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# Evaluation of commercial, customized microdilution plates for *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *Mycoplasma hominis* antimicrobial susceptibility testing and determination of antimicrobial resistance prevalence in France

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**ABSTRACT** Antimicrobial susceptibility testing (AST) of human mycoplasmas using microdilution is time-consuming. In this study, we compared the performance of MICRONAUT-S plates (Biocentric-Bruker) designed for AST of *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *Mycoplasma hominis* with the results using the Clinical & Laboratory Standards Institute (CLSI) reference method. Then, we investigated the prevalence and mechanisms of resistance to tetracyclines, fluoroquinolones, and macrolides in France in 2020 and 2021. The two methods were compared using 60 strains. For the resistance prevalence study, *U. parvum*-, *U. urealyticum*-, and *M. hominis*-positive clinical specimens were collected for 1 month each year in 22 French diagnostic laboratories. MICs were determined using the MICRONAUT-S plates. The *tet*(M) gene was screened using PCR, and fluoroquinolone resistance-associated mutations were screened using PCR and Sanger sequencing. Comparing the methods, 99.5% (679/680) MICs obtained using the MICRONAUT-S plates concurred with those obtained using the CLSI reference method. For 90 *M. hominis* isolates, the tetracycline, levofloxacin, and moxifloxacin resistance rates were 11.1%, 2.2%, and 2.2%, respectively, with no clindamycin resistance. For 248 *U. parvum* isolates, the levofloxacin and moxifloxacin resistance rates were 5.2% and 0.8%, respectively; they were 2.9% and 1.5% in 68 *U. urealyticum* isolates. Tetracycline resistance in *U. urealyticum* (11.8%) was significantly ($P < 0.001$) higher than in *U. parvum* (1.2%). No macrolide resistance was observed. Overall, the customized MICRONAUT-S plates are a reliable, convenient tool for AST of human mycoplasmas. Tetracycline and fluoroquinolone resistance remain limited in France. However, the prevalence of levofloxacin and moxifloxacin resistance has increased significantly in *Ureaplasma* spp. from 2010 to 2015 and requires monitoring.

**IMPORTANCE** Antimicrobial susceptibility testing of human urogenital mycoplasmas using the CLSI reference broth microdilution method is time-consuming and requires the laborious preparation of antimicrobial stock solutions. Here, we validated the use of reliable, convenient plates designed for antimicrobial susceptibility testing that allows the simultaneous determination of the MICs of eight antibiotics of interest. We then investigated the prevalence and mechanisms of resistance of each of these bacteria to tetracyclines, fluoroquinolones, and macrolides in France in 2020 and 2021. We showed that the prevalence of levofloxacin and moxifloxacin resistance has increased significantly in *Ureaplasma* spp. from 2010 to 2015 and requires ongoing monitoring.

**KEYWORDS** susceptibility testing, *Mycoplasma*, *Ureaplasma*, antimicrobial resistance detection, minimum inhibitory concentration determination

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*Ureaplasma* spp. and *Mycoplasma hominis* can cause urogenital, neonatal, and extragenital infections in immunocompromised patients. Only three antibiotic classes, tetracyclines, fluoroquinolones, and macrolides, and related antibiotics are potent against these bacteria. However, acquired resistance to tetracyclines and fluoroquinolones has been reported worldwide, which was associated with acquisition of the *tetracycline*(M) [*tet*(M)] gene and the selection of mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC*, and *parE* genes (1–11).

Previously, we reported a limited prevalence of tetracycline and fluoroquinolone resistance in France in 2010–2015 in *Ureaplasma* spp. and *Mycoplasma hominis*, but acquired macrolide resistance was not evaluated (12). However, determining the minimum inhibitory concentrations (MICs) using the reference broth microdilution method is time-consuming, requiring laborious preparation of antimicrobial stock solutions (13). The Biocentric–Bruker laboratory commercializes microdilution plates designed upon request that contain concentrations of lyophilized antimicrobials for MIC determination.

In this study, we first evaluated the performance of customized MICRONAUT-S plates (Biocentric–Bruker) designed upon request for antimicrobial susceptibility testing (AST) of *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *M. hominis*, and compared the results with MIC determination according to the CLSI guidelines used as reference (13). Then, we investigated the prevalence and mechanisms of resistance of each species to tetracyclines, fluoroquinolones, and macrolides in France in 2020 and 2021.

## MATERIALS AND METHODS

### Strains used for the evaluation of the MICRONAUT-S plates

For comparison purposes, 60 strains were selected (20 each of *M. hominis*, *U. parvum*, and *U. urealyticum*), including three reference strains (*M. hominis* PG21 ATCC 23114, *U. parvum* strain 27 ATCC 27815, and *U. urealyticum* T-strain 960 ATCC 27618) and 19 clinical isolates of each species previously collected at the French National Reference Centre (NRC) for bacterial sexually transmitted infections (STIs) in Bordeaux, France (Table 1). Some of the selected strains harbored the *tet*(M) gene, known to be associated with tetracycline resistance in mycoplasmas (1), or mutations in the *gyrA*, *gyrB*, *parC*, and *parE* gene QRDRs.

### Collection and culture of clinical specimens

A 1-month systematic prospective collection of remnants of *Ureaplasma*- and *M. hominis*-positive specimens was performed between September 15 and October 15, 2020 and 2021, in nine French public microbiology diagnostic laboratories and 13 private laboratories that perform *Ureaplasma* spp. and *M. hominis* detection. The remnants of *Ureaplasma*- and *M. hominis*-positive specimens were sent to the French STIs NRC at −20°C. Patient age, sex, type of sample, and sample collection site were anonymously collated by the microbiologist of each laboratory when the specimen was detected to be positive. At the French STIs NRC, the isolates were regrown from 100 µL thawed specimens inoculated in 900 µL Shepard broth and Hayflick medium supplemented with arginine, respectively (13, 14), along with two additional $10^{-1}$ dilutions in the same medium. *U. parvum* and *U. urealyticum* species were identified using real-time TaqMan PCR, as previously described (15).

### MIC determination using the MICRONAUT-S plates

The MICRONAUT-S plates (Biocentric–Bruker) are microtitration plates that are an *in vitro* diagnostic device for automated or manual AST (https://www.bruker.com/fr/products-and-solutions/microbiology-and-diagnostics/antimicrobial-susceptibility-testing.html). Susceptibility testing is based on rehydration of antibiotics by adding a standardized bacterial suspension. In our case, we used a Research Use Only ( RUO) custom plate (reference E1-LEG-MYC) designed upon request by the French STIs NRC for AST of *M.*



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**TABLE 1** Characteristics of the 60 strains used to compare the performance between the MICRONAUT-S plates and CLSI broth dilution method[b]

| Species | Isolate no. | *tet*(M) PCR | Mutations in the QRDR of | | | |
|---|---|---|---|---|---|---|
| | | | GyrA[a] | GyrB[a] | ParC[a] | ParE[a] |
| *M. hominis* | PG21 | Neg | WT | WT | WT | WT |
| | 5571 | **Pos** | WT | WT | WT | WT |
| | 5589 | **Pos** | WT | WT | WT | WT |
| | 5615 | **Pos** | WT | WT | WT | WT |
| | 5642 | **Pos** | WT | WT | WT | WT |
| | 5677 | **Pos** | WT | WT | WT | WT |
| | 5700 | Neg | **S153(83)L** | WT | **S91(80)I** & K144(134)R | WT |
| | 5738 | **Pos** | WT | WT | WT | WT |
| | 5778 | Neg | WT | WT | WT | R23(22)S & **D426(420)N** |
| | 5866 | **Pos** | WT | WT | WT | WT |
| | 5995 | **Pos** | WT | WT | K144R | WT |
| | 6188 | **Pos** | WT | WT | K144R | WT |
| | 6390 | Neg | **S153(83)L** | WT | **S91(80)I** & K144(134)R | WT |
| | 6510 | **Pos** | WT | WT | WT | WT |
| | 6558 | Neg | WT | WT | **E95(84)G** | WT |
| | 6774 | Neg | **S153(83)L** | WT | **S91(80)I** | WT |
| | MM19-001 | Neg | WT | WT | WT | WT |
| | MM19-050 | Neg | WT | WT | WT | WT |
| | MM19-084 | Neg | WT | WT | WT | WT |
| | MM19-128 | Neg | WT | WT | WT | WT |
| *U. parvum* | ATCC 27815 | Neg | WT | WT | WT | WT |
| | 5696 | Neg | WT | E502(487)Q | WT | V417(410)T & **D427(420)**N |
| | 5759 | **Pos** | WT | WT | WT | WT |
| | 6135 | **Pos** | WT | WT | WT | WT |
| | 6178 | **Pos** | WT | WT | WT | WT |
| | 6182 | **Pos** | WT | WT | WT | WT |
| | 6183 | **Pos** | WT | WT | WT | WT |
| | 6240 | Neg | WT | WT | **S83(80)L** | WT |
| | 6271 | Neg | Q104(87)K | WT | **S83(80)L** | V417(410)T |
| | 6394 | **Pos** | WT | WT | WT | WT |
| | 6405 | **Pos** | WT | WT | WT | WT |
| | 6594 | Neg | WT | WT | WT | WT |
| | 6620 | **Pos** | WT | WT | WT | WT |
| | 6793 | Neg | WT | WT | **S83(80)L** | V417(410)T |
| | 6930 | Neg | WT | WT | WT | WT |
| | MM18-236 | Neg | WT | WT | **S83(80)L** | WT |
| | MM19-002 | Neg | WT | WT | WT | WT |
| | MM19-047 | Neg | WT | WT | WT | WT |
| | MM19-061 | Neg | WT | WT | WT | WT |
| | MM19-075 | Neg | WT | WT | WT | WT |
| *U. urealyticum* | ATCC 27618 | Neg | WT | WT | WT | WT |
| | 5570 | Neg | WT | WT | WT | ND |
| | 5607 | **Pos** | WT | WT | WT | ND |
| | 5701 | Neg | WT | WT | WT | WT |
| | 6175 | Neg | WT | WT | **S83(80)L** | WT |
| | 6265 | Neg | WT | WT | WT | ND |
| | 6362 | Neg | WT | WT | WT | WT |
| | 6371 | Neg | WT | WT | WT | ND |
| | 6584 | Neg | WT | WT | WT | WT |
| | 6628 | Neg | WT | WT | WT | ND |
| | 6697 | Neg | WT | WT | WT | WT |

(*Continued on next page*)



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**TABLE 1** Characteristics of the 60 strains used to compare the performance between the MICRONAUT-S plates and CLSI broth dilution method[b] (*Continued*)

| Species | Isolate no. | *tet*(M) PCR | Mutations in the QRDR of | | | |
|---|---|---|---|---|---|---|
| | | | GyrA[a] | GyrB[a] | ParC[a] | ParE[a] |
| | 6778 | **Pos** | WT | WT | WT | WT |
| | MM18-014 | Neg | WT | WT | **S83(80)L** | WT |
| | MM18-267 | **Pos** | WT | WT | **S83(80)L** | ND |
| | MM19-003 | Neg | WT | WT | WT | ND |
| | MM19-046 | Neg | WT | WT | WT | ND |
| | MM19-065 | Neg | WT | WT | WT | WT |
| | MM19-100 | Neg | WT | WT | WT | ND |
| | MM19-110 | Neg | WT | WT | WT | WT |
| | MM19-226 | Neg | WT | WT | WT | ND |

[a]GyrA, GyrB, ParC, and ParE residues are numbered according to the *M. hominis* or *Ureaplasma* species numbering with the *E. coli* numbering in parentheses.
[b]WT, wild type; ND, not determined. Mutations known to be associated with fluoroquinolone resistance are presented in bold.

*hominis* and *Ureaplasma* spp. isolates. The plates included nine lyophilized antibiotics, eight of which were potentially potent against mycoplasmas and ureaplasmas, whereas rifampicin was intrinsically inactive on Mollicutes (Table S1). The concentration ranges were 0.031–16 µg/mL for tetracycline, 0.016–16 µg/mL for doxycycline and erythromycin, 0.008–16 µg/mL for azithromycin, 0.016–4 µg/mL for clindamycin, 0.002–8 µg/mL for levofloxacin, 0.004–8 µg/mL for moxifloxacin and telithromycin, and 0.002–4 µg/mL for rifampicin. The plates also included two wells with no antibiotic. One well was used as a positive growth control using medium inoculated with the tested strain, and the other was used as a negative control using noninoculated medium.

Calibrated bacterial suspension was first prepared by inoculating 100 µL of each isolate culture in 900 µL of Shepard for the two *Ureaplasma* species or Hayflick supplemented with arginine media for *M. hominis* before incubation (13, 14). When the colored indicator started changing color, 900 µL of suspension was aliquoted and frozen at −80°C. The remaining 100 µL was used to determine the bacterial load by performing 10-fold serial dilutions (from $10^{-1}$ to $10^{-10}$) in the proper medium. After a few days of incubation, the load of the frozen tubes was determined as the highest dilution in which the color changed. A 20-mL volume of calibrated $10^4$ colony-forming units (CFU)/mL or $10^5$ CFU/mL inoculum of each strain was then prepared by dilution from the calibrated frozen tubes. Broths inoculated with *Ureaplasma* spp. were incubated for 1 h at 37°C, whereas *M. hominis* cultures were incubated for 2 h at 37°C (13). Then, each well of the MICRONAUT-S plates was inoculated with 100-µL culture, except the negative control well, which was inoculated with 100 µL noninoculated medium. At the same time, the same calibrated culture of each strain was used to determine MICs according to the reference method (13, 14). MICRONAUT-S plates were incubated at 37°C in an ambient air incubator. The plates were read as soon as the positive growth control well without antibiotic showed a color change, i.e., after an approximately 24-h incubation for *Ureaplasma* spp. and 48-h incubation for *M. hominis*. The MIC was determined as the lowest antibiotic concentration that inhibited the color change at the time the growth control well showed a color change.

To evaluate the reproducibility of the MICRONAUT-S plates, the plates were inoculated daily for 10 days with the three reference strains using a $10^5$ UFC/mL inoculum and MIC data were compiled.

All experiments were performed using the same batch of MICRONAUT-S plates, except the reproducibility study, which used a different batch.

## Characterization of tetracycline- and fluoroquinolone-resistant strains

DNA was extracted from broth cultures using the MagNA Pure 96 DNA and viral NA small-volume kit and the MagNA Pure 96 instrument (Roche Diagnostics). The presence of the *tet*(M) gene was determined using PCR as previously described (16). The *gyrA*, *gyrB*, *parC*, and *parE* gene QRDRs were amplified and sequenced as previously described



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(12). Nucleotide sequences were compared to those of the reference strains *M. hominis* PG21, *U. parvum* strain 27, and *U. urealyticum* T-strain 960 (GenBank accession numbers FP236530, CP000942, and AAYN02000002, respectively).

### Statistical analysis

Data on antimicrobial resistance-associated mutations were presented as percentages and 95% confidence intervals (95% CI) and calculated using the exact binomial distribution. The frequencies were compared using $\chi 2$ or Fisher's exact tests, as appropriate. We considered $P < 0.05$ as significant. Statistical analyses were performed on the biostaTGV website (https://biostatgv.sentiweb.fr/).

## RESULTS

### Evaluation of the performance of the MICRONAUT-S plates

First, we compared the MICs of 60 characterized strains between the MICRONAUT-S plates and the CLSI reference method. The 60 strains included strains harboring the *tet*(M) gene and strains harboring mutations in the *gyrA*, *gyrB*, *parC*, or *parE* gene QRDRs (Table 1). Based on CLSI M43, two bacterial inocula, i.e., $10^4$ and $10^5$ CFU/mL, were assessed.

All MICs obtained using the MICRONAUT-S plates concurred with those using the CLSI method at each inoculum, with no more than a 2-fold dilution difference and no trend for one method compared with the other (Table 2; Tables S2 to S4; Fig. S1 to S3). The only exception among the 680 comparisons was the azithromycin MIC of *U. urealyticum* isolate 5570 with the $10^4$ CFU/mL inoculum, which was 0.25 and 1 µg/mL using the reference method and MICRONAUT-S plates, respectively (Table 2; Table S4; Figure S3). No strains showed a >2-fold difference in MIC.

Among the 10 *M. hominis* strains studied, a 2-fold dilution difference led once to a tetracycline categorization of "susceptible" using the MICRONAUT-S plates on a strain harboring the *tet*(M) gene compared with an accurate tetracycline categorization of "resistant" using the reference method (isolate Mh 5615, MIC = 8 and 4 µg/mL using the reference method and MICRONAUT-S plates, respectively) (Table S2). This discrepancy was not observed using the $10^5$ CFU/mL inoculum. There were no other discrepancies (Tables S2 to S4).

Among the 40 *Ureaplasma* strains studied, there were 6 discrepancies of susceptibility categorization of the 480 comparisons performed. In all cases, it was due to a 2-fold dilution MIC difference (see Tables S2 and S3 for details). When only considering the $10^5$ CFU/mL inoculum, only two discrepancies remained (*U. urealyticum* 5607 for tetracycline testing and *U. parvum* 6793 for levofloxacin testing). In both cases, the categorization of "resistant" using the CLSI method was in accordance with the presence of the *tet*(M) gene or fluoroquinolone resistance-associated mutations.

Overall, no differences were greater than a nonsignificant 2-fold dilution between the two methods in 99.5% of the 680 comparisons performed (Table 2); this validates the use of the MICRONAUT-S plates for determining the MICs for *M. hominis* and the two *Ureaplasma* species considered. Because the results were better using the $10^5$ CFU/mL than the $10^4$ CFU/mL inoculum (one versus five 2-fold dilution differences with change in susceptibility category and no versus one 4-fold dilution difference, respectively, Table 2), the $10^5$ CFU/mL inoculum was used to evaluate resistance prevalence in 2020 and 2021.

In addition, reproducibility of the MICRONAUT-S plates was assessed with the three reference strains using the $10^5$ CFU/mL inoculum for 10 days. For each species and each antibiotic, there was no more than 2-fold dilution difference (Table S5).

### Clinical isolates included in the 2020 and 2021 resistance prevalence studies

A total of 306 and 259 specimens initially deemed positive for *Ureaplasma* or *M. hominis* by culture in 2020 and 2021 were collected from the nine and 13 participating microbiology diagnostic laboratories, respectively (Table S6). Specimens were mainly



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**TABLE 2** Performance of MICRONAUT-S plates on the 60 characterized strains compared with the CLSI reference method

| Species | Antibiotics | Inoculum of $10^4$ CFU/mL | | | | Total | Inoculum of $10^5$ CFU/mL | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Number (%) of strains with identical MICs | Number (%) of strains with a 2-fold dilution difference | | Number (%) of strains with a 4-fold dilution difference | | Number (%) of strains with identical MICs | Number (%) of strains with a 2-fold dilution difference | | Number (%) of strains with a 4-fold dilution difference | |
| | | | With no change in susceptibility category | With a change in susceptibility category | | | | With no change in susceptibility category | With a change in susceptibility category | | |
| *M. hominis* | Tetracycline | 16 (80%) | 3 (15%) | 1 (5%)[a] | 0 | 20 | 14 (70%) | 6 (30%) | 0 | 0 | 20 |
| | Doxycycline | 11 (55%) | 9 (45%) | 0 | 0 | 20 | 13 (65%) | 7 (35%) | 0 | 0 | 20 |
| | Levofloxacin | 18 (90%) | 2 (10%) | 0 | 0 | 20 | 16 (80%) | 4 (20%) | 0 | 0 | 20 |
| | Moxifloxacin | 4 (20%) | 16 (80%) | 0 | 0 | 20 | 7 (35%) | 13 (65%) | 0 | 0 | 20 |
| | Clindamycin | 8 (40%) | 12 (60%) | 0 | 0 | 20 | 7 (35%) | 13 (65%) | 0 | 0 | 20 |
| | Total | 57 (57%) | 42 (42%) | 1 (1%) | 0 | 100 | 57 (57%) | 43 (43%) | 0 | 0 | 100 |
| *U. parvum* | Tetracycline | 16 (80%) | 4 (20%) | 0 | 0 | 20 | 13 (65%) | 7 (35%) | 0 | 0 | 20 |
| | Doxycycline | 17 (85%) | 3 (15%) | 0 | 0 | 20 | 16 (80%) | 4 (20%) | 0 | 0 | 20 |
| | Levofloxacin | 18 (90%) | 1 (5%) | 1 (5%)[a] | 0 | 20 | 17 (85%) | 3 (15%) | 0 | 0 | 20 |
| | Moxifloxacin | 17 (85%) | 3 (15%) | 0 | 0 | 20 | 17 (85%) | 3 (15%) | 0 | 0 | 20 |
| | Erythromycin | 15 (75%) | 5 (25%) | 0 | 0 | 20 | 14 (70%) | 6 (30%) | 0 | 0 | 20 |
| | Azithromycin | 14 (70%) | 6 (30%) | 0 | 0 | 20 | 14 (70%) | 6 (30%) | 0 | 0 | 20 |
| | Total | 97 (80.8%) | 22 (18.3%) | 1 (0.8%) | 0 | 120 | 91 (75.8%) | 29 (24.2%) | 0 | 0 | 120 |
| *U. urealyticum* | Tetracycline | 13 (65%) | 6 (30%) | 1 (5%)[a] | 0 | 20 | 15 (75%) | 4 (20%) | 1 (5%)[a] | 0 | 20 |
| | Doxycycline | 9 (45%) | 11 (55%) | 0 | 0 | 20 | 14 (70%) | 6 (30%) | 0 | 0 | 20 |
| | Levofloxacin | 12 (60%) | 6 (30%) | 2 (10%)[a,b] | 0 | 20 | 19 (95%) | 1 (5%) | 0 | 0 | 20 |
| | Moxifloxacin | 14 (70%) | 6 (30%) | 0 | 0 | 20 | 17 (85%) | 3 (15%) | 0 | 0 | 20 |
| | Erythromycin | 14 (70%) | 6 (30%) | 0 | 0 | 20 | 12 (60%) | 8 (40%) | 0 | 0 | 20 |
| | Azithromycin | 14 (70%) | 5 (25%) | 0 | 1 (5%) | 20 | 13 (65%) | 7 (35%) | 0 | 0 | 20 |
| | Total | 76 (63.3%) | 40 (33.3%) | 3 (2.5%) | 1 (0.8%) | 120 | 90 (75%) | 29 (24.2%) | 1 (0.8%) | 0 | 120 |

[a]Strain with a 2-fold dilution difference with change from resistant to susceptible category.
[b]Strain with a 2-fold dilution difference with change from susceptible to resistant category.



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cervicovaginal swabs in women and semen in men (Table S7). A total of 204 and 210 isolates were grown at the French STIs NRC in the 2020 and 2021 studies, respectively. Three cultures in the 2020 study and five cultures in the 2021 study were a mixturue of both *U. parvum* and *U. urealyticum* (Tables S6 and S7). Because the two *Ureaplasma* species could not be separated, these cultures were not used for further experiments. Ultimately, a total of 201 and 205 single isolates, including 50 and 40 *M. hominis* isolates, 123 and 125 *U. parvum* isolates, and 28 and 40 *U. urealyticum* isolates collected from 182 and 192 distinct patients were included in the 2020 and 2021 resistance prevalence studies, respectively (Tables S6 and S7). Of note, *M. hominis* and *Ureaplasma* spp. isolates were concomitantly grown from 19 and 13 specimens in 2020 and 2021, respectively (Table S7).

## Prevalence of macrolide, tetracycline, and fluoroquinolone resistance

Table 3 and Tables S8 to S10 show the data using the MICRONAUT-S plates for the 406 isolates. Besides the intrinsic resistance of the two *Ureaplasma* species to clindamycin and of *M. hominis* to 14- and 15-membered macrolides, no acquired resistance to erythromycin or to clindamycin was noted (Table 3).

In *M. hominis*, the tetracycline resistance rate was 8.0% (95% CI, 3.2–18.8) in 2020 and 15.0% (95% CI, 7.1–29.1) in 2021, with no significant difference between the years (Table 3). The resistance prevalence for levofloxacin (2%, 95% CI, 0.3–10.5) and moxifloxacin (2.5%, 95% CI, 0.4–12.9) were identical in 2020 and 2021. Regarding *U. parvum*, no tetracycline resistance was detected in 2020, and the resistance rate was 2.4% (95% CI, 0.8–6.8) in 2021. The levofloxacin resistant rate was 5.7% in 2020 and 4.8% in 2021; this

**TABLE 3** $MICs_{50}$, $MICs_{90}$, and prevalence of antibiotic resistance in *M. hominis*, *U. parvum*, and *U. urealyticum* in France in 2020 and 2021[e]

| | | *M. hominis* (50 isolates in 2020, 40 isolates in 2021) | | | *U. parvum* (123 isolates in 2020, 125 isolates in 2021) | | | *U. urealyticum* (28 isolates in 2020, 40 isolates in 2021) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Antibiotics | Year(s) of collection | $MIC_{50}$ | $MIC_{90}$ | Resistance % [95% CI] | $MIC_{50}$ | $MIC_{90}$ | Resistance % [95% CI] | $MIC_{50}$ | $MIC_{90}$ | Resistance % [95% CI] |
| Tetracycline | 2020 | 0.125 | 0.25 | 8.0 [3.2–18.8] | 0.25 | 0.5 | 0[a] | 0.5 | 2 | 10.7[a] [3.7–27.2] |
| | 2021 | 0.125 | 16 | 15.0 [7.1–29.1] | 0.25 | 0.5 | 2.4[b] [0.8–6.8] | 1 | 2 | 12.5[b] [5.5–26.1] |
| | 2020–2021 | 0.125 | 8 | 11.1 [5.4–17.9] | 0.25 | 0.5 | 1.2[c] [0.4–3.5] | 1 | 2 | 11.8[c] [6.1–21.5] |
| Doxycycline | 2020 | 0.06 | 0.06 | NA | 0.125 | 0.125 | NA | 0.25 | 0.5 | NA |
| | 2021 | 0.03 | 1 | NA | 0.125 | 0.25 | NA | 0.25 | 0.5 | NA |
| | 2020–2021 | 0.03 | 0.5 | NA | 0.125 | 0.125 | NA | 0.25 | 0.5 | NA |
| Levofloxacin | 2020 | 0.25 | 0.5 | 2.0 [0.3–10.5] | 0.5 | 1 | 5,7[d] [2.8–11.3] | 1 | 1 | 3.6 [0.6–17.7] |
| | 2021 | 0.25 | 0.5 | 2.5 [0.4–12.9] | 0.5 | 1 | 4.8[d] [2.2–10.1] | 1 | 1 | 2.5 [0.4–12.9] |
| | 2020–2021 | 0.25 | 0.5 | 2.2 [0.6–7.7] | 0.5 | 1 | 5.2 [3.1–8.8] | 1 | 1 | 2.9 [0.8–10.1] |
| Moxifloxacin | 2020 | 0.125 | 0.25 | 2.0 [0.3–10.5] | 0.25 | 0.5 | 0 | 0.5 | 0.5 | 0 |
| | 2021 | 0.125 | 0.25 | 2.5 [0.4–12.9] | 0.25 | 0.5 | 1.6 [0.4–5.7] | 0.25 | 0.5 | 2.5 [0.4–12.9] |
| | 2020–2021 | 0.125 | 0.25 | 2.2 [0.6–7.7] | 0.25 | 0.5 | 0.8 [0.2–2.9] | 0.5 | 0.5 | 1.5 [0.3–8.5] |
| Erythromycin | 2020 | - | - | - | 2 | 4 | 0 | 4 | 4 | 0 |
| | 2021 | - | - | - | 4 | 8 | 0 | 4 | 8 | 0 |
| | 2020–2021 | - | - | - | 4 | 4 | 0 | 4 | 8 | 0 |
| Azithromycin | 2020 | - | - | - | 1 | 2 | NA | 1 | 2 | NA |
| | 2021 | - | - | - | 2 | 2 | NA | 2 | 4 | NA |
| | 2020–2021 | - | - | - | 2 | 2 | NA | 2 | 4 | NA |
| Clindamycin | 2020 | 0.125 | 0.125 | 0 | - | - | - | - | - | - |
| | 2021 | 0.125 | 0.125 | 0 | - | - | - | - | - | - |
| | 2020–2021 | 0.125 | 0.125 | 0 | - | - | - | - | - | - |

[a]Significant differences between resistance percentages according to Fisher's exact tests at $P < 0.01$.
[b]Significant differences between resistance percentages according to Fisher's exact tests at $P < 0.05$.
[c]Significant differences between resistance percentages according to Fisher's exact tests at $P < 0.001$.
[d]Four *U. parvum* isolates (MM20–267 and MM20–279 collected in 2020 and MM21–237 and MM21–247 collected in 2021), which harbored the S83L substitution associated with levofloxacin resistance but had a susceptible MIC of 2 µg/mL, were considered to be resistant to levofloxacin.
[e]NA, not applicable (no interpretation criteria according to CLSI); -, intrinsic resistance.

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included two and two isolates collected in 2020 and 2021, respectively, with a susceptible MIC of 2 µg/mL, but they were considered resistant because of the presence of a levofloxacin resistance-associated mutation (see below). No moxifloxacin resistance was detected in 2020; the resistance rate was 1.6% (95% CI, 0.4–5.7) in 2021. For *U. urealyticum*, the rates were 10.7% and 12.5% for tetracycline, 3.6% and 2.5% for levofloxacin, and 2.5% and 0% for moxifloxacin for the two years, respectively. Of note, tetracycline resistance was significantly more prevalent in *U. urealyticum* than in *U. parvum* in 2020 ($P < 0.001$) and 2021 ($P < 0.05$). By contrast, no significant difference in levofloxacin or moxifloxacin resistance was observed between the two *Ureaplasma* species.

### Molecular characterization of resistant isolates

All 406 isolates collected in 2020 and 2021 were screened by PCR for the presence of the *tet*(M) gene. Isolates categorized as susceptible to tetracycline did not harbor the gene. The *tet*(M) gene was found in all tetracycline-resistant *M. hominis* and *U. parvum* isolates. Among the 68 *U. urealyticum* isolates studied, 2 (2.9%) tetracycline-resistant isolates with tetracycline MICs > 16 µg/mL harbored the *tet*(M) gene, but 6 (8.8%) tetracycline-resistant isolates with tetracycline MICs between 2 and 4 µg/mL did not harbor it.

Regarding levofloxacin susceptibility, 83.3% (75/90) of the *M. hominis* isolates had a MIC of ≤0.25 µg/mL (Table S8), whereas 94.0% (233/248) of the *U. parvum* and 95.6% (65/68) of the *U. urealyticum* isolates had a MIC of ≤1 µg/mL (Tables S9 and S10). To characterize potential isolates with fluoroquinolone resistance-associated mutations, we sequenced the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of the 15 *M. hominis* and 18 *Ureaplasma* isolates with a levofloxacin MIC of >0.25 and >1 µg/mL, respectively (Table 4). No mutations were found in the *gyrA* and *gyrB* QRDRs except a D427N mutation (*Escherichia coli* numbering) in GyrB of the MM21–109 *U. parvum* isolate , along with a S80L mutation in ParC.

In *M. hominis*, the two levofloxacin- and moxifloxacin-resistant isolates harbored mutations in ParC:S80I and K134R substitutions in MM20–002 and K134R only in MM21–219. However, the latter K134R mutation in ParC was present in four other *M. hominis* isolates categorized as susceptible to both antibiotics (Table 4).

In *Ureaplasma* species, all of the levofloxacin-resistant isolates harbored the ParC S80L substitution. Four *U. parvum* isolates (MM20–267, MM20–2789, MM21–237, and MM21–247) also harbored the ParC S80L substitution but were categorized as susceptible to levofloxacin with MICs of 2 µg/mL, which is the levofloxacin breakpoint. In addition, one levofloxacin-resistant *U. parvum* isolate harbored a ParC E84K mutation. The three moxifloxacin-resistant *Ureaplasma* isolates harbored the S80L substitution in ParC.

## DISCUSSION

### Evaluation of the MICRONAUT-S microdilution plates

According to the CLSI guidelines (13), when determining MICs, broth microdilution assays should use an inoculum of $10^4$–$10^5$ CFU/mL and an incubation period of 48–72 h for *M. hominis* and 16–24 h for *Ureaplasma*. Thus, we assessed each inoculum separately using incubation periods of approximately 24 and 48 h for *Ureaplasma* and *M. hominis*, respectively, i.e., as soon as the positive growth control well without an antibiotic showed a color change. Of note, MICs of erythromycin and azithromycin were not evaluated for *M. hominis* because of the intrinsic resistance of this species to 14- and 15-membered macrolides (17). Likewise, the MIC of clindamycin was not evaluated in *Ureaplasma* spp., which are intrinsically resistant to lincosamides. As reported in the results, we validated the MICRONAUT-S plates for all species tested and found that the $10^5$ CFU/mL inoculum was preferred for further tests. As recommended by the CLSI guidelines (13), frequent examination of the positive growth control was also essential because of the tendency for the MIC end point to shift, in tandem with the rapid growth of all species tested. Overall, the use of the MICRONAUT-S plates was less time-consuming than the reference method because it does not require the purchase of antibiotic



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**TABLE 4** Origin, MICs, and molecular characterization of *M. hominis* isolates with a levofloxacin MIC of ≥0.5 μg/mL and *U. parvum* and *U. urealyticum* isolates with a levofloxacin MIC of ≥2 μg/mL collected in 2020 and 2021[a,b]

| Strain no. | Year of collection | Species | Sex | Age (years) | Specimen type | Fluoroquinolone MIC (μg/mL) | | Mutation in the QRDRs of | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | LVX | MXF | GyrA | GyrB | ParC | ParE |
| ATCC 23114 | Reference strain | *M. hominis* | | | | 0.5 | 0.125 | WT | WT | WT | WT |
| MM20-002 | 2020 | *M. hominis* | W | 15 | Oro-pharyngeal swab | **4** | **0.5** | WT | WT | S91(80)I & K144(134)R | WT |
| MM20-323 | 2020 | *M. hominis* | W | 42 | Vaginal swab | 1 | 0.25 | WT | WT | S91(80)N & A154(144)V | WT |
| MM21-219 | 2021 | *M. hominis* | W | 31 | Endocervical swab | **2** | **0.5** | WT | WT | K144(134)R | WT |
| MM21-230 | 2021 | *M. hominis* | W | 38 | Vaginal swab | 1 | 0.25 | WT | WT | WT | ND |
| MM21-258 | 2021 | *M. hominis* | W | 25 | Vaginal swab | 1 | 0.125 | WT | WT | S91(80)C | ND |
| ATCC 27815 | Reference strain | *U. parvum* | | | | 0.5 | 0.25 | WT | WT | WT | WT |
| MM20-014 | 2020 | *U. parvum* | W | 25 | Placenta | **4** | 1 | WT | WT | S83(80)L | WT |
| MM20-179 | 2020 | *U. parvum* | W | 31 | Vaginal swab | **4** | 1 | WT | WT | S83(80)L | WT |
| MM20-218 | 2020 | *U. parvum* | W | 48 | Vaginal swab | **4** | 1 | WT | WT | S83(80)L | WT |
| MM20-267 | 2020 | *U. parvum* | W | 30 | Vaginal swab | 2 | 1 | WT | WT | S83(80)L | WT |
| MM20-279 | 2020 | *U. parvum* | W | 20 | Vaginal swab | 2 | 1 | WT | WT | S83(80)L | WT |
| MM20-338 | 2020 | *U. parvum* | W | 32 | Vaginal swab | **4** | 1 | WT | ND | S83(80)L | WT |
| MM20-369 | 2020 | *U. parvum* | W | 24 | Vaginal swab | 2 | 1 | WT | WT | WT | WT |
| MM20-396 | 2020 | *U. parvum* | W | 47 | Vaginal swab | **4** | 1 | WT | WT | S83(80)L | WT |
| MM20-401 | 2020 | *U. parvum* | W | 49 | Vaginal swab | 2 | 1 | WT | WT | WT | WT |
| MM21-109 | 2021 | *U. parvum* | W | 25 | Vaginal swab | **>8** | **8** | WT | D443(427)N | S83(80)L | WT |
| MM21-200 | 2021 | *U. parvum* | M | 39 | Semen | **4** | 1 | ND | WT | E87(84)K | WT |
| MM21-229 | 2021 | *U. parvum* | W | 57 | Vaginal swab | **8** | **8** | WT | WT | S83(80)L | WT |
| MM21-237 | 2021 | *U. parvum* | W | 58 | Vaginal swab | 2 | 1 | WT | WT | S83(80)L | WT |
| MM21-247 | 2021 | *U. parvum* | W | 52 | Vaginal swab | 2 | 0.5 | WT | WT | S83(80)L | WT |
| MM21-271 | 2021 | *U. parvum* | W | 45 | Vaginal swab | **4** | 2 | WT | WT | S83(80)L | WT |
| ATCC 27618 | Reference strain | *U. urealyticum* | | | | 1 | 0.5 | WT | WT | WT | WT |
| MM20-178 | 2020 | *U. urealyticum* | W | 20 | Vaginal swab | **8** | 2 | WT | WT | S83(80)L | ND |
| MM21-042 | 2021 | *U. urealyticum* | M | 31 | Semen | **8** | **4** | WT | WT | S83(80)L | WT |
| MM21-048 | 2021 | *U. urealyticum* | M | 34 | Semen | 2 | 1 | WT | WT | WT | ND |

[a]LVX, levofloxacin; MXF, moxifloxacin; M, man; W, woman; WT, wild type; ND, not determined.
[b]Isolates resistant to levofloxacin according to CLSI are presented in bold. Discrepancies between the phenotypic susceptibility and presence of mutations associated with levofloxacin resistance are highlighted in gray. GyrA, GyrB, ParC, and ParE residues are numbered according to *M. hominis* or *Ureaplasma* species numbering with *E. coli* numbering in parentheses.



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powders, preparation of accurate dilution ranges according to the tested antibiotics, and distribution of each antibiotic dilution in plate wells. Moreover, the MICRONAUT-S plates avoid the risk of antibiotic dilution errors and allow the MICs of eight antibiotics of interest to be determined simultaneously. In addition, MICRONAUT-S plates could be preserved at room temperature (15–25°C) for more than a year. These commodities come at significant increase in cost because the MICRONAUT-S plate price is approximately 15 euros excluding taxes for eight MIC determinations.

## Resistance prevalence

In *M. hominis*, the overall tetracycline resistance for 2020–2021 (11.1%, 10/90) was not significantly different from that previously reported for 2010–2015 (14.8%, 27/183, $P$ = 0.4) (12); the same was true for levofloxacin and moxifloxacin. The resistance rates for tetracycline and fluoroquinolone against *M. hominis* are also in accordance with those reported in England and Wales (5, 6). However, between the two *Ureaplasma* species, tetracycline resistance was significantly higher in *U. urealyticum* than in *U. parvum* in 2020 (10.7% vs. 0%, $P < 0.01$) and 2021 (12.5% vs. 2.4%, $P = 0.02$) (Table 3). To our knowledge, such a significant difference has only been reported once in the literature (9), but only a few studies have accurately investigated this point in *U. parvum* and *U. urealyticum* since the two *Ureaplasma* species have been separated (3, 8, 18). Interestingly, in both *Ureaplasma* species, the overall rates for levofloxacin and moxifloxacin resistance were 4.7% (15/316) and 0.9% (3/316) in 2020–2021 (Tables S9 and S10), which were significantly higher than 1.2% (10/831, $P < 0.001$) and 0.1% (1/831, $P < 0.05$) in our similar previous study in 2010–2015, respectively (12).

Our results are in line with data from other European countries, Cuba, and the USA, in which levofloxacin resistance has ranged between 0% and 7.1% (3, 4, 8, 9, 19–21). By contrast, two studies from China have reported dramatically higher levofloxacin resistance rates of 47.5% and 84.7% (2, 7), which may be associated with the intensive use of fluoroquinolones in China.

## Tetracycline-resistant *U. urealyticum* isolates not harboring the *tet*(M) gene

A total of 8.8% (6/68) *U. urealyticum* tetracycline-resistant isolates with MICs ranging between 2 and 4 µg/mL did not harbor the *tet*(M) gene. We did not search for tetracycline resistance-associated mutations in the 16S rRNA, so they cannot be ruled out. To the best of our knowledge, these mutations have not yet been reported from clinical isolates from humans but are frequently associated with tetracycline resistance in animal *Mycoplasma* species (1). In addition, *in vitro* selection studies in *M. hominis* have selected low-level tetracycline-resistant mutants harboring one to three mutations in the 16S rRNA gene (22).

## Molecular characterization of fluoroquinolone-resistant isolates

Of the two *M. hominis* isolates resistant to levofloxacin and moxifloxacin, one (MM20–002) (Table 4) harbored the S80I mutation, which has previously been reported to be associated with resistance (2, 23), whereas the other (MM21–219) harbored only a K134R mutation in ParC, which was not located in the QRDR (23). This mutation was not associated with fluoroquinolone resistance in four other *M. hominis* isolates in our study, and it was previously concluded that this substitution is a nonresistance polymorphism (5). Thus, the mechanism underlying fluoroquinolone resistance in MM21–219 remains unknown. Overexpression of putative efflux pumps, previously reported in *in vitro*-selected strains, should be investigated (24, 25).

In *Ureaplasma* spp., besides ParC E84K, which has previously been associated with levofloxacin resistance (2, 4, 8, 10, 23), the S80L mutation was the most frequent mutation causing fluoroquinolone resistance in our study. This mutation has been reported worldwide and is usually associated with levofloxacin MICs of >4 µg/mL (2, 4, 8, 10, 12, 26, 27). However, in many studies, only phenotypically resistant isolates, i.e., with a levofloxacin MIC of >4 µg/mL, have been sequenced. Here, we sequenced isolates



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with a levofloxacin MIC of 2 µg/mL, which is the MIC breakpoint used to categorize an isolate as susceptible. Of the seven *Ureaplasma* isolates with a levofloxacin MIC of 2 µg/mL, four *U. parvum* isolates harbored the S80L mutation associated with levofloxacin resistance (Table 4). Comparing the MICRONAUT-S and CLSI reference methods, one isolate (*U. parvum* 6793) harbored the S80L mutation, and the levofloxacin MIC differed between the two methods (4 and 2 µg/mL using the CLSI and MICRONAUT-S methods, respectively). Therefore, we checked the levofloxacin MICs of these four isolates using the CLSI method in parallel with the MICRONAUT-S method using the same strain inoculum. Both methods gave identical levofloxacin MICs for isolates MM21–237 and MM21–247 (2 µg/mL, i.e., susceptible) and isolate MM20–267 (4 µg/mL, i.e., resistant), whereas for isolate MM20–279 the levofloxacin MIC was 4 µg/mL with the CLSI method and 2 µg/mL with the MICRONAUT-S method. Because the S80L mutation is consistently associated with levofloxacin resistance in the literature (2, 4, 8, 10, 12, 26, 27) and because the levofloxacin MIC was only one dilution below the levofloxacin-resistant breakpoint, these four isolates were considered levofloxacin resistant to calculate the prevalence of levofloxacin resistance in the prevalence assessment. In addition, in complicated or severe *Ureaplasma* infections, we suggest that *Ureaplasma* isolates with a levofloxacin MIC of 2 µg/mL should benefit from *parC* gene QRDR sequencing to look for fluoroquinolone resistance-associated mutations whatever the MIC determination method used.

In conclusion, compared with the CLSI reference method, the MICRONAUT-S plates were a reliable, convenient tool for determining the MICs of *M. hominis* and *Ureaplasma* spp.. No acquired resistance to erythromycin or clindamycin was found in France in 2020–2021. The prevalence of tetracycline and fluoroquinolone resistance remained moderate overall, although that of the latter has significantly increased in *Ureaplasma* spp. since the 2010-2015 study. These results underline the need to monitor the antibiotic susceptibility of human urogenital mycoplasmas.

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Laurier-Nadalié, Data curation, Formal analysis | Marie Gardette, Data curation, Investigation | Cécile Bébéar, Conceptualization, Writing – review and editing.

## ETHICS APPROVAL

Remnants of specimens were preserved at the Centre de Ressource Biologique-Bordeaux Biothèque Santé (CRB-BBS) of Bordeaux University Hospital under collection number BB-0033-00094 and authorization AC-2014-2166 from the French Ministry of Higher Education and Research with no information regarding patient identity. All patient data were reported anonymously.

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Supplemental figures (JCM00226-24-s0001.pdf).** Figures S1 to S3.
**Supplemental tables (JCM00226-24-s0002.pdf).** Tables S1 to S10.

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