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SHORT REPORT

Clonal Spread of Tetracycline Resistance Among *Mycoplasma hominis* Clinical Strains, Tunisia

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Abstract: Antimicrobial resistance in a number of bacterial pathogens has been shown to spread clonally. To our knowledge, data about the phylodistribution of drug resistance in *Mycoplasma hominis* are very scarce. The aims of this study were to assess the antimicrobial susceptibility of *Mycoplasma hominis* clinical strains in Tunisia, to identify the molecular basis of antibiotic resistance, and to investigate the phylogenetic relationships of resistant strains. This study included 65 molecularly typed *Mycoplasma hominis* clinical strains recovered from Tunisian patients over 18 years (2000–2018). The antimicrobial susceptibility was tested against nine antibacterial agents using the broth microdilution method. Minimum spanning tree was constructed to establish the phylogenetic relationships among resistant isolates. Fluoroquinolones, doxycycline, and josamycine were found to be the most effective antibacterial agents. However, 22 strains belonging to 11 expanded multilocus sequence types (eSTs) proved resistant to tetracycline. The majority of these eSTs were genetically related, indicative of clonal expansion of tetracycline resistance. The present study provides relevant information on the antibiotic susceptibility of Tunisian *M. hominis* clinical strains, lending support to a clonal transmission of tetracycline resistance. This is likely to have an important implication in monitoring the spread of drug resistance among *M. hominis*.

Keywords: *Mycoplasma hominis*, antibiotic resistance, tetracycline, clonal transmission, expanded multilocus sequence type

Introduction

Mycoplasma hominis has an etiological role in genitourinary tract infections (pelvic inflammatory disease, cervicitis, bacterial vaginosis) and appears to be associated with severe complications in pregnancy and serious infections in newborns and immunocompromised hosts.[1] Ample evidence from recent studies conducted in Tunisia indicates that *M. hominis* is one of the potential causative agents that play an important role in the pathogenesis of human infertility with a prevalence of 83%.[2] And only 17% were associated with gynecological infections.[2] However, *M. hominis* is less frequent among women suffering from complications during pregnancy (2,5%), in Tunisia.[3]

In comparison with *Ureaplasma* spp. and *Mycoplasma pneumoniae, Mycoplasma hominis* displays high levels of genetic and antigenic heterogeneity among clinical isolates as disclosed by several molecular typing methods,[2,4] which lends further support to the virulence of this species.

In recent years, due to the abuse of antibiotics, the resistance of *M. hominis* to antibiotics has been shown to have an increasing trend.[5] The prevalence of this

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pathogen and the extent of its antibiotic resistance profiles vary geographically and may be related to local antibiotic use regulations.[6] Thus, antimicrobial surveillance should be diligent and thorough for effective antimicrobial therapy, and to monitor the spread of resistant strains.

The present study aimed to evaluate the antibiotic susceptibility of *M. hominis* to various antimicrobial agents over 18 years, in Tunisia, and to identify the involved resistance markers. The association of antimicrobial susceptibility with genetic diversity was also assessed to highlight the evolution of antibiotic resistance in *M. hominis*.

Materials and Methods

Out of 1180 samples, sixty-five (5,5%) *M. hominis* clinical strains isolated from outpatients (48 non-pregnant women and 17 men) presenting to the diagnostic center of the Pasteur Institute of Tunis (Grand Tunis, Tunisia), from January 2000 to December 2018, were included in this study. As control, *M. hominis* strain PG21 (ATCC 23,114) has been considered. The patient group, whose ages ranged between 25 and 48 years, experienced symptoms of urogenital tract infections (10 cases) or infertility (55 cases).

From the 65 isolates, 59 were previously characterized using an Expanded MultiLocus Sequence Typing (eMLST) scheme, including ten genes (*uvrA*, *gyrB*, *ftsY*, *tuf*, *gap*, *p120'*, *vaa*, *lmp1*, *lmp3*, *p60*).[2] The remaining six isolates were genetically typed in this study by the same method. The sequences generated by eMLST were submitted to the *Mycoplasma hominis* MLST website (https://pubmlst.org/mhominis/) sited at the University of Oxford.[7]

Nine antimicrobial agents were tested for antibiotic susceptibility: tetracycline 'TET', doxycycline 'DOX', ofloxacin 'OFX', ciprofloxacin 'CIP', levofloxacin 'LVX', moxifloxacin 'MXF', erythromycin 'ERY', azithromycin 'AZM', and josamycin 'JOS' (Sigma-Aldrich, Germany). Antimicrobial susceptibility was determined using a broth microdilution method for *M. hominis* PG21 and clinical strains, as detailed previously.[8] The specific breakpoints (mg/liter) indicating susceptibility (S) or resistance (R) are: tetracycline S ≤4, R ≥8; doxycycline S≤4, R≥8; ciprofloxacin S≤1, R≥2; ofloxacin S≤1, R≥4; levofloxacin S ≤1, R ≥2; moxifloxacin S ≤0.25, R≥0.5; erythromycin S ≤1, R ≥4; azithromycin S≤0.125, R≥4; and josamycin S≤2, R≥8.[9,10]

To confirm the occurrence of tetracycline resistance among *M. hominis* isolates, *tet*(M) and Int-Tn genes were amplified from both tetracycline-susceptible and -resistant strains, as previously detailed.[11] Simultaneously, macrolide-resistant strains underwent PCR amplification of domain II and domain V of 23S rRNA, as well as the genes encoding ribosomal proteins L4 and L22.[12] Amplicons were then sequenced.[2] Sequences were analyzed using BioEdit and translated into amino acids. Amino acid substitutions in L4 and L22, as well as nucleotide mutations in domain II and domain V of 23S rRNA, were identified by comparison with the corresponding sequences of *Escherichia coli* ER1709 (GenBank accession number: CP030240.1).

A minimum spanning tree (MST) analysis based on eMLST data has been constructed, using BioNumerics software (version 7.0, Applied Maths), to illustrate the distribution of antimicrobial resistance among *M. hominis* clinical strains.[2]

Results and Discussion

Minimum inhibitory concentration (MIC) results for the 65 *M. hominis* clinical isolates are shown in Supplementary Table 1. All *M. hominis* isolates were sensitive to DOX, OFX, CIP, LVX, MXF, and JOS (MIC ≤ 1 mg/liter). The high sensitivity rate of *M. hominis* to fluoroquinolones is fairly consistent with that reported by Meygret et al in France,[13] but distinctly different from the studies carried out in China, which reported a high resistance rates to OFX (59%) and CIP (64%).[5] Interestingly, Tunisian genital mycoplasmas (*Ureaplasma* spp. and *M. hominis*) showed a difference in their resistance patterns to fluoroquinolones. Indeed, *Ureaplasma* spp. strains proved more resistant to CIP, OFX, and LVX (100%, 38.62%, 17.82%, respectively) than did *M. hominis* strains.[14]

Since *M. hominis* is known to be naturally resistant to 14- and 15-membered macrolides, all isolates were resistant to ERY (MIC ≥ 16 mg/liter) and AZM (MIC ≥ 4 mg/liter).[12] No mutations were found in either L4 or L22 ribosomal proteins; however, all resistant strains harbored a G2057A transition in their 23S rRNA sequences. This is in agreement with prior reports indicating that the G2057A transition accounts for the intrinsic resistance to macrolides in *M. hominis*.[12]

Regarding tetracycline susceptibility, 43 (66.15%) strains were sensitive. This rate is distinctly different from that of other countries, which reported a higher (>80%) sensitivity rate to TET.[15] We noticed through the studied period a decreased susceptibility to tetracycline, most likely as a consequence of its use as a first-line drug against *M. hominis* in Tunisia. Indeed, a previous study conducted in our laboratory in 2012 showed that 75% of *M. hominis* strains were sensitive to tetracycline.[11] Screening of the *tet*(M) determinant, which is associated with tetracycline resistance, as well as the Int-Tn gene, were carried out, and all tetracycline-resistant isolates were positive, while no tetracycline-sensitive isolates

harbored these genes. As mentioned previously, a unique sequence of the *tet*(M) amplicon was shared by the tetracycline-resistant strains, suggesting that they originally acquired the *tet*(M) determinant from a common source.[11]

As demonstrated previously, the eMLST method segregated *M. hominis* strains into two distinct genetic lineages, which were differentially associated with infertility (lineage A) or gynecological infections (lineage B).[2] Based on the MST, we found that tetracycline resistance was widespread in lineage A. Indeed, all *M. hominis* isolates from lineage B were sensitive to tetracycline, whereas 41.5% of isolates from lineage A were resistant. Interestingly, the MST showed that tetracycline resistance was linked to specific clones: eST3, eST6, eST14, eST17, eST20, eST21, eST23, eST24, eST25, and eST26 (Figure 1). The depiction of tetracycline resistance on the MST clearly showed its clonal expansion among the majority of clones (82%). However, the remaining tetracycline-resistant clones (eST14, eST17, eST21, eST24) were positioned at the edges of the MST, indicating a very distant genetic relatedness to the major resistant clones, which suggests the convergent acquisition of tetracycline resistance by these isolates.

Although horizontal transfer mediated by mobile elements was considered as the major process underlying the

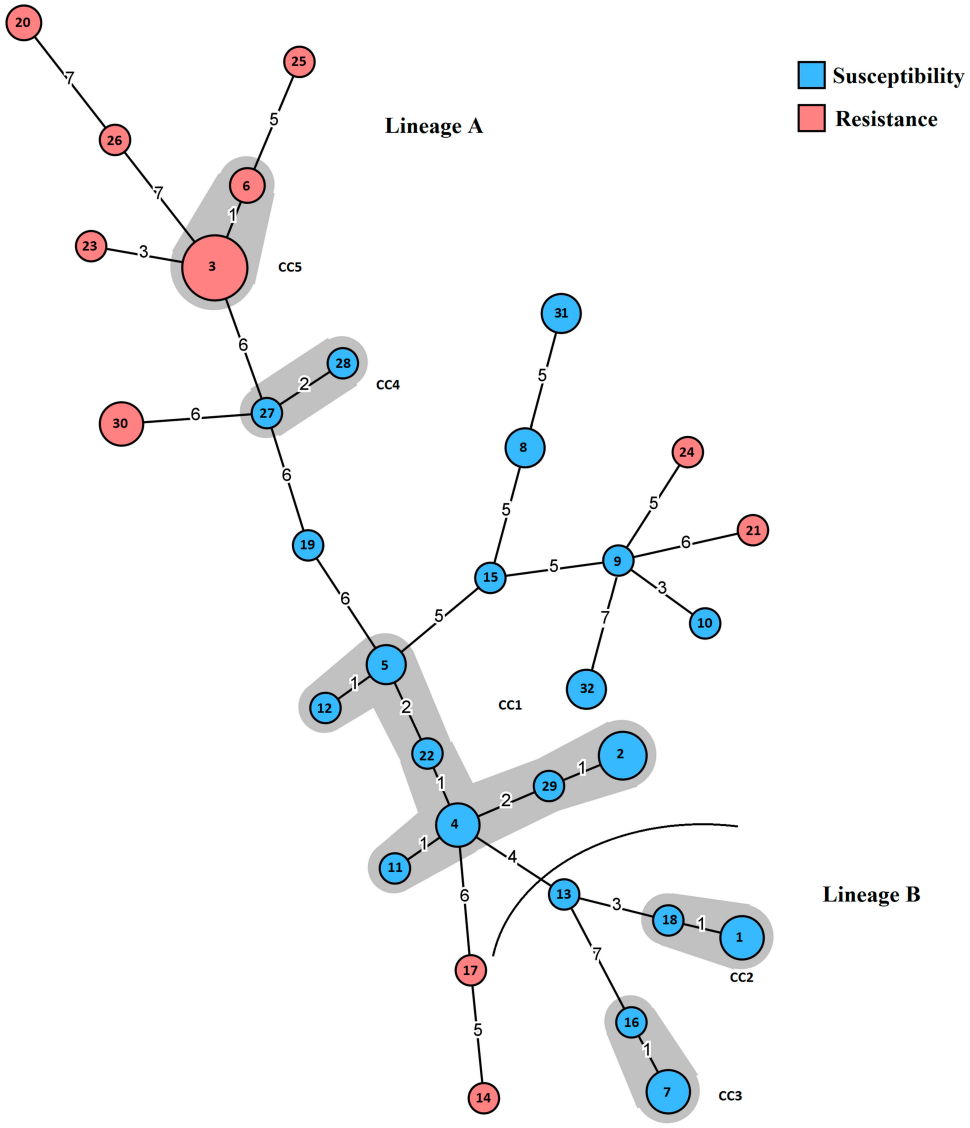

Figure 1 Minimum spanning tree analysis of the 65 *M. hominis* isolates based on eMLST data. Each circle corresponds to a distinct allelic profile, and the circle size corresponds to the number of isolates sharing the same profile. The circle was coded by assigning the color blue to sensitive-tetracycline eSTs and the color pink to resistant-tetracycline eSTs. The shaded zones between certain groups of circles indicate that these profiles belong to the same clonal complex (CC). Numerals connecting the circles indicate the number of allelic differences between the profiles.

worldwide dissemination of tetracycline resistance, our study indicates that clonal spread of tetracycline resistance might account significantly in particular settings. Moreover, the observation that tetracycline-resistant *M. hominis* strains shared a common *tet*(M) sequence type further support the clonal expansion of tetracycline resistance. However, it has been previously shown that this *tet* (M) sequence type was also harbored by *Ureaplasma parvum* tetracycline-resistant strains, indicating that it might be particularly competent to spread between these species.[11]

The clonal dissemination of tetracycline resistance observed here is in sharp disagreement with previous studies. Férandon et al have reported that 68 *M. hominis* isolates resistant to tetracycline were not related and grouped into 25 MLVA types (Multi Locus VNTR Analysis).[4] Similarly, a previous study from our group, based on p120′ gene fragment polymorphisms, found no genetic relationships among tetracycline-resistant *M. hominis* strains.[11] Hence, aside from segregating *M. hominis* strains into two genetically differentiable urogenital pathotypes, the eMLST scheme used herein proved to be a useful epidemiological tool to track the clonal transmission of tetracycline resistance in *M. hominis* strains. Similar results have been reported for other bacteria. For example, a combination of multilocus sequence typing scheme with molecular evolutionary analysis, such as the minimum spanning tree, provided reliable insight into the evolutionary pathways and the transmission of antibiotic resistance among *Neisseria gonorrhoeae* strains.[16]

Conclusion

In conclusion, the upward trend in the incidence of tetracycline-resistant *M. hominis* strains in Tunisia appears to be mainly due to clonal spread. The identification of resistant clones associated with tetracycline resistance could contribute to investigate novel genetic biomarkers for predicting drug resistance in *M. hominis* strains, which would have an impact on controlling resistance and disease monitoring.

However, this finding should prompt additional validation studies using larger and geographically diverse, strain collections. Hence, we recommend the use at first intention of fluoroquinolones, doxycycline and josamycin for treating *M. hominis* infections in Tunisian patients and break down the transmission of tetracycline resistance. Furthermore, the appropriate treatment of *M. hominis* may prove to be important in controlling infertility in Tunisia.

Data Sharing Statement

All data generated or analyzed during this study are included in this article and its additional file.

Ethical Approval

Neither human/animal subjects nor human cell lines/tissues were involved in this study. Only fully anonymized data were processed, and hence no ethical approval was required.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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