Drift of *tet*M Determinant in Urogenital Microbiocenosis Containing Mycoplasmas during Treatment with a Tetracycline Antibiotic

A. E. Taraskina, A. M. Savicheva, T. A. Akopian*, A. E. Soroka*, K. T. Momynaliev*, and V. M. Govorun*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 134, No. 7, pp. 69-72, July, 2002
Original article submitted June 6, 2001

We studied the correlation between genetic transfer of *tet*M determinant in Tn*916* conjugative transposon by urogenital mycoplasmas (*Mycoplasma hominis* and *Ureaplasma urealyticum*) and changes in the bacterial repertoire during treatment with a tetracycline antibiotic. Basic conditions favoring the nonspecific transfer of *tet*M determinant into mollicute cells are determined and the allele polymorphism of *tet*M determinant in clinical strains of *M. hominis* and *U. urealyticum* is evaluated. The structure of *tet*M gene in clinical mycoplasma and ureaplasma strains is characterized by a peculiar mosaic pattern and differs from all previously described alleles of this gene. The results suggest that tetracycline resistance in mollicutes is determined by mechanisms alternative to genetic transfer of *tet*M determinant.

Key Words: *Mycoplasma hominis; Ureaplasma urealyticum; antibiotic resistance; tet*M *determinant; allele polymorphism*

Tetracyclines are traditionally used as antibiotics of choice in the treatment of gynecological disorders, including bacterial vaginosis caused by mycoplasmas. They bind to bacterial ribosome 30S subunit and inhibit its binding to aminoacyl-tRNA [1,2,4].

Until present, mycoplasma resistance to tetracyclines was explained by transfer of *tet*M gene, the resistance determinant in Tn*916* conjugative transposon, from microbiocenosis neighbors into the mollicute genome. The product of *tet*M gene reacts with the ribosome 30S subunit and prevents tetracycline binding without preventing binding of aminoacyl-tRNA to the specific site of the ribosome [4,11, 13]. The *tet*M determinant is therefore considered to be the key gene, whose product ensures mycoplasma survival during tetracycline therapy.

Laboratory of Microbiology, D. O. Ott Institute of Obstetrics and Gynecology, Russian Academy of Medical Sciences, St. Petersburg; *Laboratory of Gene Engineering and Immunogenetics, Institute of Physicochemical Medicine, Ministry of Health of Russian Federation, Moscow

The aim of our study was to characterize the main parameters of *tet*M determinant transfer into *M. hominis* and *U. urealyticum* cells during symptomatic antibiotic therapy.

MATERIALS AND METHODS

Thirty nonpregnant women aged 18-46 years with nonspecific urogenital inflammations (26.6% with bacterial vaginosis and 73.3% with vaginitis) associated with *M. hominis* and/or *U. urealyticum* were observed. The disease was in many cases (about 70%) characterized by a torpid course and autoimmune complications; 10% of examined patients were previously treated for mycoplasma infection.

The patients received doxicyclin hydrochloride orally in a single dose of 0.2 g on day 1, then 0.1 g twice a day for 10 days in parallel with combined therapy described previously [1].

Vaginal discharge samples for studies of microbiocenosis (species composition, *tet*M gene content in

0007-4888/02/1341-060$27.00

the isolated microorganisms) were collected before the therapy, on days 5-6 of doxicyclin therapy (middle of the course), immediately after therapy (days 11-12), 2-3 weeks after antibiotic therapy, and then several times during the subsequent 2-6 months (eradication control).

M. hominis and *U. urealyticum* were isolated using liquid accumulation medium with arginine (or urea), agar medium with tetracycline (1 μg/ml) or doxicyclin (1 μg/ml), and control solid medium without antibiotics with appropriate selective additives for each type of mollicutes. Other representatives of the urogenital flora were isolated by inoculation in universal diagnostic media (5% blood agar, thioglycol broth, agar+ broth wort) and the isolated microflora was identified by routine methods [4].

The *tet*M fragment was synthesized using 5'-CGA GTACCAGCAGGAAAGCGTGATT-3' and 5'-GCC ACTTTTGGTCTTTGGTGTTTCA-3' primers, allowing amplification of a 660 b.p. site. The parameters of amplification were as follows: 95°C, 3 min (1 cycle), 95°C, 20 sec, 57°C, 20 sec, 72°C, 20 sec (30 cycles). In order to evaluate the approximate percentage of *tet*M dissemination among microorganisms of each species in the studied biocenosis, we measured the concentration of bacterial suspension whose DNA was used as the matrix in PCR. The content of DNA in the studied samples was evaluated by the fluorescence intensity of DNA fragments stained with ethidium bromide after gel electrophoresis.

Allele polymorphism of the *tet*M determinant was analyzed by PCR in 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 2.5 μM each dNTP (Promega), 0.5 μM each primer, 1 Unit *Taq* polymerase (Promega), and 50 ng *M. hominis* or *U. urealyticum* DNA. The amplification protocol in Gene Amp 2400 programmed thermostat (Perkin Elmer) was as follows: 94°C (5 sec), 50°C (10 sec), 72°C (15 sec), a total of 30 cycles. The product of amplification was identified in 2.5% agarose gel with ethidium bromide and purified using Wizard PCR Pres DNA Purification System (Promega).

The nucleotide sequence of *tet*M amplicons was determined as described previously [15] using fmol DNA Cycle Sequencing System (Promega). Three fragments constituting a full-sized gene copy, synthesized by PCR on *M. hominis* and *U. urealyticum* DNA using 6 primers selected in the conservative areas of gene alleles with 100% homology of nucleotide sequences, served as the matrices.

RESULTS

Before treatment *M. hominis* and/or *U. urealyticum* containing no *tet*M in their genomes and sensitive to tetracycline and doxicyclin (minimum inhibitory concentration less than 1 μg/ml *in vitro*) were detected in the vaginal discharge of all patients. Concomitant bacterial flora contained *tet*M in 50% cases.

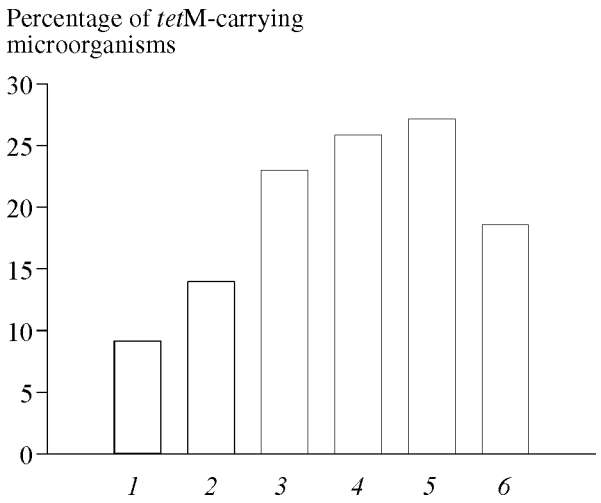

Fig. 1. Time course of changes in *tet*M determinant concentration in vaginal biocenosis before, during and after tetracycline treatment. *1*) before treatment; *2*, *3*) days 5 and 11 of antibiotic therapy; *4-6*) 2 weeks, 1-2 months, and 3-6 months after antibiotic therapy, respectively.

Tetracycline stimulates *tet*M transcription in Tn*916* conjugative transposon [9,11]. In our study the number of bacteria carrying *tet*M gene drastically increased as early as on day 5 of antibiotic therapy (Fig. 1). Gradual accumulation of *tet*M in the vaginal biocenosis persisted after the end of doxicyclin therapy. Long-term follow-up (up to 6 months) showed that the percentage of *tet*M-carrying clinical strains of bacteria remained high and then trended to decrease.

We characterized 13 variants of *tet*M detected in clinical strains of *M. hominis* and 5 variants in clinical strains of *U. urealyticum* resistant to tetracycline. The *tet*M determinant in clinical strains of mycoplasma and ureaplasma was characterized by a peculiar mosaicism and resembled none previously described allele of this gene (Fig. 2). The mosaic structure of *tet*M in 11 of the 13 studied tetracycline-resistant strains of *M. hominis* was completely identical, but belonged to a new allele type. One more new allele variant of *tet*M was detected in 2 *M. hominis* strains. Allele polymorphism of *tet*M genes in clinical strains of *M. hominis* is similar to that of *tet*M genes of *Gardnerella vaginalis* and pOZ101 plasmid of *Neisseria gonorrhoeae*. Comparison of *tet*M determinant sequences determined for 5 clinical strains of *U. urealyticum* showed a gene variant identical to *tet*M sequence previously described for ureaplasma in 3 cases and to an unknown allele variant in 2 cases (Fig. 2).

Eleven of 13 tetracycline-resistant *M. hominis* strains with new allele variants of *tet*M could get the

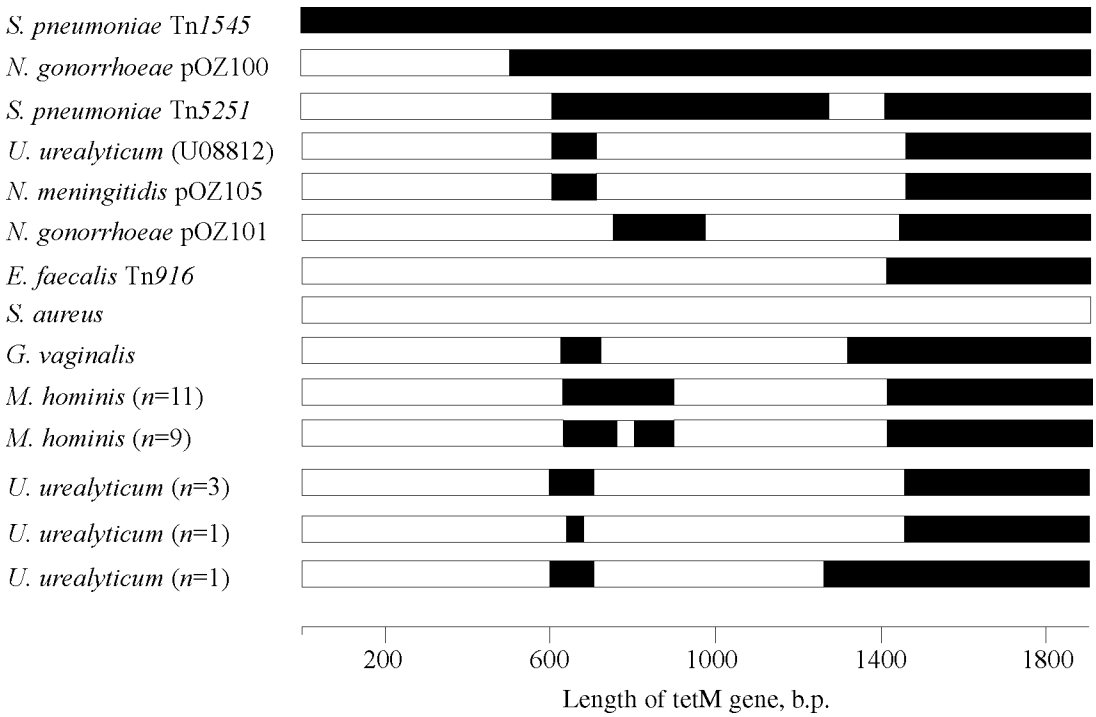

Fig. 2. Schematic presentation of mosaic structure of compared *tet*M genes. Two alleles with maximum divergence are colored black (*S. pneumoniae* Tn1545) and white (*S. aureus*).

resistance gene from the same host, while 2 other strains accepted it from another source. A similar conclusion can be drawn from the results of evaluation of *tet*M nucleotide polymorphism in *U. urealyticum*.

The acquisition of *tet*M determinant is not the only program of the formation of tetracycline resistance in mycoplasmas. Repeated manifestation of *M. hominis* and/or *U. urealyticum* after therapy was observed in 20 (66.7%) patients, but mycoplasmas acquired the *tet*M resistance genes in only 4 cases. The potential donors of *tet*M were lacto-, bifido-, and corinebacteria, *Enterococcus faecalis*, *Escherichia coli*, and different streptococci (α-hemolytic streptococcus, group B streptococcus). In other cases persistent forms of urogenital mycoplasmas in recurrent vaginosis developed without *tet*M determinant, that is, the appearance of resistant mycoplasmas in most cases was due to other changes in the microorganism under the effect of antibiotic.

It seems that mycoplasmas, particularly under conditions of their natural environment, possess additional mechanisms modulating their resistance to antibiotics, and these mechanisms are always realized, being obligatory during the formation of resistance, and often precede acquisition of *tet*M determinant. For example, in one patient with recurrent mycoplasma infection the agent had no *tet*M 4 months after the end of antibiotic therapy, but this gene reappeared 5.5 months after treatment. Hence, *tet*M was acquired during mycoplasma adaptation to this antibiotic and its effect on the survival of cells during tetracycline pressing was not decisive.

In other patient *tet*M-carrying *M. hominis* strain was isolated 2 weeks after the end of treatment. Eubiotic agents were prescribed instead of the 2nd course of antibiotic therapy. Vaginal flora was examined 3 months after this therapy. Though the patient's status was far from ideal (with *Candida* fungi predominating), no mycoplasma were isolated from the sample. This probably resulted from incomplete optimization of cell metabolism under novel conditions or this biotype was subsided by a more aggressive bacterium. This fact proves that even if *tet*M was acquired during adaptation to tetracycline treatment, but cell metabolism was not adapted to modified environmental conditions, this persistent mollicute form is destined to be eliminated by host immunological factors and symbiotic flora in a generally healthy patient.

Study of the regularities of *tet*M prevalence in the urogenital tract microbiocenosis containing mycoplasmas during tetracycline therapy showed that acquisition of *tet*M determinant in the conjugative transposon was only an intermediate stage in general adaptation of mollicutes to these antibiotics.

A high percentage (22.0-42.2) of *tet*M-containing microorganisms in the vaginal biocenosis provide conditions for *tet*M the transfer of determinant into mycoplasmas cells; dystrophic changes in the urogenital mucosa, desquamation of the epithelium also promote adhesion of microorganisms (specifically mycoplasmas).

The processes of the formation of tetracycline resistance in urogenital mollicutes under *in vivo* conditions during antibiotic therapy should be further studied on adequate experimental models.

REFERENCES

1. N. N. Zakharevich, L. N. Novikova, E. A. Mikhnina, and A. E. Taraskina, *Zh. Akush. Zhensk. Bolezn.*, No. 2, 30-34 (2001).
2. C. Bebear, B. de Barbeyrac, C. M. Bebear, *et al.*, *Wien Klin. Wochenschr.*, **109**, 594-599 (1997).
3. G. Christiansen, L. T. Jensen, T. Boesen, *et al.*, *Ibid.*, 557-561.
4. M. C. Cumming and W. M. McCormack, *J. Antimicrob. Chemother.*, **34**, No. 12, 2297-2299 (1990).
5. R. Huang, D. M. Gascoyne-Binzi, P. M. Hawkey, *et al.*, *Ibid.*, **40**, No. 4, 561-565 (1997).
6. *Manual of Clinical Microbiology*, Ed. A. Balou, Washington (1991).
7. K. B. Mullis and F. Faloora, *Methods Enzymol.*, **155**, 335-350 (1987).
8. M. R. Oggioni, C. G. Dowson, J. M. Smith, *et al.*, *Plasmid*, **35**, No. 3, 156-163 (1996).
9. C. Poyart, J. Celli, and P. Triew-Cuot, *J. Antimicrob. Chemother.*, **39**, No. 2, 500-506 (1995).
10. S. Razin, D. Yogev, and Y. Naot, *Microbiol. Mol. Biol. Rev.*, **62**, No. 4, 1094-1156 (1998).
11. L. B. Rice, *J. Antimicrob. Chemother.*, **42**, No. 8, 1871-1877 (1998).
12. M. C. Roberts and G. E. Kenny, *J. Bacteriol.*, **169**, No. 8, 3836-3839 (1987).
13. M. C. Roberts, *Antimicrob. Agents Chemother.*, **34**, No. 3, 476-478 (1990).
14. F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467 (1977).