, whereas 200 μL of transport medium from eSwabs or 200 μL of urine or semen were added and mixed with R1 for other sample types. Inoculated R1 broths have been validated to be stable without infectivity loss for 5 h at 18°C–25°C or 48 h at 2°C–8°C. With as short a delay as possible, the assay was set up and inoculated as per the manufacturer's instructions (Supplementary Materials and methods and Figure S1a), starting with a suspension of 3 mL of R1 being added into the R2 vial containing lyophilized growth medium followed by the inoculation of three non-confluent 10 μL drops of the R1 + R2 suspension onto the surface of A7 agar (bioMérieux, France) for species identification and enumeration. The R1 + R2 vials were incubated alongside the assay and at the first observation of colour change, a 10-fold dilution series in growth medium was performed and frozen for transport to Cardiff University for quantitative PCR (qPCR) and MIC determination.

Gold standard comparison methods

Quality control for gold standards utilized the recommended strains ATCC 23114 (*M. hominis*), ATCC 27815 (*Ureaplasma parvum*) and ATCC 33175 (*Ureaplasma urealyticum*); they were used to evaluate each batch of MYCOPLASMA IST3 assays (bioMérieux), each batch of A7 agar (bioMérieux) and run in parallel during each experiment for determining the MIC for clinical samples. MICs were determined for all isolates using broth microdilution methods compliant with CLSI M43A guidelines (thresholds detailed in Supplementary Materials and methods).[10,11,17] Confirmation of species was performed by colony morphology determination on A7 agar[18] and by specific multiplex qPCR relative to a standardized plasmid containing defined copies of all primer/probe targets as previously published.[15] Viability and quantification of *M. hominis and Ureaplasma* spp. in the initial clinical sample were determined by A7 agar (bioMérieux) colony enumeration. Plates were incubated microaerophilically at 37°C and the two distinctive colony types were independently counted at ×100 magnification by light microscopy, in three non-overlapping fields per drop (nine fields). Average colony numbers were used to stratify bacterial load (>0 but <1 average colonies, 10^3 cfu/mL; 1–4 colonies, 10^4 cfu/mL; 5–15 colonies, 10^5 cfu/mL; and >15 colonies, $\geq 10^6$ cfu/mL).

Validation values and statistical analysis

Sensitivity, specificity, positive predictive value and negative predictive value were calculated using a 2×2 contingency table and for each value a 2-sided CI was determined. The Electronic Data Capture (EDC) system Medidata Rave was used for data management activity, with direct entry at 0, 24 and 48 h timepoints, as well as for confirmatory data generated for qPCR and MIC at Cardiff University for calculation of accuracy. Significance was set at $P < 0.05$ and analysis was carried out for each species independently. The antimicrobial susceptibility testing (AST) performance, as assessed by categorical agreement (CA, %), major error rate (ME, %) and very major error counts (VME, *n*), was calculated by comparing the MYCOPLASMA IST3 category (susceptible or resistant) with MIC results interpreted using CLSI M43-A interpretive breakpoints.

Underlying mechanisms of resistance

WGS was performed on an Illumina MiSeq with scaled-up cultures of *Ureaplasma and Mycoplasma* strains, following genomic DNA extraction as described in previously published methods for both reference and endogenous strains (see Supplementary Materials and methods and Table S1 for more details).[15,19]

Results

Collected sample demographics

Clinical samples were collected from consenting participants in Wales ($n = 143$), France ($n = 213$) and Serbia ($n = 160$). Sample types investigated included semen ($n = 88$), male urine ($n = 142$), urethral swab ($n = 26$ by eSwab and $n = 30$ by dry swab) and vulvovaginal or endocervical swab ($n = 61$ by eSwab and $n = 169$ by dry swab), as detailed in Table 1. A panel of 80 pre-characterized samples were also added ('spiked') into samples in an experimenter-blinded fashion, which included 7 antimicrobial-susceptible *Ureaplasma* spp. strains, 8 antimicrobial-resistant *Ureaplasma* strains (4 macrolide-resistant strains, 2 tetracycline-resistant strains and 2 fluoroquinolone-resistant strains), 49 antimicrobial-susceptible *M. hominis* strains and 16 antimicrobial-resistant *M. hominis* strains (2 moxifloxacin-resistant strains and 14 tetracycline-resistant strains).

MYCOPLASMA IST3 detection and identification of *M. hominis* and *Ureaplasma* spp.

Species identification by the MYCOPLASMA IST3 was compared with A7 agar identification by colony morphology[18] for *M. hominis* ('fried egg', i.e. central colony with lower surrounding coronal dispersion across the surface of the agar) and *Ureaplasma* species ('sea urchin', i.e. small intense colony, visualization aided by brown precipitate, without colony corona), with discrepancies confirmed via qPCR. Overall, the accuracy of species identification was very high for both *Ureaplasma* spp. (98.4% sensitivity, 99.7% specificity) and *M. hominis* (95.7% sensitivity, 100% specificity), with minimal false positive or false negative results (Table 2). Furthermore, all samples spiked with viable characterized strains (15 *Ureaplasma* spp. and 65 *M. hominis*) were correctly identified. In total, 108 new *Ureaplasma* spp. isolates and 21 new *M. hominis* isolates were detected and collected from unspiked clinical specimens.

MYCOPLASMA IST3 enumeration of *M. hominis* and *Ureaplasma* spp.

The accuracy of the assay's determination of bacterial load, which is a requirement for CLSI-compliant AST, was evaluated relative to colonies formed on A7 agar measured at 24 and 48 h post-inoculation relative to colour change in the $\geq 10^3$ (*Ureaplasma* only), $\geq 10^4$ and $\geq 10^6$ cupules. This evaluates the capacity of the assay to estimate the number of bacteria per mL, in the absence of physical dilution; the data are presented in Table 3 and show that the assay and growth on agar were in complete agreement (agar colonies/mL rounded to nearest 10) between 83.7% (*M. hominis*) and 86.3% (*Ureaplasma* spp.) of the time and they were within one 10-fold dilution (i.e. within ±10 cfu/mL agreement) between 94.2% (*M. hominis*) and 100% (*Ureaplasma* spp.) of the time. Data were excluded for those samples where cfu/mL was out of the range of the assay or where other bacterial or fungal contamination growth obscured colony counting on A7 agar.

MYCOPLASMA IST3 accuracy for detecting antimicrobial resistance

From the 516 samples collected, MICs of levofloxacin, moxifloxacin, tetracycline and clindamycin were determined for all 85 *M. hominis*-positive samples (endogenous and spiked) detected by the MYCOPLASMA IST3 assay; likewise, MICs of levofloxacin, moxifloxacin, tetracycline, erythromycin and telithromycin were determined for 124 *Ureaplasma* spp. strains (Table 4). These results were used to validate the prediction accuracy (Table 5) of antimicrobial resistance identified by the MYCOPLASMA IST3 assay. Comparison of the results for the 65 characterized *M. hominis* strains spiked into various negative sample types correctly identified all 15 strains carrying *tet*(M) resistance genes (MIC = 16 or 32 mg/L). MYCOPLASMA IST3 also correctly identified two strains with resistance-mediating mutations in both the *gyrA and parC*

Table 1. Details of sampling type, geographic location and addition of characterized strains

Sample	No growth	Endogenous	Negative and spiked	Total	From Wales	From Serbia	From France
Urine	90	16	36	142	104	0	38
Female swab	115	87	28	230	39	130	61
Male swab	45	5	6	56	0	30	26
Semen	66	12	10	88	0	0	88

All urine samples were from male patients. All male swabs were urethral swabs; all female swabs were either vulvovaginal or endocervical swabs. All spiked urine samples and vulvovaginal/endocervical swabs were tested in Wales, while all spiked urethral swabs and semen samples were tested in France.

Table 2. Statistics for correct identification of *Ureaplasma* spp. and *M. hominis*

Isolate	True positive	False positive	True negative	False negative	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive predictive value, %	Negative predictive value, %
Ureaplasma spp.	125	1	384	2	98.4 (94.4–99.6)	99.7 (98.5–100.0)	99	99
M. hominis	88	0	419	4	95.7 (89.3–98.3)	100.0 (99.1–100.0)	100	99

A total of five samples were excluded from comparisons: one sample was excluded from *M. hominis* analysis due to the presence of fungus in the original sample, preventing quantification of viable organisms; and four samples containing erythromycin-resistant *Ureaplasma* spp. were excluded, as directed by the MYCOPLASMA IST3 *in vitro* diagnostics instructions for use.

Table 3. Summary of MYCOPLASMA IST3 bacterial load enumeration accuracy

Isolate	Exact agreement, % (95% CI) *n/N*	Within ±10 cfu/mL agreement, % (95% CI) *n/N*
Combined species[a]	86.0 (80.3–90.3) 160/186	97.8 (94.6–99.4) 182/186
Ureaplasma spp.	86.3 (78.9–91.4) 101/117	100.0 (96.9–100.0) 117/117
M. hominis	83.7 (74.5–90.0) 72/86	94.2 (87.1–97.5) 81/86

[a]For mixed samples, a concordant sample may be concordant for *Ureaplasma* spp. and/or *M. hominis*.

genes for moxifloxacin (MIC = 4 mg/L) and levofloxacin (MIC = 8 mg/L) and correctly identified 49 fully susceptible control *M. hominis* strains. Clindamycin resistance was not found in any samples (control or endogenous strains), in full agreement with MIC values for all strains (maximum MIC = 0.125 mg/L). In 4 of the 21 samples with endogenous *M. hominis*, the *M. hominis* was tetracycline resistant [MIC = 32 or 64 mg/L, all carrying the *tet*(M) resistance gene], all from vulvovaginal or endocervical swabs: 1 from France and 3 from Serbia. Importantly, 17/21 of these samples were also positive for *Ureaplasma* spp. (i.e. a mixed infection) and the MYCOPLASMA IST3 assay correctly associated the tetracycline resistance only with the *M. hominis* infection, which the predecessor assay (MYCOPLASMA IST2) was unable to do. This is because the MYCOPLASMA IST3 has separate selective areas for each species that allows differential interrogation of antimicrobial susceptibility (see Figure S2 for further details).

MIC determination (Table 4) and assay performance for *Ureaplasma* spp. was similar (Table 5). Of the 15 characterized *Ureaplasma* spp. strains, MYCOPLASMA IST3 correctly identified the 2 *tet*(M)-positive strains (MIC = 16 mg/L). Two of the endogenous *Ureaplasma* spp. were found to have MICs above the resistance threshold (one at 2 mg/L and one at 4 mg/L), but the assay only identified growth at 1 mg/L for the latter strain at 24 h. However, neither of these strains was found to carry the *tet*(M) gene and the mechanism of these intermediate tetracycline MICs is unknown. The assay identified the only moxifloxacin-resistant sample (MIC = 8 mg/L), a vaginal/cervical swab sample from France (C031), correctly. The underlying resistance mechanism was identified by WGS as a dual mutation of S83L in ParC in combination with an E482K mutation in GyrB (no mutations in GyrA or ParE), which is adjacent to the QRDR identified for other bacteria (Figure S1). This strain also had a levofloxacin MIC of 32 mg/L that was also correctly identified by MYCOPLASMA IST3. Table 4 shows three *Ureaplasma* spp. isolates with a levofloxacin MIC of 4 mg/L (one characterized strain DF99 and two vaginal/cervical swab samples from Serbia; S084 and S138) and six isolates with a levofloxacin MIC of 2 mg/L [one characterized strain (U6); one male urine (U052) from the UK; three vaginal/cervical swabs (C007, C079 and C105) from France; and one vaginal/cervical swab (S042) from Serbia]. Four of these samples showed growth in the 2 mg/L cupule of the assay at 24 h and one also showed growth in the 4 mg/L cupule at 24 h, and all but one showed growth in the 2 mg/L cupule at 48 h, but this was 24 h after the ID well had turned positive. WGS data were available for 7/9 of all strains (not C007 or C079) with an MIC of 2 mg/L; they all had the S83L mutation in ParC, which was unable to mediate true resistance, i.e. MIC $\geq$ 4 mg/L.

Finally, four characterized macrolide-resistant *Ureaplasma* strains were included for evaluation as spiked strains: three with dual 23S rRNA operon mutations A2058G (strains 234, 284 and 342; erythromycin MIC = 128 mg/L, telithromycin MIC = 16 mg/L) and one with a 6 bp deletion in the L4 accessory protein (O10; erythromycin MIC = 64 mg/L, telithromycin MIC = 0.125 mg/L). The *Ureaplasma* sector of the MYCOPLASMA IST3 assay correctly identified erythromycin resistance for all four strains and telithromycin resistance for three strains (the mutation in O10 leaves it susceptible to telithromycin). However, the selective agent for the *Mycoplasma* sector of the plate is also erythromycin; therefore, despite the absence of *Mycoplasma* in the sample, the assay also showed colour change for *M. hominis* ID cupules and the clindamycin cupules (as *Ureaplasma* spp. are inherently resistant). This pattern of growth has been identified in the manufacturer's instructions as representing macrolide-resistant *Ureaplasma* in the absence of *M. hominis* and recommends further investigation by alternative methods.

Overall accuracy of the AST is presented in Table 5. For *Ureaplasma* spp., the highest ME rate was for levofloxacin (5/120; 4.2%) (discrepancy but MIC near threshold), with an overall ME rate across all antibiotics of 1.8%, and the only VME (complete discrepancy between MIC and assay) was found for one sample for tetracycline. For *M. hominis*, ME rates ranged from 0% to 1.5% (overall ME rate of 1.5%), with one VME for one sample for tetracycline and one sample for moxifloxacin.

Table 4. MIC (mg/L) distribution for *Ureaplasma* spp. and *M. hominis*

Isolate and antimicrobial	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}
Ureaplasma spp., *n* (%)																
Levofloxacin				4 (3)	33 (27)	109 (88)	114 (92)	120 (97)	**123** (99)			**124** (100)			0.5	1
Moxifloxacin			4 (3)	62 (50)	112 (90)	121 (98)	123 (99)			**124** (100)					0.125	0.25
Tetracycline		2 (2)	6 (5)	37 (30)	78 (63)	112 (90)	120 (97)	**121** (98)	**122** (99)		**124** (100)				0.25	0.5
Erythromycin					13 (10)	57 (46)	108 (87)	119 (96)	120 (97)				**121** (98)	**124** (100)	1	2
Telithromycin		44 (35)	80 (65)	103 (83)	118 (95)	121 (98)					124 (100)				0.06	0.25
M. hominis, *n* (%)																
Levofloxacin				9 (11)	46 (54)	82 (96)	83 (97)			**85** (100)					0.25	0.5
Moxifloxacin		6 (7)	73 (86)	78 (92)	82 (96)	83 (97)			85 (100)						0.06	0.125
Tetracycline				52 (61)	54 (64)	60 (71)	66 (78)				**70** (82)	**79** (93)	**85** (100)		0.125	32[a]
Clindamycin	6 (7)	32 (38)	72 (84)	85 (100)											0.06	0.125

The cumulative number of isolates is listed from lowest to highest dilution, with the cumulative percentage shown in brackets below. The isolates above the resistance threshold are shown in bold. MICs of antimicrobials were determined using broth microdilution methods as defined by the CLSI publication M43.

[a]The MIC_{90} for tetracycline is skewed due to the large number of *tet*(M)-containing reference and endogenous strains present. Excluding *tet*(M) strains, the MIC_{90} would be 0.5 mg/L.

Discussion

The MYCOPLASMA IST2 assay had been a market leader for evaluating *Ureaplasma* spp. and *Mycoplasma* presence and antimicrobial susceptibility since 2004. However, international guidelines instituted by the CLSI in 2011[10] necessitated an updated version of this assay. Beyond updating the choice and concentrations of the antimicrobials evaluated, the MYCOPLASMA IST3 can independently evaluate *Ureaplasma* spp. and *M. hominis* and provides cupules to evaluate bacterial load without the requirement for titration of the sample. Our evaluation against gold standard methodology in a multinational trial showed good performance for various types of clinical samples.

MYCOPLASMA IST3 had high sensitivity (98.4% and 95.7%) and specificity (99.7% and 100%) for *Ureaplasma* spp. and *M. hominis*, respectively. These results are slightly higher than those reported for the MYCO WELL D-ONE assay (CPM SAS) (*Ureaplasma* spp. sensitivity of 91.98% and specificity of 96.44% and *M. hominis* sensitivity of 78.23% and specificity of 98.84%)[15] and the MYCOFAST RevolutioN, although the 77.3% sensitivity and 80% specificity values for MYCOFAST RevolutioN were for combined genital mycoplasmas rather than separately for each species.[20] These represent the most valid comparisons as these are the only other two assays available that evaluate AST separately for *Ureaplasma and Mycoplasma* using CLSI-compliant antimicrobial concentrations. However, it should be noted that the other assays were evaluated relative to PCR detection, which detects non-viable bacteria in the samples.

Accuracy of the MYCOPLASMA IST3 to independently assess the susceptibility of the two species was of primary interest, as the absence of this ability was the major shortcoming of MYCOPLASMA IST2. Therefore, we supplemented investigation of WT infection of clinical samples from France, Serbia and the UK with known characterized (often fully genome-sequenced) susceptible and resistant strains, blinded to the staff performing the assay. All characterized *M. hominis and Ureaplasma* spp. (predominantly from previously published investigations)[16,17,19] carrying the *tet*(M) resistance genes were identified correctly, in addition to four clinical *M. hominis* samples (19% WT prevalence of tetracycline resistance) with endogenous *tet*(M) and tetracycline resistance. Out of the 108 clinical samples with endogenous *Ureaplasma* spp., only 1 isolate was found to carry the *tet*(M) gene (MIC = 16 mg/L), as well as a second isolate with MIC = 2 mg/L (just at the threshold) that did not carry *tet*(M) resistance (2% WT prevalence of tetracycline resistance). The mechanism for this low-level resistance in the latter isolate was not identified.

No endogenous fluoroquinolone resistance was observed in the 21 clinical samples carrying *M. hominis*, but the MYCOPLASMA IST3 correctly identified both of the characterized strains carrying moxifloxacin/levofloxacin resistance-mediating mutations in both the *gyrA and parC* genes.[19] Evaluating fluoroquinolone resistance in *Ureaplasma* spp. was more complicated, but not due to the

Table 5. Summary of MYCOPLASMA IST3 AST accuracy

	Isolate status (BMD)			Performance index		
Isolate and antimicrobial	Total (*n*)	S (*n*)	R (*n*)	CA, % (actual)	ME[a], % (actual)	VME[b], *n*
Ureaplasma spp.						
Levofloxacin	124	120	4	96.0 (119/124)	4.2 (5/120)	0
Moxifloxacin	124	123	1	98.4 (122/124)	1.6 (2/123)	0
Tetracycline	124	120	4	97.6 (121/124)	1.7 (2/120)	1
Erythromycin	124	120	4	99.2 (123/124)	0.8 (1/120)	0
Telithromycin	125[c]	122	3[d]	99.2 (124/125)	0.8 (1/122)	0
All drugs combined	621	605	16	98.1 (609/621)	1.8 (11/605)	1
M. hominis						
Levofloxacin	84	82	2	98.8 (83/84)	1.2 (1/82)	0
Moxifloxacin	84	81	3	97.6 (82/84)	1.2 (1/81)	1
Tetracycline	84	65	19	97.6 (82/84)	1.5 (1/65)	1
Clindamycin	84	84	0	100.0 (84/84)	0.0 (0/84)	N/A
All drugs combined	336	312	24	98.5 (331/336)	1.0 (3/312)	2
All drugs and species combined	957	917	40	98.2 (940/957)	1.5 (14/917)	3

BMD, broth microdilution; S, susceptible; R, resistant; N/A, not applicable.
[a]ME is defined as disagreement between MIC and assay regarding intermediate resistance and full resistance.
[b]VME is defined as complete disagreement of assignment of strain as susceptible or resistant comparing MIC data with assay result.
[c]125 *Ureaplasma* spp. strains had telithromycin data available, while only 124 strains had data for all antimicrobials tested.
[d]No resistant breakpoints are published and these isolates were found to be non-susceptible with MICs 32-fold higher than the maximum of the remaining *Ureaplasma* spp. strains.

MYCOPLASMA IST3 assay. MIC determination of the combined endogenous and characterized strains identified one isolate (C031) with a levofloxacin MIC of 32 mg/L and a moxifloxacin MIC of 8 mg/L, which was correctly identified by the assay (resistance prevalence = 1%). This isolate was found to carry the S83L ParC mutation and a GyrB E482K mutation, which is very similar to a previously reported S83L/E482D dual mutation with MICs of 16 mg/L (levofloxacin) and 4 mg/L (moxifloxacin).[21] Resistance to moxifloxacin has previously been noted to require a mutation in both the gyrase and topoisomerase genes for *Streptococcus pneumoniae*, while levofloxacin resistance can be mediated by mutation in only one of these enzyme complexes.[22] The location and alteration of amino acid charge are critical to mutations disrupting the tertiary structure elements of the QRDR of *gyrA and parC*,[23] as well as *gyrB and parE* (the latter two being extrapolated from data on *M. hominis and Mycobacterium tuberculosis*).[24,25] For *Escherichia coli*, QRDR mutations are additive: S83L in GyrA gives a median 24-fold increase in ciprofloxacin MIC, while GyrA S83L + ParC S80I gives a 62.5-fold increase (reviewed by van der Putten *et al.*[26]). However, we found the isolated *Ureaplasma* spp. S83L ParC mutation altered the MIC of 0.5 mg/L (for five fully sequenced susceptible strains) to either 2 mg/L (intermediate resistant; four fully sequenced strains) or 4 mg/L (resistant; three fully sequenced strains). Moreover, no secondary SNP could be identified that was consistent for the 4 mg/L group. If we consider MIC > 2 mg/L as resistance, then the endogenous prevalence of levofloxacin resistance was the highest observed 8/108 (7.4%), while an additional mutation in gyrB was required for moxifloxacin resistance.

Finally, MYCOPLASMA IST3 correctly identified four characterized strains with erythromycin MICs of 64–128 mg/L that were incorporated (investigator-blinded) into the sample panels. However, telithromycin MIC was only elevated to 16 mg/L for isolates where the mechanism of resistance was due to A2058G mutation in both 23S rRNA operons (data not shown), while strain O10[17] (where the mechanism of erythromycin resistance was a R66Q67 deletion in L4) was still susceptible to telithromycin

(MIC = 0.125 mg/L relative to the MIC_{90} = 0.5 mg/L) as expected from previous research.[27]

In conclusion, the MYCOPLASMA IST3 assay was found to be extremely sensitive and specific for the identification of *M. hominis and Ureaplasma* spp., and has eliminated the largest inadequacies of its predecessor, MYCOPLASMA IST2, through its ability to accurately evaluate AST for both species independently in a mixed infection and compliance with CLSI resistance thresholds. The capacity to estimate bacterial load, in the absence of performing a dilution series of the sample, was surprisingly accurate. Concordance between true MIC determinations and growth in the cupules was very high, although it suggests there may be some controversy in using S83L as a hallmark of fluoroquinolone resistance for *Ureaplasma* spp. However, the utility of the MYCOPLASMA IST3 assay may be limited in areas where macrolide-resistant *Ureaplasma* spp. (and potentially clindamycin-resistant *M. hominis*) become prevalent.

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Transparency declarations

Y.B. and L.D. are employees of bioMérieux. All other authors: none to declare.

Supplementary data

Supplementary Materials and methods, Results, Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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