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Identification of 16S rRNA mutations in *Mycoplasma genitalium* potentially associated with tetracycline resistance *in vivo* but not selected *in vitro* in *M. genitalium* and *Chlamydia trachomatis*

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Objectives: Tetracyclines are widely used for the treatment of bacterial sexually transmitted infections (STIs) and recently have been used successfully for post-exposure prophylaxis of STIs in MSM. We investigated the *in vitro* and *in vivo* development of tetracycline resistance in *Chlamydia trachomatis* and *Mycoplasma genitalium* and evaluated 16S rRNA mutations associated with acquired resistance in other bacteria.

Methods: *In vitro* selection of resistant mutants of reference strains of *C. trachomatis* and *M. genitalium* was undertaken by serial passage in medium containing subinhibitory concentrations of tetracycline or doxycycline, respectively. The 16S rRNA gene of the two microorganisms was amplified and sequenced at different passages, as were those of 43 *C. trachomatis*- and 106 *M. genitalium*-positive specimens collected in France from 2013 to 2019.

Results: No tetracycline- or doxycycline-resistant strains of *C. trachomatis* and *M. genitalium*, respectively, were obtained after 30 serial passages. The tetracycline and doxycycline MICs were unchanged and analysis of the 16S rRNA gene, the molecular target of tetracyclines, of *C. trachomatis* and *M. genitalium* revealed no mutation. No mutation in the 16S rRNA gene was detected in *C. trachomatis*-positive specimens. However, six *M. genitalium*-positive specimens harboured a mutation potentially associated with tetracycline resistance without known prior tetracycline treatment for patients.

Conclusions: Tetracyclines did not select *in vitro*-resistant mutants of *C. trachomatis* or *M. genitalium*. However, 16S rRNA mutations either responsible for or associated with tetracycline resistance in other bacteria, including mycoplasma species, were identified in several *M. genitalium*-positive specimens.

Introduction

Increased rates of bacterial sexually transmitted infections (STIs) (*Chlamydia trachomatis, Neisseria gonorrhoeae* and *Treponema pallidum*) have been reported worldwide,[1] particularly in MSM and in users of HIV pre-exposure prophylaxis (PrEP).[2] Furthermore, people who engage in high-risk sexual behaviour and who have elevated exposure to antibiotics, such as PrEP users, are more likely to become infected by macrolide- or fluoroquinolone-resistant *Mycoplasma genitalium*.[3,4]

Tetracyclines are the first-line treatment for chlamydia infection and doxycycline has been recently used successfully for post-exposure prophylaxis (PEP) of STIs in high-risk MSM using PrEP. Doxycycline PEP significantly reduced the incidence of chlamydia and syphilis in this population.[5] A few *C. trachomatis* strains with decreased susceptibility to tetracyclines have been reported, but the molecular mechanisms were not elucidated.[6] Doxycycline has poor clinical efficacy against *M. genitalium* infections, with microbiological cure rates of 30%–40%, and is recommended as the third-line therapy in the European guideline on *M. genitalium* infection.[7] However, an initial use of doxycycline that can significantly lower the *M. genitalium* bacterial load is the first step of the resistance-guided therapy approach recommended for *M. genitalium* infections in Australian and British guidelines.[8,9]

Because *M. genitalium* and *C. trachomatis* possess only one and two *rrn* operons, respectively, target-related resistance to tetracyclines due to 16S rRNA mutations could be expected, as such mutations have previously been described in other mycoplasma species.[10,11] Most of the 16S rRNA mutations associated with tetracycline resistance are located in the primary tetracycline-binding site formed by 16S rRNA nucleotides 964–967 of helix 31 (H31) and nucleotides 1054–1056 and 1196–1200 of helix 34 (H34).[10–12] The purpose of this study was to evaluate the resistance mechanism in the *C. trachomatis* L2 and *M. genitalium* G37 reference strains by selecting for tetracycline- and doxycycline-resistant mutants *in vitro*, respectively, and sequencing the 16S rRNA genes of the mutants. We also evaluated 16S rRNA mutations in *C. trachomatis*- or *M. genitalium*-positive clinical specimens.

Materials and methods

In vitro selection of tetracycline-resistant mutants of C. trachomatis and doxycycline-resistant mutants of M. genitalium

Growth and tetracycline susceptibility testing of the *C. trachomatis* L2/434/Bu (ATCC VR-902B) reference strain were performed on McCoy mouse fibroblast (ATCC CRL1696) cells with an inoculum of 10^5 inclusion forming units/mL, as described previously.[13] To select tetracycline-resistant mutants, *C. trachomatis* L2/434/Bu (10^7 inclusion-forming units/mL) was cultivated for 30 passages in the presence of constant subinhibitory concentration of tetracycline (Sigma–Aldrich). Tetracycline was chosen because this molecule is less active than doxycycline against *C. trachomatis* and thus would be more able to select resistance *in vitro*.

The growth conditions and doxycycline susceptibility testing of *M. genitalium* G37 (ATCC 33530) were as reported previously.[14] Broth selection was conducted in 1 mL of SP4 broth containing constant subinhibitory concentrations of doxycycline for 30 passages, as described previously for *Mycoplasma pneumoniae*.[15]

Clinical specimens

Regarding *C. trachomatis*, 23 L2b-positive anorectal samples were sequentially collected at the French National Reference Centre for Bacterial STIs from 2013 to 2017 from nine patients who presented with recurrent *C. trachomatis* rectal infections and who were treated with doxycycline for 3 weeks. Two or three sequential samples were available per patient. Moreover, 18 *C. trachomatis*-positive anorectal and 2 *C. trachomatis*-positive pharyngeal specimens (5 L, 8 non-L and 7 not typed) were obtained and amplified for 16S rRNA in 2015 and 2016 from 18 patients (7 in the PEP arm and 11 in the no-PEP arm) during the Ipergay substudy of doxycycline PEP.[5]

For *M. genitalium*, a total of 106 DNA extracts (47 urines, 21 rectal swabs, 11 vaginal swabs, 16 endocervical swabs, 6 urethral swabs, 2 semen specimens, 1 pharyngeal swab and 2 others) was collected from 103 patients (74 men and 29 women) at the French National Reference Centre for Bacterial STIs from 2017 to 2019. Regarding previous antibiotic treatments, 67 specimens were obtained from patients without known previous antibiotic treatment and 39 from patients known to have received antibiotics (12 azithromycin, 15 moxifloxacin and 12 doxycycline).

Specimens collected at the French National Reference Centre for Bacterial STIs were preserved at the Centre de Ressource Biologique-Bordeaux Biothèque Santé of Bordeaux University Hospital under the collection number BB-0033-00094 and authorization number AC-2014-2166 from the French Ministry of Higher Education and Research.

Sequencing of the 16S rRNA gene

DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel) from 200 µL of a culture of *M. genitalium* G37 or *C. trachomatis* L2 at different passages. Three pairs of primers were designed to sequence the 1500 bp 16S rRNA gene of *C. trachomatis*. Primers MG16-439F and MG16-1301R were used to amplify an 889 bp fragment of the *M. genitalium* 16S rRNA, as described previously (Table 1).[16] Five and ten microlitres of DNA extracted from cultures and clinical specimens, respectively, were used for PCR. The PCR products were sent to Eurofins Genomics (Germany) for Sanger sequencing.

Results and discussion

In vitro selection of mutants resistant to tetracyclines

The MIC of tetracycline for the *C. trachomatis* L2/434/Bu reference strain was 0.25 mg/L at the first passage. The subinhibitory concentration used for the selection corresponded to one-quarter of the MIC determined, i.e. 0.06 mg/L. Thirty passages were carried out without increasing the tetracycline concentration in the medium and no mutant with reduced susceptibility to tetracycline was obtained. The tetracycline MIC was 0.25 mg/L at passage 30. Sequencing of the 16S rRNA gene of *C. trachomatis* L2/434/Bu at passages 1, 5, 10, 15, 20, 25 and 30 revealed no mutation.

In vitro, antimicrobial resistance has been selected by serial passage of *C. trachomatis* strains in subinhibitory concentrations of sulphonamides, penicillins, rifampicin and fluoroquinolones.[17] However, the selection of mutants resistant to tetracycline has not been reported. All genovars of *C. trachomatis* are naturally susceptible to tetracyclines, with MICs ranging from 0.125 to 1 mg/L,

Table 1. Primers used for the sequencing of the 16S rRNA gene of *C. trachomatis* and *M. genitalium*

Target	Name	Sequence (5′→3′)	Tm (°C)	Size (bp)	Reference
16S rRNA positions 40–554	CT16S-F1	GTGGATGAGGCATGCAAGT	56.7	515	this study
	CT16S-R1	GCTAGCACCCTCCGTATTAC	59.4		this study
16S rRNA positions 407–989	CT16S-F2	CGTGTGTGATGAAGGCTCTA	57.3	582	this study
	CT16S-R2	TAAGGTCCTTCGCGTTGCAT	57.3		this study
16S rRNA positions 899–1499	CT16S-F3	ACTCGCAAGGGTGAAACTCA	57.3	600	this study
	CT16S-R3	TTCATCCTAGTCATCAGCCTC	57.9		this study
16S rRNA positions 458–1346	MG16-439F	GAATGACTCTAGCAGGCAATGGCTG	54.2	889	16
	MG16-1301R	CTGATTCGCGATTACTAGTGATTCCAG	53.1		16

Table 2. Mutations in the 16S rRNA of *M. genitalium*-positive specimens

Specimen number	Collection date (dd/mm/yyyy)	Sex	Specimen type	Previous antibiotic treatment	23S rRNA mutations	16S rRNA mutations[a]
7103507102	03/06/2017	F	endocervical	moxifloxacin	ND	G1166A
7103507104	20/07/2017	F	endocervical	moxifloxacin	ND	G1166A
BCT0001018	30/03/2018	M	semen	unknown	no	*C1192T*
BCT0001084	24/04/2018	M	urine	moxifloxacin	yes	A746G
BCT0001087	24/04/2018	M	rectal	moxifloxacin	ND	A746G
BCT0001153	22/02/2018	M	urine	unknown	no	A746G
BCT0001264	04/06/2018	M	urine	azithromycin	yes	C912T
BCT0001415	17/07/2018	M	urine	doxycycline	yes	C580T
BCT0001428	24/07/2018	M	urine	moxifloxacin	yes	A746G
BCT0001518	28/08/2018	M	urine	doxycycline	yes	A746G
BCT0001724	25/09/2018	M	rectal	azithromycin	yes	A746G
BCT0001806	10/10/2018	M	rectal	moxifloxacin	yes	A746G
BCT0001915	24/10/2018	M	rectal	doxycycline	yes	A746G
BCT0002503	05/12/2018	M	rectal	azithromycin	yes	A746G
BCT0002609	13/12/2018	M	rectal	unknown	yes	A746G
BCT0002631	07/12/2018	M	rectal	unknown	yes	A746G
BCT0002647	14/12/2018	M	rectal	doxycycline	yes	A746G
BCT0002649	02/01/2019	F	vaginal	unknown	yes	A746G
BCT0002691	07/01/2019	M	rectal	azithromycin	yes	A746G
BCT0002699	10/01/2019	M	urine	unknown	yes	**G966T** **C967T**
BCT0002729	17/01/2019	M	urine	unknown	no	A746G
BCT0002733	15/01/2019	M	urine	unknown	yes	A746G
BCT0002736	22/01/2019	M	rectal	unknown	yes	A746G
BCT0002758	16/01/2019	M	urine	unknown	yes	A746G
BCT0002834	06/02/2019	M	urine	unknown	yes	A746G
BCT0002841	11/02/2019	M	urine	unknown	yes	*G1193A*
BCT0002866	16/02/2019	M	urine	moxifloxacin	yes	A746G
BCT0002883	20/02/2019	M	urine	unknown	yes	A746G
BCT0002905	21/02/2019	M	urine	moxifloxacin	yes	A746G
BCT0002930	01/03/2019	M	rectal	unknown	yes	A746G
BCT0002934	02/03/2019	F	endocervical	unknown	no	**G966T** **C967T**
BCT0002991	06/02/2019	M	rectal	azithromycin	yes	A746G
BCT0003025	16/03/2019	M	urine	azithromycin	yes	A746G
BCT0003049	18/03/2019	M	urine	azithromycin	yes	A746G C897T
BCT0003055	20/03/2019	M	urine	azithromycin	yes	**G966T** **C967T**
19032164642	02/04/2019	M	urine	doxycycline	yes	A746G
19032241222	09/04/2019	M	urine	unknown	no	**G966T** **C967T**
19032388559	12/04/2019	M	urine	unknown	no	T850C
19032430134	16/04/2019	M	urine	moxifloxacin	yes	A746G

F, female; M, male; ND, not determined.
Mutations responsible for tetracycline resistance in *H. pylori* or associated with tetracycline resistance in other mycoplasma species are indicated in bold and italic, respectively.
[a]*E. coli* numbering.

depending on the antibiotic, the genovar and the duration of exposure to antibiotics in infected cells.[18] Only 17 clinical strains have been reported to have decreased susceptibility to tetracycline, but either the resistance has not been genotypically characterized[6,19] or no evidence of tetracycline-resistance genes was revealed by genomic analysis.[20]

The MIC of doxycycline for *M. genitalium* G37 was 0.125 mg/L at the first passage, as reported previously.[14] During 30 serial passages in subinhibitory concentrations of doxycycline (0.03–0.06 mg/L), the MIC ranged from 0.06 to 0.125 mg/L. No mutant with reduced susceptibility to doxycycline was obtained *in vitro* and 16S rRNA sequencing at passages 1, 5, 10, 15, 20, 25 and 30 did not reveal any mutation.

Detection of 16S rRNA mutations in *C. trachomatis*- and *M. genitalium*-positive clinical specimens

No mutation was identified in the 16S rRNA genes of the 23 *C. trachomatis* L2b-positive anorectal specimens collected at the French National Reference Centre for Bacterial STIs from nine patients with recurrent *C. trachomatis* infections after treatment for 3 weeks with doxycycline. Similarly, no mutation was discovered in the 20 *C. trachomatis*-positive anorectal and pharyngeal specimens obtained from 18 MSM in the Ipergay substudy on the efficacy of doxycycline PEP for STI prevention.[5] Regarding doxycycline exposure of the 18 MSM, 9 subjects (2 in the PEP arm and 7 in the no-PEP arm) had a known previous STI treatment with doxycycline.

Among the 106 clinical *M. genitalium*-positive DNA extracts subjected to 16S rRNA sequencing, 67 harboured a WT sequence and 39 exhibited one or several SNPs (Table 2). SNPs at positions 580, 746, 840, 850, 897, 912, 1009 and 1166 were identified in *M. genitalium*-positive clinical specimens (Table 2), but were located outside H31 and H34, in 16S rRNA regions far from tetracycline-binding sites. Six specimens harboured one or two SNPs reported as being responsible for tetracycline resistance in *Helicobacter pylori*[12] or reportedly associated with tetracycline resistance in mycoplasmas.[10,11] Two mutations—G966T and C967T (*Escherichia coli* numbering)—were described in four specimens: three male urines and one female cervix specimen. Two of these patients also harboured azithromycin-resistant *M. genitalium* (Table 2). Such mutations in H31 were shown to confer tetracycline resistance on *H. pylori* by transformation and recombination.[11] These mutations were also described *in vitro* for *Mycoplasma hominis* and were associated with an 8–16-fold increase in the doxycycline MIC.[10] Position 967 was mutated in field strains of *Mycoplasma bovis*, an animal mycoplasma, and associated with high-level tetracycline resistance with an increase in the oxytetracycline MIC from 1 to 32 mg/L.[11] One patient had a urine specimen positive for an azithromycin-resistant strain of *M. genitalium* harbouring the mutation G1193A, located close to H34. This mutation was associated with an 8-fold increased MIC of doxycycline for an *M. pneumoniae* mutant selected *in vitro* with doxycycline.[10] The final specimen, a semen specimen, harboured the mutation C1192T. A C1192A mutation has been described in *M. bovis* tetracycline-resistant isolates, but associated with other 16S rRNA mutations.[11] However, these mutations at positions 1193 and 1192 were only found associated with tetracycline resistance in *M. pneumoniae* and *M. bovis*, respectively. None of the six patients had a history of known prior tetracycline treatment. In a very recent study,[3] mutations C1192G, G966T and C967T were observed in *M. genitalium*-positive specimens of two MSM, enrolled in the open-label phase of the Ipergay trial with on-demand PrEP and on doxycycline PEP, who had received doxycycline (one for prophylaxis and the other for lymphogranuloma venereum treatment). These data add further evidence to support the potential role of these 16S rRNA mutations in tetracycline resistance in *M. genitalium*, although stronger evidence, particularly phenotypic confirmation of resistance, is clearly needed.

Conclusions

In summary, we failed to select *in vitro C. trachomatis* mutants resistant to tetracycline and no mutations associated with tetracycline resistance were detected in *C. trachomatis*-positive clinical specimens from patients of the Ipergay substudy or with therapeutic failure. Although we were unable to select *in vitro* doxycycline-resistant mutants of *M. genitalium*, 16S rRNA mutations potentially associated with tetracycline resistance were identified in *M. genitalium*-positive clinical specimens. However, according to the extant clinical information, there is no correlation between antibiotic treatment history and these mutations, and no MIC data are available to confirm doxycycline resistance. Our findings emphasize the need to monitor the doxycycline susceptibility of STI pathogens, particularly in doxycycline PEP trials, and to obtain *M. genitalium* clinical strains after treatment failure.

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Transparency declarations

None to declare.

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