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Clinical Evaluation of Three Commercial PCR Assays for the Detection of Macrolide Resistance in *Mycoplasma genitalium*

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ABSTRACT As macrolide resistance in *Mycoplasma genitalium* is increasing worldwide, macrolide resistance-associated mutations should be assessed in *M. genitalium*-positive specimens. New commercial kits are available for detection of macrolide resistance concurrently with *M. genitalium*. We prospectively evaluated the handling and clinical performances of three commercial kits for detection of macrolide resistance in *M. genitalium*. Between August and December 2018, remnants of all urogenital specimens determined to be *M. genitalium* positive using an in-house real-time PCR assay were prospectively collected at the French National Reference Center for Bacterial Sexually Transmitted Infections, Bordeaux University Hospital, Bordeaux, France. The internal control of each kit was added to the primary specimen before DNA extraction, and the absence of amplification inhibition associated with the addition of the three internal controls was assessed. Specimens were evaluated with four assays: the ResistancePlus MG assay (SpeeDx), the S-DiaMGRes assay (Diagenode), the RealAccurate TVMGres assay (PathoFinder), and amplification and sequencing of the 23S rRNA gene (the reference assay). Overall, 195 *M. genitalium*-positive specimens were assessed. The positive agreement of *M. genitalium* detection for each kit ranged between 94.8% and 96.4%. Among 154 specimens with *M. genitalium* positivity as detected by the three commercial kits and 23S rRNA sequencing data, the clinical sensitivity and specificity ranges of the three commercial kits for detecting macrolide resistance-associated mutations were 95 to 100% and 94.6 to 97.3%, respectively. The sensitivity and specificity values were similar among the kits. The launch of three easy-to-use sensitive and specific commercial kits for simultaneous detection of *M. genitalium* and macrolide resistance will be useful for resistance-guided therapy.

KEYWORDS *Mycoplasma genitalium*, macrolide, resistance, detection, commercial kits, antibiotic resistance, macrolides

Mycoplasma genitalium is a sexually transmitted organism involved in nongonococcal urethritis in men and is associated with cervicitis and pelvic inflammatory disease in women (1, 2). First-line treatments for *M. genitalium* infection recommended in Europe and Australia include azithromycin, used either alone via a 5-day regimen (500 mg on day 1, followed by 250 mg on the following 4 days) (3) or as part of resistance-guided sequential treatment. Recommended sequential treatments in the United Kingdom and Australia are composed of 100 mg doxycycline two times daily for 7 days to reduce the *M. genitalium* load (4), followed by 2 or 2.5 g azithromycin over 3 or 4 days (5; http://www.sti.guidelines.org.au/sexually-transmissible-infections/mycoplasma-genitalium). However, macrolide resistance is increasing worldwide (6–9), thus compromising the effectiveness of azithromycin in treating *M. genitalium* infection (10). Consequently, detection of macrolide resistance is recommended in all *M.*

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genitalium-positive specimens, based on the European guideline for *M. genitalium* infection (3) and the BASHH United Kingdom national guideline for the management of *M. genitalium* (5). Macrolide resistance in *M. genitalium* is mediated by point mutations in the peptidyl transferase loop of 23S rRNA, mainly at positions A2058 and A2059 (*Escherichia coli* numbering) and occasionally at position A2062 (11–13). The classical 23S rRNA amplification and sequencing method is laborious and time-consuming; therefore, several in-house assays based on real-time PCR or pyrosequencing have been developed to detect macrolide resistance-associated mutations (14–18). A few years ago, the ResistancePlus MG assay (SpeeDx, Australia) was developed and was the first commercial kit capable of simultaneously detecting *M. genitalium* and the five most frequent 23S rRNA mutations associated with macrolide resistance (19). Additional commercial real-time PCR-based kits that detect *M. genitalium* and four or five macrolide resistance-associated mutations have since been developed.

The aim of the present study was to evaluate and compare the performances of three commercial real-time PCR kits for the detection of *M. genitalium* and macrolide resistance-associated mutations: the ResistancePlus MG assay (SpeeDx, Australia), the S-DiaMGRes assay (Diagenode, Belgium), and the RealAccurate TVMGres assay (Pathofinder, The Netherlands). Only *M. genitalium*-positive specimens were included, and the positive agreement of *M. genitalium* detection was assessed in comparison with an in-house TaqMan PCR assay targeting the MgPa adhesin gene (20). The clinical sensitivity and specificity of each of the three kits for detecting macrolide resistance-associated mutations were determined in comparison with the in-house 23S rRNA real-time PCR (14) and 23S rRNA Sanger sequencing assay used as references.

MATERIALS AND METHODS

Clinical specimens. Between 1 August 2018 and 31 December 2018, remnants of all clinical specimens found to be positive for *M. genitalium* using an in-house real-time PCR assay targeting the MgPa adhesin gene (20) were collected systematically and prospectively at the French National Reference Center for Bacterial Sexually Transmitted Infections in the Bacteriology Department of Bordeaux University Hospital (Bordeaux, France). The specimens were stored at −20°C until they were retested. The 195 *M. genitalium*-positive specimens collected for the study included 43 vaginal and 30 endocervical swabs; 58 first-void urine samples; and 54 rectal, 5 urethral, and 5 pharyngeal swabs from 119 men and 76 women. A total of 126 (64.6%) specimens were collected from southwest France and 73 (35.4%) from other French regions, from symptomatic or asymptomatic patients, with no information on the proportion of each category.

Addition of ICs and DNA extraction. Internal controls (ICs) provided in each kit were prepared and added to the specimens in accordance with the manufacturer's instructions. Nucleic acid extraction was performed using 200 μl of a mixture containing 162 μl of the clinical sample, 20 μl of IC cell solution provided with the ResistancePlus MG kit, 15 μl M13 IC provided with the RealAccurate TVMGres kit, and 3 μl PCR extraction and inhibition control Dia-EIC (Diagenode). DNA extraction was performed using the MagNA Pure 96 DNA and viral NA small-volume kit and the MagNA Pure 96 instrument (Roche Diagnostics).

To compare the detection of each IC when adding only the IC of the evaluated kit to the specimen or adding the three ICs together, several mixtures with a total volume of 200 μl were extracted and evaluated: (i) 180 μl phosphate-buffered saline (PBS) plus 20 μl IC cells (ResistancePlus MG), (ii) 185 μl PBS plus 15 μl M13 IC (RealAccurate TVMGres PCR kit), (iii) 197 μl PBS plus 3 μl Dia-EIC (Diagenode), and (iv) 162 μl PBS plus each of the three ICs listed above.

To evaluate the detection of *M. genitalium* when adding only the IC of the evaluated kit versus adding the three ICs together, a 10-fold serial dilution of *M. genitalium* G37 (ATCC 33530) culture was used, and four 10-fold dilutions of interest were quantified as genome equivalents per microliter using the in-house real-time PCR targeting the MgPa adhesin gene (20). Mixtures prepared for extraction were composed of 162 μl of each *M. genitalium* G37 culture dilution with the addition of one of the following: (i) 38 μl PBS; (ii) 20 μl IC cell solution (ResistancePlus MG) plus 18 μl PBS; (iii) 15 μl M13 IC (RealAccurate TVMGres) plus 23 μl PBS; (iv) 3 μl Dia-EIC (Diagenode) plus 35 μl PBS; or (v) 20, 15, and 3 μl of the three ICs, respectively. All comparison experiments were performed in triplicate.

Amplification and sequencing. The three commercial assays were performed in accordance with the manufacturer's instructions using the LightCycler 480 II instrument (Roche Diagnostics). For each evaluated kit, a color compensation file was generated previously using a color compensation kit provided by the manufacturer: the color compensation kit for LC480 Roche (Diagenode), RealAccurate Quadruplex Color Compensation v2 (Pathofinder), and PlexPCR Color Compensation (SpeeDx). The in-house 23S rRNA real-time PCR assay (14) was performed using the Cobas z 480 analyzer of the Cobas 4800 platform (Roche Diagnostics). Data were analyzed using Roche LightCycler 480 software in accordance with each manufacturer's instructions, with the exception of the SpeeDx assay results, which were analyzed using UgenTec FastFinder 3.2.12 software provided by the manufacturer. The PCR

TABLE 1 Comparison of PCR C_T values for detection of the IC of the evaluated kit when added to the primary specimen alone versus in addition to the other two ICs[a]

Commercial kit	C_T (mean ± SD) for IC amplification		ΔC_T
	Specific IC alone in the specimen	All three ICs in the specimen	
S-DiaMGRes Diagenode	34.88 ± 0.03	34.66 ± 0.36	−0.22
RealAccurate TVMGres Pathofinder	26.49 ± 0.11	26.53 ± 0.76	+0.05
ResistancePlus MG SpeeDx	25.73 ± 0.45	25.82 ± 0.22	+0.09

[a]Experiments were performed in triplicate. SD, standard deviation.

products obtained from the in-house 23S rRNA real-time PCR were sent to Eurofins Genomics (Germany) for Sanger sequencing. Sequencing data were analyzed using BioEdit 7.2.5 software (Isis Pharmaceuticals).

Data analysis. A maximum difference in cycle threshold (C_T) values of +1 between the assay performed using the IC of the evaluated kit only and the assay performed using the three ICs added together to the primary specimen was considered nonsignificant. The positive agreement of *M. genitalium* detection was calculated for each kit using the in-house real-time PCR assay targeting the MgPa adhesin gene (20) as a reference. Sanger sequencing of the 23S rRNA PCR products was used as the reference standard for detection of macrolide resistance-associated mutations. The clinical sensitivity and specificity of each kit for detecting macrolide resistance were calculated. Statistical analysis was performed using the biostaTGV website (https://biostatgv.sentiweb.fr/).

RESULTS

Validation of the addition of three ICs to primary specimens. Before comparing the performances of the three commercial kits, we first confirmed that the addition of the three ICs to the primary specimen did not inhibit the amplification of each specific IC and did not alter the detection of *M. genitalium*. To assess any potential inhibition of IC amplification, we compared the C_T values obtained from PCR amplification of the specific IC when added to the primary specimen (before DNA extraction) alone versus its addition along with the other two ICs (Table 1). Concurrent addition of the three ICs did not increase the C_T value (ΔC_T [the difference in the mean C_T value for amplification of the specific IC when added to the primary specimen {before DNA extraction} alone versus in addition to the other two ICs] $\leq$ 0.09), suggesting that IC amplification was not inhibited in any of the commercial kits.

To assess any potential inhibition of *M. genitalium* amplification, four dilutions of *M. genitalium* culture were used with each kit, adding each IC to the specimen (before DNA extraction) either alone or in addition to the other two ICs (Table 2). Concurrent addition of the three ICs increased the C_T by less than 0.8, 1.0, and 0.4 when using the S-DiaMGRes, RealAccurate TVMGres, and ResistancePlus MG kits, respectively (Table 2), suggesting that amplification of *M. genitalium* was not affected by concurrent addition of the three ICs to the primary clinical specimen. Consequently, evaluation of the performances of the three commercial kits was performed on DNA extracts obtained from clinical specimens in which the three ICs were added together.

Assessment of *M. genitalium* detection. All of the 195 selected *M. genitalium*-positive specimens were found to be *M. genitalium* positive after thawing, addition of the three ICs, DNA extraction, and retesting using the in-house real-time PCR targeting

TABLE 2 Comparison of PCR C_T values for *M. genitalium* detection when the IC of the evaluated kit was added to the specimen (before DNA extraction) alone versus in addition to the other two ICs[a]

Serial dilution of *M. genitalium* G37 culture (genome equivalent/μl)	Mean C_T ± SD								
	S-DiaMGRes (Diagenode)			RealAccurate TVMGres (Pathofinder)			ResistancePlus MG (SpeeDx)		
	Diagenode IC alone	Three ICs	ΔC_T	Pathofinder IC alone	Three ICs	ΔC_T	SpeeDx IC alone	Three ICs	ΔC_T
7.2×10^4	20.80 ± 0.21	21.43 ± 0.04	+0.63	20.71 ± 0.32	21.73 ± 0.39	+1.02	13.01 ± 0.00	12.96 ± 0.01	−0.05
1.2×10^3	26.48 ± 0.26	25.86 ± 0.20	−0.62	24.64 ± 0.40	24.81 ± 0.85	+0.17	17.02 ± 0.01	16.54 ± 0.14	−0.48
3.0×10^2	28.94 ± 0.52	28.00 ± 0.27	−0.94	27.29 ± 0.12	27.83 ± 0.17	+0.54	22.04 ± 0.02	21.98 ± 0.02	−0.07
2.8×10^1	34.38 ± 0.35	35.16 ± 0.55	+0.78	33.75 ± 0.04	34.44 ± 0.30	+0.69	26.47 ± 0.42	26.89 ± 0.54	+0.42

[a]Experiments were performed in triplicate.

TABLE 3 *M. genitalium* detection among 195 *M. genitalium*-positive specimens using the three commercial kits compared with the in-house real-time PCR assay (20)

Commercial kit	No. of *M. genitalium*-positive specimens (n = 195)			% positive agreement (95% CI[a])
	Positive	Negative	Invalid IC	
S-DiaMGRes Diagenode	183	10	2	94.8 (90.7–97.2)
RealAccurate TVMGres Pathofinder	188	7	0	96.4 (92.8–98.3)
ResistancePlus MG SpeeDx	185	9	1	95.4 (91.4–97.5)

[a]CI, confidence interval.

the MgPa adhesin gene. Among them, the IC was not detected in two specimens (one first-void urine and one rectal swab specimen from two men) using the S-DiaMGRes (Diagenode) and in one specimen (the same rectal swab mentioned above) using the ResistancePlus MG (SpeeDx) (Table 3). The positive agreements of *M. genitalium* detection for the three commercial kits, in comparison with the in-house assay, were 94.8%, 96.4%, and 95.4% for the S-DiaMGRes, RealAccurate TVMGres, and ResistancePlus MG kits, respectively.

Clinical performance of macrolide resistance detection. Among the 195 *M. genitalium*-positive specimens assessed in this study, 21 were not found to be *M. genitalium* positive by the three commercial kits and were not included in the macrolide resistance comparison. Among the 174 remaining specimens, 20 specimens could not be analyzed because the sequencing assay did not provide results. Thus, 154 specimens were used to assess the performance of macrolide resistance detection. Among them, 74 (48.1%) specimens had no 23S rRNA mutations, whereas 80 (51.9%) harbored a mutation at position A2058 (A2058C, A2058G, or A2058T [*E. coli* numbering]) or A2059 (A2059G) (Table 4). The S-DiaMGRes (Diagenode) and ResistancePlus MG (SpeeDx) kits had clinical sensitivities of 100% for macrolide resistance detection, whereas the RealAccurate TVMGres kit (Pathofinder) identified 95.0% of the specimens containing a macrolide-resistant strain. The clinical specificities were 94.6%, 97.3%, and 97.3% for the ResistancePlus MG (SpeeDx), S-DiaMGRes (Diagenode), and RealAccurate TVMGres (Pathofinder) kits, respectively (Table 4). In all cases with discordant results relative to those of 23S rRNA sequencing, the results from the other two commercial kits were concordant with those of 23S rRNA sequencing, with the exception of a single rectal swab specimen. This specimen was determined to have no mutation by 23S rRNA sequencing and the RealAccurate TVMGres (Pathofinder) kit but was determined to possess a mutation by the S-DiaMGRes (Diagenode) and ResistancePlus MG (SpeeDx) kits. The sequencing chromatograms of this specimen were checked to determine whether the specimen contained a mixture of macrolide-susceptible and macrolide-resistant strains, but no double peaks were observed at positions 2058 and 2059. In addition, apart from this study, this clinical specimen had previously been found not to be mutated using the ResistancePlus MG (SpeeDx) kit used in our routine diagnosis activity. Altogether, these data suggest that the S-DiaMGRes (Diagenode) and Resis-

TABLE 4 Clinical performance of the three commercial kits in detecting macrolide resistance among 154 specimens with *M. genitalium* positivity as detected by the three commercial kits and 23S rRNA sequencing data

Commercial kit	23S rRNA mutation	23S rRNA sequencing		% sensitivity (95% CI)	% specificity (95% CI)
		No. mutated	No. WT[a]		
S-DiaMGRes Diagenode	Present	80	2	100 (95.4–100)	97.3 (90.7–99.3)
	Absent	0	72		
RealAccurate TVMGres Pathofinder	Present	76	2	95.0 (87.8–98.0)	97.3 (90.7–99.3)
	Absent	4	72		
ResistancePlus MG SpeeDx	Present	80	4	100 (95.4–100)	94.6 (86.9–97.9)
	Absent	0	70		

[a]WT, wild type.

TABLE 5 Handling, testing duration, and cost of the three evaluated commercial kits

Commercial kit	No. of reagents per reaction mixture	Internal control	Thermal-cycling duration (min)	Positive and negative controls	Data analysis	List price/reaction excluding. taxes (€)
S-DiaMGRes Diagenode	4	Included[a]	1 h 45 min	Included	LightCycler 480 software (Fit point analysis[d])	30
Real Accurate TVMGres Pathofinder	2	Included[b]	1 h 35 min	Included	LightCycler 480 software (Abs Quant/2nd Derivative Max[d])	15
ResistancePlus MG SpeeDx	4	Included[b]	1 h 25 min	Not included[c]	UgenTec FastFinder ResistancePlus MG	22

[a]Inhibition control.
[b]Extraction and inhibition control.
[c]ResistancePlus MG positive-control kit is optional.
[d]In accordance with the manufacturer's instructions.

tancePlus MG (SpeeDx) results obtained for the specimen in the present study may be false-mutated results.

DISCUSSION

In the present study, the clinical performances of three commercial kits for detection of macrolide resistance in *M. genitalium* were assessed. Against the background of rising macrolide resistance in many regions, evaluated assays are necessary to increase the rate of *M. genitalium*-positive specimens tested for mutations and to maintain the use of macrolide treatments when possible. Alternatively, such assays could be used to monitor the prevalence of macrolide resistance in each setting.

The ResistancePlus MG (SpeeDx) assay has previously been evaluated by several research groups (6, 11, 19, 21). The sensitivity for macrolide resistance detection of 100% found in this study is in accordance with those reported in previous studies (95.4 to 100%) (6, 11, 19). Likewise, the specificity of 94.6% is in accordance with previously reported values: 95.4% (11), 97.4% (19), and 100% (6). Notably, this is the first published evaluation of the S-DiaMGRes (Diagenode) and RealAccurate TVMGres (Pathofinder) kits. Similar to the ResistancePlus MG (SpeeDx) kit, the S-DiaMGRes (Diagenode) kit detects five 23S rRNA mutations associated with macrolide resistance, whereas the RealAccurate TVMGres (Pathofinder) assay detects only four such mutations. However, the one mutation (A2059C) not detected by the RealAccurate TVMGres (Pathofinder) assay is rare and was not present in the specimens evaluated in the present study. Overall, the three kits showed good agreement with the in-house PCR assay for *M. genitalium* detection and equivalent clinical sensitivities and specificities for the detection of macrolide resistance, as the 95% confidence intervals of the sensitivities and specificities of the three kits overlapped (Tables 3 and 4). However, it should be noted that false-susceptible results were obtained for four samples using the Pathofinder assay, leading to a risk of macrolide treatment failure, whereas no false-susceptible results were obtained with the other assays. On the other hand, false-mutated results were obtained for four samples using the SpeeDx assay, leading to a risk of moxifloxacin overuse and side effects associated with the fluoroquinolone.

Although assay sensitivity and specificity are important considerations when evaluating the reliability of a kit, additional criteria should not be disregarded (e.g., feasibility, ease of use, handling time, and cost). A color compensation file, generated in an additional run, is required before testing clinical specimens using each kit. All of the kits have a short workflow sequence requiring the mixing of only two to four reagents (Table 5). The S-DiaMGRes (Diagenode) kit includes only an inhibition control that detects amplification inhibition, whereas the other two kits include an extraction and inhibition control. This difference is because the S-DiaMGRes (Diagenode) kit was designed to be used primarily as a reflex assay (i.e., on *M. genitalium*-positive specimens previously detected using the S-DiaMGTV [Diagenode] kit, which already includes an extraction and inhibition control). The results are available within 85 to 105 min, depending on the kit (Table 5). Regarding data analysis, results generated on the

LightCycler 480 instrument using the ResistancePlus MG (SpeeDx) kit must be analyzed using the UgenTec FastFinder 3.2.12 software provided by the manufacturer at no extra charge. For the other two kits, investigators can perform data interpretation using the LightCycler 480 software (fit point analysis or Abs Quant/2nd Derivative Max analysis, based on the manufacturer's instructions); this analysis step may depend on the technician's skills.

A limit of the present study is that only *M. genitalium*-positive specimens were included. Thus, only the percentage of positive agreement with the in-house PCR could be determined for *M. genitalium* detection. However, the sensitivity and specificity of macrolide resistance detection were calculated for the three commercial kits, as mutated and nonmutated *M. genitalium* isolates were collected.

Importantly, as previously reported for the ResistancePlus MG (SpeeDx) assay (11), the three kits evaluated in this study do not include a 23S rRNA amplification control. Thus, if there is a failure of 23S rRNA amplification in the presence of a successful IC amplification, false macrolide-susceptible results may be released because no mutation would be detected by the kit. Subsequently, macrolide antibiotics would certainly be used to treat the patient, leading to a risk of treatment failure if the nonamplified 23S rRNA was actually mutated. A 23S rRNA amplification control would be beneficial in future versions of the three commercial assays.

Conclusions. In conclusion, the three kits showed excellent performance for the simultaneous detection of *M. genitalium* and 23S rRNA mutations, with high sensitivities and specificities for detection of macrolide resistance-associated mutations compared with the gold standard method of 23S rRNA sequencing. The launch of these easy-to-use commercial kits will enable rapid choice of an effective first-line antibiotic therapy and may prevent the spread of antimicrobial resistance in *M. genitalium* sexually transmitted infections.

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