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Evidence for the predominance of a single *tet*(M) gene sequence type in tetracycline-resistant *Ureaplasma parvum* and *Mycoplasma hominis* isolates from Tunisian patients

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Resistance to tetracyclines in genital mycoplasmas is due mainly to acquisition of the *tet*(M) determinant, which is frequently associated with conjugative transposon elements of the Tn*916*/Tn*1545* family. The aim of the present work was to evaluate the prevalence of *tet*(M) in Tunisian isolates and to gain an insight into its origin and evolution. Twenty *Ureaplasma parvum*, two *Ureaplasma urealyticum* and 48 *Mycoplasma hominis* isolates, recovered from Tunisian patients with urogenital and infertility disorders, were evaluated for their resistance to tetracyclines and interrogated by PCR amplification for the presence of *tet*(M) and *int-Tn*, the gene encoding the integrase of Tn*916*/Tn*1545*-like transposons. The resistance rates to tetracyclines were 22.72 and 25.0 % among *U. parvum* and *M. hominis* isolates, respectively, with high-level resistance observed in 11 of the 12 resistant *M. hominis* isolates. All resistant isolates harboured both *tet*(M) and *int-Tn* sequences. Nucleotide sequence analysis of the *tet*(M) amplicon revealed a unique sequence shared by all tetracycline-resistant clinical isolates of both species. Molecular typing indicated that the tetracycline-resistant *U. parvum* and *M. hominis* isolates were not clonal. Taken together, these data indicate that a single *tet*(M) gene sequence type, most probably transmitted via a Tn*916*/Tn*1545*-like transposon, contributes to most of the tetracycline resistance in *U. parvum* and *M. hominis* isolates in Tunisia. Because this *tet*(M) gene sequence type was harboured by different *Mycoplasma* spp. and by phylogenetically distinct isolates within these species, one could reasonably argue that it may have benefited from an efficient horizontal transfer context, making it highly competent to spread.

INTRODUCTION

Mycoplasmas are among the smallest self-replicating micro-organisms capable of independent growth. They are wide-spread in nature, and several species have been isolated from humans. Genital mycoplasmas represent a complex group of micro-organisms that have been associated with a wide array of infectious diseases in adults and children. *Mycoplasma hominis*, *Ureaplasma urealyticum* and/or *Ureaplasma parvum* are suspected of being causative agents of non-gonococcal urethritis (Horner *et al.*, 2001; Taylor-Robinson *et al.*, 1985), pregnancy complications and prenatal infections (Abele-Horn *et al.*, 2000; Gerber *et al.*, 2003; Judlin, 2003; Yoon *et al.*, 2003). Recent evidence using a primate model has confirmed their implication as aetiological agents in genital pathologies (Novy *et al.*, 2009). The pathogenic potential of *Mycoplasma genitalium* has been recognized recently (McGowin & Anderson-Smits, 2011; Taylor-Robinson & Jensen, 2011).

As occurs in a wide range of bacteria, acquisition of high-level resistance to tetracyclines in genital mycoplasmas is due mainly to the presence of the *tet*(M) determinant (de Barbeyrac *et al.*, 1996; Dégrange *et al.*, 2008; Roberts, 1990). The product of the *tet*(M) gene renders ribosomes resistant to tetracyclines, a mechanism of action referred to as ribosome protection (Burdett, 1991; Chopra & Roberts, 2001).

The *tet*(M) gene is usually located on the chromosome and has been associated with a number of conjugative transposons, including Tn*1545*, Tn*916* and Tn*919*, although it may also be plasmid-encoded (Bentorcha *et al.*, 1992; Burdett, 1990; de Barbeyrac *et al.*, 1996; Gascoyne-Binzi *et al.*, 1993). Excisive recombination of these transposons requires two

Abbreviations: MBA, multiple-banded antigen; SP4-U, SP4 medium with urea.

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transposon-encoded proteins, Xis-Tn and Int-Tn. The latter protein alone is sufficient for integration (Poyart-Salmeron *et al.*, 1990). Comparative sequence analyses of *tet*(M) genes from different bacterial species, including genital mycoplasmas, have revealed its mosaic structure (Gascoyne-Binzi *et al.*, 1993; Huang *et al.*, 1997; Oggioni *et al.*, 1996; Soroka *et al.*, 2002). Here, we have provided evidence that a single *tet*(M) sequence type predominates in tetracycline-resistant genital mycoplasmas isolated from Tunisian patients, suggesting its increased ability to spread.

METHODS

Mycoplasma strain collection. Three reference mycoplasma strains and two ureaplasma strains purchased from the American Type Culture Collection (ATCC) were used: *M. hominis* PG21 (ATCC 23114), *Mycoplasma fermentans* (ATCC 19989), *M. genitalium* (ATCC 33530), *U. parvum* serotype 3 (ATCC 27815) and *U. urealyticum* serotype 8 (ATCC 27618). An additional set of 27 *M. hominis* (MH1–MH27) isolates that have been characterized previously (Mardassi *et al.*, 2007) were included in this study.

Clinical specimens and data collection. The study included patients from three geographically distinct regions in Tunisia (Tunis, Bizerte and Sousse) whose clinical specimens were processed in the Laboratory of Mycoplasmas of the Institut Pasteur de Tunis between 2005 and 2009. Each patient represented a unique case, and mycoplasma isolation was not attempted on consecutive specimens. The clinical specimens included: (i) cervical swabs from female patients suffering from sexually transmissible diseases; (ii) vaginal samples from women presenting a number of complications associated with pregnancy, including chorioamnionitis and pre-term birth; (iii) urethral specimens obtained from men with urethritis; and (iv) semen samples from clinical cases associated with infertility. All specimens were tested for the presence of genital mycoplasmas (*M. hominis*, *M. genitalium*, *M. fermentans* and *Ureaplasma* spp.) by broth and solid culture.

Information was obtained prospectively on gender, age and associated clinical data of the patients. This study was conducted in close conformity with ethical aspects, which were established by the ethical committee of the Tunisian Ministry of Health. Samples were collected in the context of the routine diagnostic activity of the Laboratory of Mycoplasma with the consent of patients. All patient files were kept confidential.

Specimen processing and mycoplasma isolation. The collected specimens were inoculated onto 1.8 ml SP4 broth medium and then transferred to the laboratory at 4 °C within 24 h. Semen samples were also placed in a tube with 1.8 ml SP4 medium for cultivation. The specimens were processed immediately upon arrival or after storage at −80 °C. After filtration through a 0.45 μm pore-size single-use syringe filter, 200 μl of the specimen was inoculated into SP4 medium (Tully *et al.*, 1977) supplemented with 5 % CMRL 1066 (Sigma), 2000 U penicillin G ml^{-1}, 500 U polymyxin B ml^{-1}, 2.5 μg amphotericin B ml^{-1}, 10 % fresh yeast extract (Amersham Biosciences), 15 % horse serum (Gibco-BRL) and 0.5 % phenol red. The medium was further supplemented with 0.5 % glucose, 0.5 % arginine or 0.5 % urea (SP4-U), depending on the nutritional needs of the species being cultivated. All broth cultures were incubated at 37 °C and examined daily for turbidity and pH changes. Growth confirmation was performed on SP4 agar plates maintained at 37 °C with 5 % CO_2, and the plates were regularly observed microscopically for the appearance of mycoplasma colonies. Growth of ureaplasmas was observed as a change in colour of the SP4-U medium (hydrolysis of urea with the release of ammonia, signalled by a colour change of a pH indicator). The presence of characteristic brownish colonies on SP4 agar plates further confirmed the isolation of ureaplasmas. Given its ability to hydrolyse arginine, growth of *M. hominis* was accompanied by alkalinization of the SP4 liquid medium in the presence of arginine, typically recognized as a change in colour of the phenol red indicator. No acidification of the SP4 liquid medium was observed, indicating the absence of mycoplasma species that ferment glucose such as *M. genitalium*, *M. fermentans* and *Mycoplasma pneumoniae*. Identification of the isolated mycoplasma and ureaplasma species was further confirmed by PCR amplification (see below).

For mycoplasma titration, dilutions and subcultures were performed as described elsewhere (Taylor-Robinson & Furr, 1981).

Mycoplasma detection by PCR. *M. hominis* and ureaplasmas were also detected by PCR, targeting DNA fragments of the P120′ and multiple-banded antigen (MBA) genes, respectively. The primer pairs Mhp120′F/Mhp120′R (Mardassi *et al.*, 2007) and UMS-125/UMA226 (Knox *et al.*, 1998) were used to amplify the P120′ and MBA fragments, respectively. The characteristics of these primers are given in Table 1. Based on the amplicon size of the PCR targeting the MBA gene, ureaplasmas were assigned to *U. parvum* (403 or 404 bp) or *U. urealyticum* (448 bp).

Tetracycline susceptibility testing. Tetracycline susceptibility was determined using a quantitative broth microdilution technique, as described by Beeton *et al.* (2009), with some modifications. Serial twofold dilutions of tetracycline hydrochloride (Sigma) in 100 μl SP4 or SP4-U broth medium, from 51.5 to 0.025 μg ml^{-1}, were prepared from a stock solution of 1030 μg ml^{-1} and dispensed into a test 96-well polystyrene plate. The inoculum suspension (100 μl) of *M. hominis* or *Ureaplasma* spp. isolates at 10^5 c.f.u. ml^{-1} was then inoculated into the wells containing the antibiotic. Plates were covered with plastic wrap and incubated at 37 °C for 24–48 h. Culture controls containing SP4 or SP4-U broth plus organisms without antibiotic were included in all tests. As a negative control for each isolate, a row of the 96-well plate was filled with uninoculated medium (SP4 or SP4-U). The lowest concentration of antibiotic that completely inhibited visual growth of mycoplasma (no pH change) was recorded as the MIC. Control of inoculum density was achieved by viable counts in SP4 or SP4-U broth.

Detection of *tet*(M) and *int-Tn* genes. *M. hominis* or ureaplasma culture volumes (5–8 ml) were used to prepare DNA for PCR amplification. The cultures were centrifuged at 42 654 ***g*** for 30 min, washed in PBS (pH 7.4) and resuspended in 200 μl PBS. This preparation was treated with SDS (1 % final concentration) and RNase A (5 μg ml^{-1}) for 30 min at 37 °C. Next, 5–15 μl (corresponding to 10^4 bacterial cells) was boiled for 5 min and used for PCR amplification.

Detection of *tet*(M) and *int-Tn* sequences was performed using the primer pairs tet1/tet2 and int1/int2, respectively (Table 1). The cycling parameters consisted of 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 or 50 °C [for the *tet*(M) and *int-Tn* primers, respectively] and 1 min extension at 72 °C, followed by a final extension step at 72 °C for 10 min. The PCR amplicons were visualized by agarose gel electrophoresis, as described by Sambrook *et al.* (1989).

Nucleotide sequencing of the *tet*(M) amplicon. The PCR products were separated by electrophoresis in a 1.5 % low-melting-point agarose gel, excised from the gel and purified using a GFX PCR DNA and Gel Band Purification system (Amersham Biosciences). Determination of the nucleotide sequence was performed using a Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit on an ABI PRISM 377 DNA sequencer (Applied Biosystems). Each sample was sequenced from two independent PCR amplifications.

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Table 1. Characteristics of the primers used in this study

Primer pair designation	Target gene/region	Primer sequence (5′→3′)	Primer orientation	Primer position	Amplicon size (bp)	Reference
tet1	*tet*(M)	GAACTGTATCCTAATGTGTG	Sense	697–716	377	de Barbeyrac *et al.* (1996)
tet2		GATACTCTAACCGAATCTTCG	Reverse	1054–1073		
int1	*int-Tn*	TGACACTCTGCCAGCTTAC	Sense	758–777	579	de Barbeyrac *et al.* (1996)
int2		CCATAGGAACTTGACGTTGG	Reverse	1317–1336		
UMS-125	MBA	GTATTTGCAATCTTTATATGTTTTCG	Sense	−150 to −124	403/404 (*U. parvum*)/448 (*U. urealyticum*)	Knox *et al.* (1998)
UMA226		CAGCTGATGTAAGTGCAGCATTAAATTC	Reverse	225–253		
Mhp120′F	P120′	GAGGAATTCAACTGGTGTCC	Sense	83–103	510	Mardassi *et al.* (2007)
Mhp120′R		CTGTTGTAATAGCATTTAAG	Reverse	573–592		

The sequence data were aligned using CLUSTAL W (Thompson *et al.*, 1994) and edited with the software program BioEdit (Hall, 1999).

Phylogeny of *Ureaplasma* spp. and *M. hominis* clinical isolates. The phylogenies of *Ureaplasma* spp. and *M. hominis* clinical isolates were reconstructed based on sequence polymorphisms within fragments of their MBA and P120′ genes, respectively, as described previously (Knox *et al.*, 1998; Mardassi *et al.*, 2007). The PCR amplicons were subjected to nucleotide sequencing, and phylogenetic reconstructions were performed in MEGA5 (Tamura *et al.*, 2011). A dendrogram was obtained using the neighbour-joining method with 1000 bootstrapping replicates.

RESULTS

Mycoplasma identification and tetracycline resistance

Based on the MBA amplicon size, 90.90 % (20/22) of ureaplasma isolates were assigned to the species *U. parvum* and the remaining two isolates (9.1 %) to *U. urealyticum* (Table 2). Tetracycline resistance was observed in five of the 20 *U. parvum*, yielding a resistance rate of 22.72 % among all ureaplasmas. All *U. parvum* clinical isolates were of intermediate resistance (MIC of 12.87 mg l^{-1}; Table 2). Twelve of the 48 *M. hominis* isolates (25 .0%) were resistant to tetracycline (Table 2). In contrast to *U. parvum*, high-level resistance was observed in the majority (11/12; 91.67 %) of *M. hominis* isolates, of which 36.36 % (4/11) showed MIC values of 51.5 mg l^{-1}.

Detection of *tet*(M)/*int-Tn* in *U. parvum* and *M. hominis* clinical isolates

The molecular survey aiming at detecting *tet*(M) and *int-Tn* sequences revealed that all tetracycline-resistant *U. parvum* and *M. hominis* clinical isolates simultaneously harboured the two sequences (Table 2). The PCR products of *tet*(M) and *int-Tn* all migrated at the expected sizes of 377 and 579 bp, respectively (data not shown). Neither *tet*(M) nor *int-Tn* sequences could be amplified from clinical isolates that were susceptible to tetracycline.

Evidence for the predominant spread of a single *tet*(M) sequence type among *U. parvum* and *M. hominis* clinical isolates in Tunisia

To gain insight into the nature and evolution of *tet*(M), we determined its nucleotide sequence from all *U. parvum* and *M. hominis tet*(M)-positive isolates. As shown in Fig. 1, a unique sequence of the *tet*(M) amplicon was shared by all *U. parvum* and *M. hominis* clinical isolates, suggesting that these isolates originally acquired the *tet*(M) determinant from a common source. BLASTN analysis revealed equivalent identities (~96–97 %) with the sequence of *tet*(M) of *Gardnerella vaginalis*, the *Streptococcus pneumoniae* Tn*916* integrative and conjugative element, *Enterococcus faecalis*, *Lactobacillus salivarius* and others. Genome BLAST analysis revealed 94 % identity with the corresponding *tet*(M)

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Table 2. Resistance of *M. hominis* and *Ureaplasma* spp. isolates to tetracycline and correlation with the presence of *tet*(M) and *int-Tn* sequences

+, Positive PCR result; −, negative PCR result.

Mycoplasma isolates (*n*)	Designation	MIC of tetracycline (mg l^{-1})	*tet*(M) PCR	*int-Tn* PCR
U. parvum (15)	Up01, Up03, Up04, Up20, Up33, Up43, Up08, Up41, Up51, Up56, Up60, Up61, Up66, Up68, Up90	3.2	−	−
U. parvum (5)	Up16, Up53, Up58, Up70, Up81	12.87	+	+
U. urealyticum (2)	Uu10, Uu91	3.2	−	−
M. hominis (36)	MH2, MH3, MH5, MH7, MH8, MH9, MH10, MH12, MH13, MH14, MH15, MH16, MH17, MH19, MH20, MH21, MH22, MH23, MH24, MH27, MH28, MH29, MH30, MH31, MH32, MH33, MH35, MH36, MH37, MH38, MH39, MH43, MH44, MH46, MH47, MH48	3.2	−	−
M. hominis (1)	MH6	12.87	+	+
M. hominis (7)	MH1, MH18, MH25, MH34, MH36, MH40, MH45	25.75	+	+
M. hominis (4)	MH4, MH11, MH41, MH42	51.5	+	+

sequence of *U. urealyticum* serovar 9 (GenBank accession no. AAYQ02000002.1). The differences between the corresponding two sequences consisted of four single-nucleotide deletions, a dinucleotide (AA) insertion and 15 mutations (data not shown), resulting in a total of 21 amino acid changes, 14 of which were the consequence of indels (data not shown). Outside of these indels, the Tunisian *tet*(M) sequence proved much more homologous to that of *S. pneumoniae* (one amino acid difference) than to *U. urealyticum* serovar 9 (seven amino acid differences).

The unique *tet*(M) sequence type does not cluster within the ureaplasma and *M. hominis* Tunisian clinical isolate populations

The finding that a single *tet*(M) sequence type was shared by all tetracycline-resistant *U. parvum* and *M. hominis* Tunisian clinical isolates prompted us to explore the genetic relationships of these isolates. For this purpose, we determined the nucleotide sequences of their MBA and P120′ gene fragments, respectively. The phylogenetic relationships of the ureaplasma and *M. hominis* isolates are depicted in Figs 2 and 3, respectively. Of the 20 *U. parvum* isolates, 11 grouped with serovar 3, four with serovar 1 and five with serovar 6. Interestingly, the presence of *tet*(M) was not confined to a single cluster (Fig. 2). Likewise, *M. hominis* isolates genotyped according to sequence polymorphism in the surface-exposed domain of the P120′ protein showed a *tet*(M) distribution along the different branches of the phylogenetic tree (Fig. 3).

DISCUSSION

For a number of decades, tetracycline has been at the forefront of the antibiotic treatment arsenal of genital mycoplasma infections in Tunisia. However, curiously, data regarding the prevalence of resistance to this drug and the dynamics of resistance transmission are lacking. To our knowledge, this is the first study dealing with this aspect in this country.

We showed that tetracycline resistance rates in *U. parvum* and *M. hominis* clinical isolates (~23 and 25 %, respectively) in Tunisia are rather high, especially for ureaplasmas, in comparison with European countries, probably due to the frequent and systematic use of tetracycline in recent decades. A study carried out in France revealed that 24 of 128 (18.77 %) *M. hominis* and six of 276 (2.17 %) *U. urealyticum* isolates, collected over a 4-year period, were tetracycline resistant (Dégrange *et al.*, 2008). In the UK, among 61 ureaplasma isolates collected between 2003 and 2009, one isolate proved resistant to tetracycline (1.64 %) (Beeton *et al.*, 2009). In Hungary, in isolates collected between May 2008 and July 2010, 6.07 % (15/247) and 11.54 % (3/26) of *U. urealyticum* and *M. hominis* isolates were resistant to tetracycline, respectively (Farkas *et al.*, 2011). Similar rates of tetracycline resistance were obtained in Germany according to a study involving 469 mycoplasma isolates (179 ureaplasmas and 290 *M. hominis*) collected over a 20-year period (Krausse & Schubert, 2010), with observed tetracycline resistance rates of 1–3 and 10–13 %, respectively. However, higher tetracycline resistance rates of *U. urealyticum* isolates (17 %) were reported in China (Zhou *et al.*, 2011), and the 45 % (45/100) prevalence of *tet*(M) in ureaplasma isolates collected from a broad geographical area of the USA between 2000 and 2004 (Waites *et al.*, 2005) suggests resistance rates even higher than those reported in Tunisia. Taken together, these data indicate a global variation in tetracycline resistance rates, probably due to different practices and policies.

All tetracycline-resistant clinical isolates harboured the *tet*(M) determinant along with *int-Tn*, indicating transposon-mediated transfer of tetracycline resistance. Dissemination

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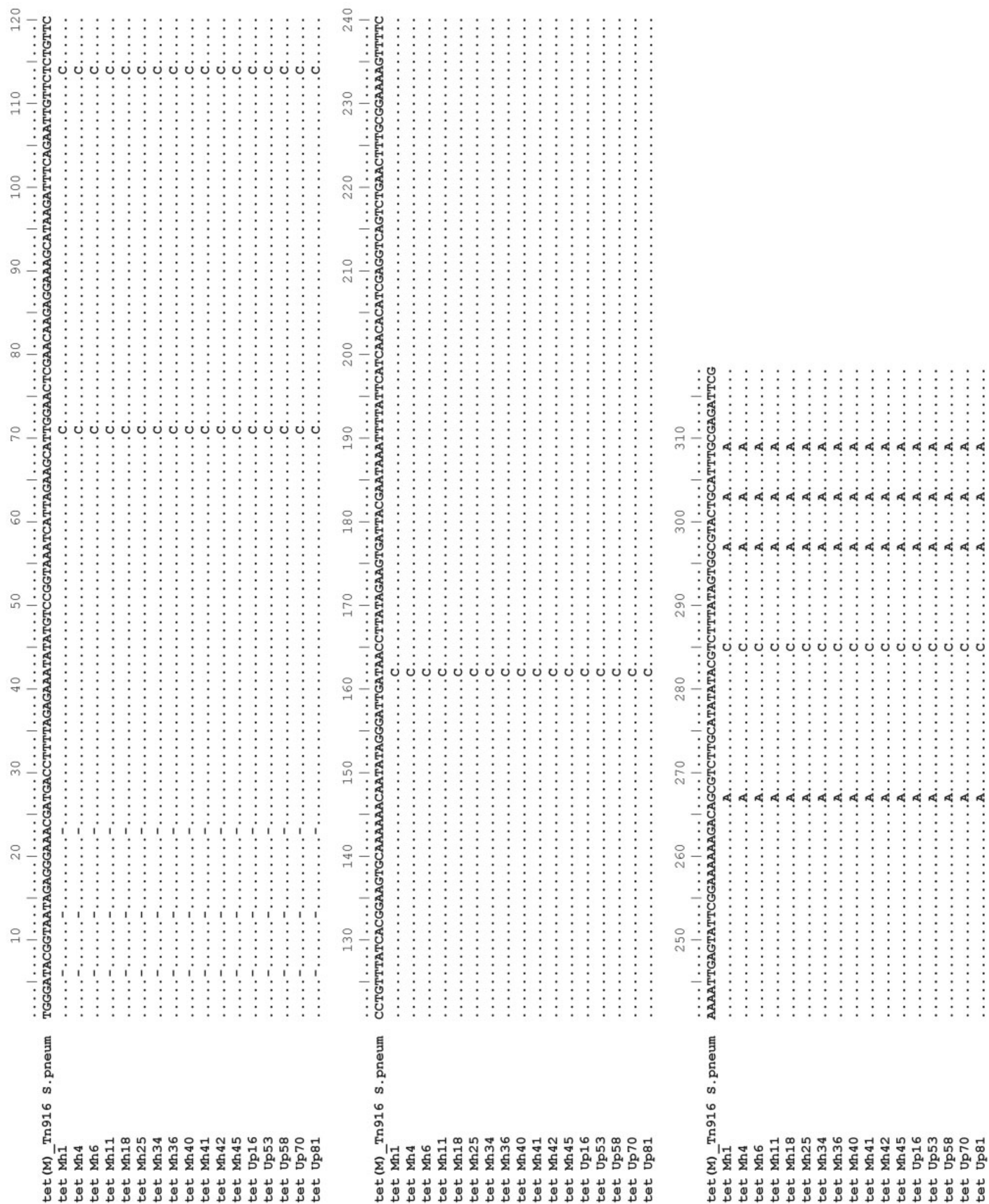

Fig. 1. Nucleotide sequence alignment of the *tet*(M) DNA fragment amplified from Tunisian *U. parvum* and *M. hominis* clinical isolates and the corresponding sequence of the *tet*(M)_Tn*916* integrative and conjugative element from *Streptococcus pneumoniae* (GenBank accession no. FR671414.1). The dots indicate identical nucleotides and dashes indicates spaces between adjacent nucleotides introduced for maximum alignment.

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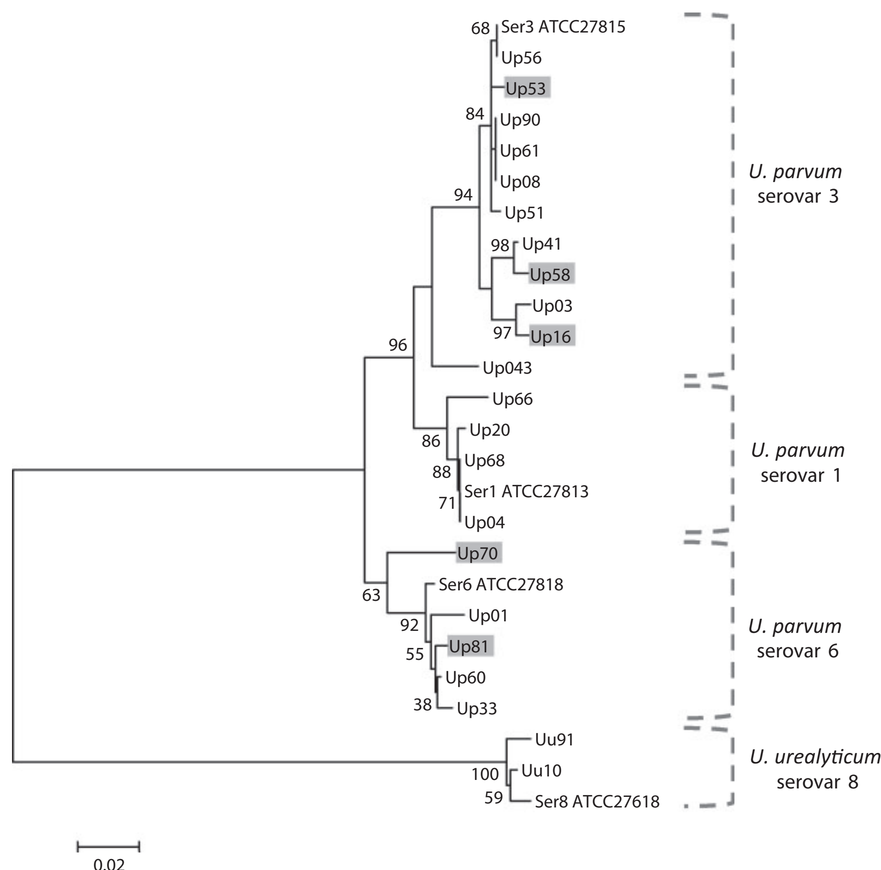

Fig. 2. MBA-based phylogenetic tree of Tunisian *Ureaplasma* spp. clinical isolates. Tetracycline-resistant isolates are highlighted in grey. Bootstrap values (%) were obtained from 1000 resamplings, and those >50 % are shown next to their corresponding node. Bar, 0.02 nucleotide substitutions per site.

of *tet*(M) has been shown to occur generally via a broad-host-range conjugative transposon such as Tn*916* and Tn*1545* (Bentorcha *et al.*, 1992; Burdett, 1990). The fact that all tetracycline-resistant Tunisian isolates harboured both *tet*(M) and *int-Tn* strongly suggests that resistance was acquired through the transposition of a Tn*916*/Tn*1545*-like element. Strikingly, analysis of the *tet*(M) nucleotide sequence pointed to the widespread transfer of a single *tet*(M) sequence type, a finding generally associated with the clonal expansion of resistant strains. However, phylogenetic analyses revealed that *tet*(M) was harboured by genetically diverse *U. parvum* and *M. hominis* isolates, a finding compatible with resistance transmission, probably through horizontal transfer. The predominance of a single *tet*(M) gene sequence in different mycoplasma species and diverse isolates within each species may reflect an increased transmission competence of the conjugative element bearing this particular *tet*(M) sequence. Previous studies dealing with *tet*(M) evolution in other mycoplasmas have reported the predominance of certain sequence types. For instance, when the nucleotide sequence of the *tet*(M) gene was examined in five *G. vaginalis* of different biotypes, four isolates showed identical sequences along the whole *tet*(M) gene (Huang *et al.*, 1997). Comparative sequence analysis of *tet*(M) in several mycoplasma isolates from Russia showed that the gene was completely identical in 11 of 13 *M. hominis* clinical strains (Soroka *et al.*, 2002). By contrast, using a high-resolution restriction analysis, *tet*(M) variation in *S. pneumoniae* was shown to occur at both the inter- and intraclone levels (Doherty *et al.*, 2000), suggesting that *tet*(M) may evolve differently depending on the bacterial species. However, comparative analyses of several *S. pneumoniae tet*(M) sequences available in GenBank revealed a divergence rate of no more than 2 % (data not shown).

Despite the fact that both *U. parvum* and *M. hominis* clinical isolates shared the same *tet*(M) sequence type, resistance levels to tetracycline were significantly higher in *M. hominis*. This finding is consistent with the fact that regulation of tetracycline resistance takes place at the transcriptional level. Hence, it is possible that the transcriptional environment in *M. hominis* is more favourable to the expression of a high-level resistance phenotype.

In conclusion, we have provided evidence for the inter- and intraspecies dissemination of a unique *tet*(M) sequence type in genetically diverse, tetracycline-resistant clinical isolates of *U. parvum* and *M. hominis* from Tunisia. The

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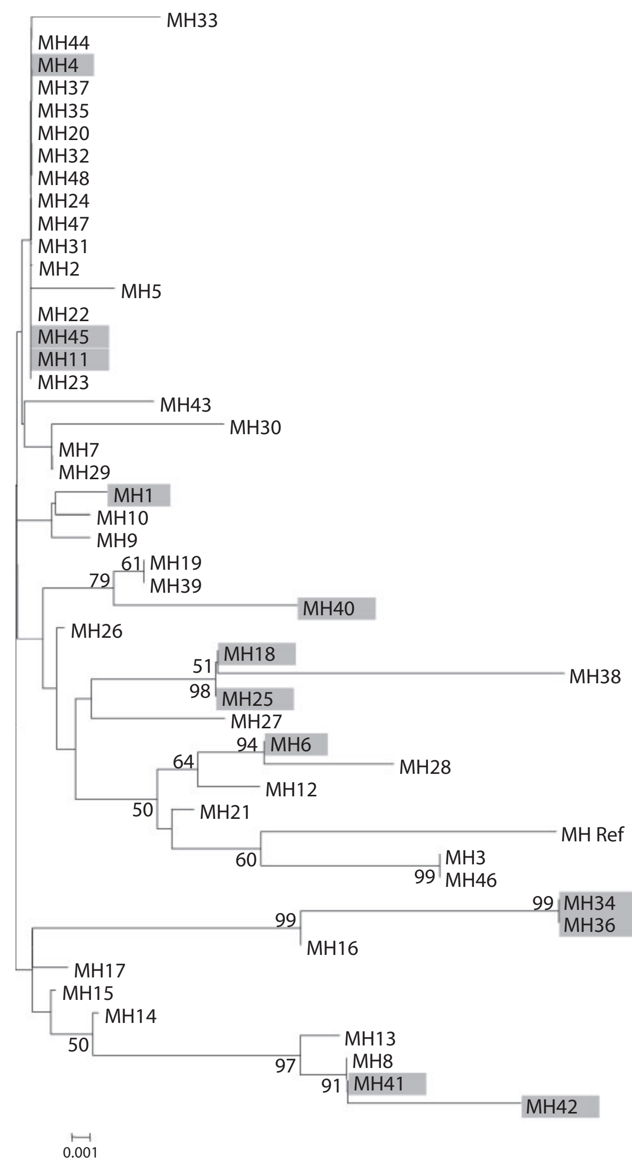

Fig. 3. P120′-based phylogenetic tree of Tunisian *M. hominis* clinical isolates. Tetracycline-resistant isolates are highlighted in grey. Bootstrap values (%) were obtained from 1000 resamplings, and those >50 % are shown next to their corresponding node. Bar, 0.001 nucleotide substitutions per site.

fact that this unique *tet*(M) sequence type was invariably associated with *int-Tn* argues for the transfer of a conjugative element that might be intrinsically endowed with an increased ability for transfer. It may be of interest to characterize this element fully in order to gain insight into the molecular basis underlying its success.

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