GENETICS

Genetic Heterogeneity of *Mycoplasma hominis* Clinical Isolates Detected during Observation of Patients with Recurrent Urogenital Inflammation

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A rapid reproducible effective method for molecular typing of *Mycoplasma hominis* strains based on random amplified polymorphic DNA (RAPD) technique was developed. RAPD detected genetic heterogeneity of genomes of *Mycoplasma hominis* clinical isolates and showed changes in the genomes of *Mycoplasma hominis* clinical isolates from patients with chronic infection.

Key Words: *Mycoplasma hominis; RAPD; genetic heterogeneity*

Mycoplasma (Mollicutes) are the most primitive prokaryote microorganisms with genomes of 540-2200 thousand base pairs (b.p.) able to divide on artificial nutrient media. Usually Mycoplasma infections are routinely treated with tetracyclines. Tetracycline resistance of mycoplasma in most cases is associated with the presence of *tet*(M) determinant in conjugative transposons, for instance, Tn916. However, the absence of *tet*(M) gene in urogenital mycoplasma does not guarantee positive effect of antibiotic therapy. *Mycoplasma hominis* is characterized by high genetic heterogeneity, which manifests by point nucleotide and amino acid substitutions and genome mutations leading to essential inter-strain variations in genome size within the same species. The following methods are now used for differentiation of clinical isolates of mycoplasma: restriction analysis, reverse field electrophoresis, restriction fragment length polymorphism (RFLP) analysis, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), and insertion sequence typing. These analyses take long time, require much biological material, and their results are difficult to interpret. Therefore, a rapid easily reproducible method is needed, which can effectively type mycoplasma and detect genome changes during therapy.

Here we investigated changeability of *M. hominis* genome during its long-term persistence in humans. Our aim was to develop a rapid easily reproducible effective method for detection of intraspecies heterogeneity of *M. hominis* isolates from patients and to detect genome rearrangements during persistence of mycoplasma and during antibiotic treatment under clinical conditions and during laboratory culturing.

MATERIALS AND METHODS

Clinical strains of *M. hominis* were isolated from patients with nonspecific urogenital inflammations and laboratory strain H-34 was a gift from Professor I. V. Rakovskaya (N. F. Gamaleya Institute of Epidemio-

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logy and Microbiology, Moscow). The strains were cultured as described previously [1].

DNA was isolated from *M. hominis* and *U. urealyticum* cell cultures as described previously [1].

RAPD was carried out in a reaction mixture containing: 66 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.1% Tween-20, 200 μmol each dNTP, 10 pmol MC7 primer, 2 U *Taq* polymerase (Fermentas), and 50 ng *M. hominis* DNA. The reaction was carried out in a GeneAmp 2400 programmed thermostat (Perkin Elmer): 10 sec at 93°C, 45 sec at 36°C, and 90 sec at 72°C, a total of 35 cycles.

The primers used in the study are listed in Table 1.

The reaction products were separated and identified in 1.5% agarose gel. The results were documented using a Gel Doc 1000 video system and processed using Molecular Analyst Software (Bio-Rad).

Restriction was carried out as specified in the manufacturer's instructions on a CHEF-DR III device (Bio-Rad), PFGE was carried out on the same device. Agarose gel was stained with ethidium bromide. The conditions of PFGE were as follows: 1% agarose (Bio-Rad), 0.5-fold Tris-borate buffer (47.5 mM Tris-HCl, 47.5 mM boric acid, 1 mM EDTA, pH 8.3), 14°C, pulse duration 1-10 sec, PFGE duration 11 h, angle 120°, voltage 6 V/cm.

At the second stage of the study we investigated clinical isolates of *M. hominis* from patients with chronic or recurrent Mycoplasma infection. Thirty women aged 18-46 years with nonspecific urogenital inflammations were examined. Urogenital microflora included *M. hominis* in all cases. The patients were treated with doxycycline for 10 days. For quantitative and qualitative evaluation of changes in microbiocenosis under the effect of antibiotic therapy, vaginal smears were taken before, on days 5-6 of doxycycline therapy (middle of the course), immediately after the course (days 11-12), 2-3 weeks after treatment, and then during 2-6-month follow-up. Mycoplasma were inoculated into liquid arginine-containing growth medium, agarized medium with tetracycline (1 μ/ml), agarized medium with doxycycline (1 μg/ml), and control medium without antibiotics. Clinical isolates cloned in agarized nutrient medium were reinoculated in liquid nutrient medium, DNA was isolated, and RAPD typing was carried out.

RESULTS

Mycoplasma genome is characterized by low content of G+C pairs, and therefore AT-enriched primers should be used for *M. hominis* DNA amplification. Amplification with so-called universal primers, *i.e.* oligonucleotides consisting of 10 nucleotides most often used in RAPD analysis [6], failed and we selected original smaller primers, enabling less specific amplification. Four nucleotides were attached to the 5' terminal of these eight six-nucleotide primers for increasing the annealing temperature (Table 1).

Only primer MC7 was chosen for further analyses (Fig. 1), because amplification of this primer yielded reproducible results. RAPD was carried out with DNA from *M. hominis* H34 laboratory strain. The results were reproducible with different concentrations of DNA (0.2-10 ng/sample). Only the yield of synthesized fragments, but not the amplification profile changed.

Many factors are essential for RAPD-PCR: DNA concentration, temperature of annealing, concentrations of Mg^{2+} and other salts, quality of *Taq* polymerase, length and nucleotide sequence of the primer. RAPD was never before used for *M. hominis* typing, and therefore we first choose optimal conditions for this analysis. The number of amplification cycles, primer concentrations in the amplification mixture, DNA and *Taq* polymerase volumes were changed until optimal conditions for RAPD-PCR were selected (see Materials and Methods). The length of the amplified fragments varied from 300 to 4000 b.p.

Genotyping of clinical strains of *M. hominis* by RAPD showed their high heterogeneity (Fig. 2, *a*).

In order to confirm the differences between clinical isolates of *M. hominis* detected by RAPD, DNA restricted by *Bam*HI was analyzed by PFGE in parallel genotyping experiments. These experiments con-

TABLE 1. Structure of Primers for RAPD

Primer	Nucleotide sequence (5'-3')
MYC1	AATTAGAAAA
MYC2	AAAAGAAGTT
MYC3	AATTAGAAAC
MYC4	TAAAACAAGC
MYC5	TGACAAAGAT
MYC6	GAATTAAAAC
MYC7	TTGTTTGTTC
MYC8	TATTTCTATT
MYC9	TTTTTAATCT
MYC10	TGATTTTTTA
MYC11	TTAATTTTGT
MC1	CATGAAAGAT
MC2	CATGAAACAA
MC3	CATGATTAGA
MC4	CATGAAAAAT
MC5	CATGAAATTA
MC6	CATGTTTCAA
MC7	CATGAGCAAA
MC8	CATGGAATTA

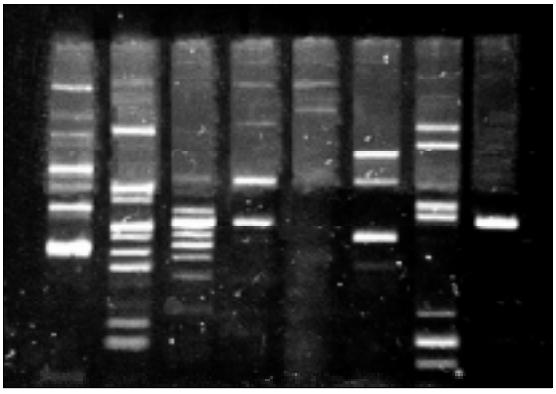

Fig. 1. RAPD-PCR. Selection of 10-nucleotide primer.

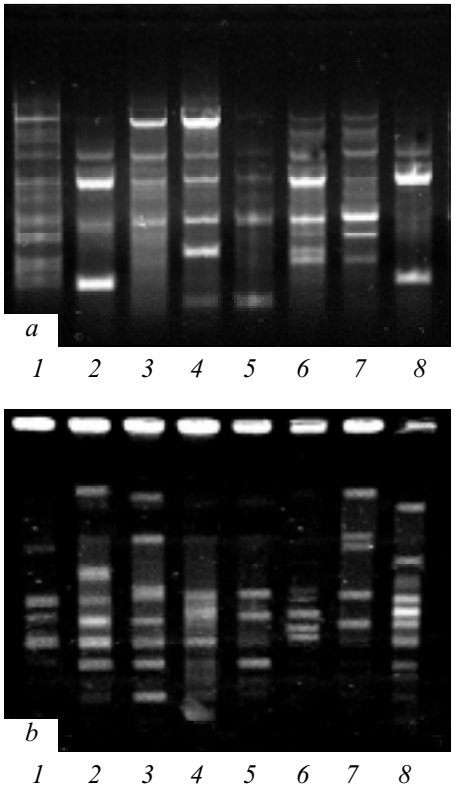

Fig. 2. RAPD-PCR of clinical isolates of *M. hominis* (*a*) and pulsed-field gel electrophoresis of *Bam*HI-restricted DNA from *M. hominis* clinical isolate (*b*). Numbers of tracks correspond to patients' numbers.

firmed the differences in genomes of different clinical isolates (Fig. 2, *b*).

These findings suggest that RAPD is a promising method for identification of mycoplasma isolates both at the species and strain levels. The method is reproducible, simple, and rapid, and therefore can be recommended for evaluation of variability of natural mycoplasma populations and genotyping of mycoplasmata and detection of superinfection or relapses in clinical practice.

In 9 women (36%) *M. hominis* was repeatedly detected at various terms (2 weeks-5 months) after antibiotic therapy. Clinical strains isolated before and after treatment were compared by RAPD analysis. In 2 of 9 cases the profiles of amplification products did not change, in the rest cases RAPD pictures before and after treatment were quite different. Determinant *tet*M appeared after treatment in only 2 clinical isolates. The mycoplasma clones survived after antibiotic treatment contained no *tet*M, and one isolate lost it after additional cifran therapy. The detected genetic polymorphism of *M. hominis* clinical isolates can be due to deletion-insertion of *tet*M determinants and to genome rearrangements during mycoplasma persistence.

The phenomenon of genetic heterogeneity of mycoplasma isolates from various sources is well known. However, induced or spontaneous genetic rearrangements in the mycoplasma population during their *in vivo* persistence remain poorly studied. In the present study we describe the formation of genetic heterogeneity of clinical isolates during chronic Mycoplasma infection, which is a marker of developing Mycoplasma tolerance to organism defense and antibiotics used in the treatment of Mycoplasma infection.

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