Identification of *Mycoplasma hominis, Ureaplasma urealyticum,* and *Chlamydia trachomatis* by the Polymerase Chain Reaction

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A procedure for identifying chlamydia, mycoplasmas, and ureaplasmas by the polymerase chain reaction is described which uses a system of four primers complementary to fragments of the 16S RNA gene, one of which detects all three microorganisms and the other three are each specific for a particular organism. With this primer system, chlamydia, mycoplasmas, and ureaplasmas are detectable in a single reaction. The procedure may be used for routine diagnoses in diagnostic laboratories.

Key Words: *polymerase chain reaction; Mycoplasma hominis; Ureaplasma urealyticum; Chlamydia trachomatis*

It is now generally recognized that chlamydial, mycoplasmal, and ureaplasmal infections are implicated in the pathogenesis of many human and animal diseases. Their epidemiological significance is increasing with every passing year. Some members of the *Chlamydia* genus, notably certain strains of *C. trachomatis* and *C. pneumoniae*, as well as mycoplasmas are capable of inducing acute and chronic forms of respiratory disease, urethritis, pyelonephritis, arthritis, and perinatal infections [1,2,7,11]. Since microbiological methods of determining these organisms are difficult to apply, expensive, and poorly reproducible, reliable, highly sensitive diagnostic techniques need to be developed and introduced into practice for the detection of carrier states and the timely institution of appropriate antibacterial therapy.

Today, one promising approach involves the use of the polymerase chain reaction (PCR) technique in its various modifications [8]. Its success in detecting *Mycoplasma pneumoniae, M. fermentans,* and *Ureaplasma urealyticum* has already been reported [3,4,13].

The present investigation was undertaken to define a system of primers suitable for the detection and identification of such difficult-to-culture organisms as *C. trachomatis, M. hominis, and U. urealyticum* in clinical samples using PCR.

MATERIALS AND METHODS

DNA preparations used in the study are listed in Table 1. The DNAs were isolated as previously described [13].

DNAs from clinical specimens, which represented scrapings from cervical or urethral epithelium, were isolated by two methods - either as described by Wenzel and Hermann [13] or by our modification of the more frequently used method of Saito *et al.* [9].

In this modification, 300 μl of a solution containing 6 M guanidine thiocyanate (Sigma), 50 mM EDTA (pH 8.0), and 5 μl of an aqueous suspension of the macroporous MPS-1000V-GKh glass (Russia) or of calcined diatomaceous earth (Sigma) were added to each 1.5-ml test tube containing the material

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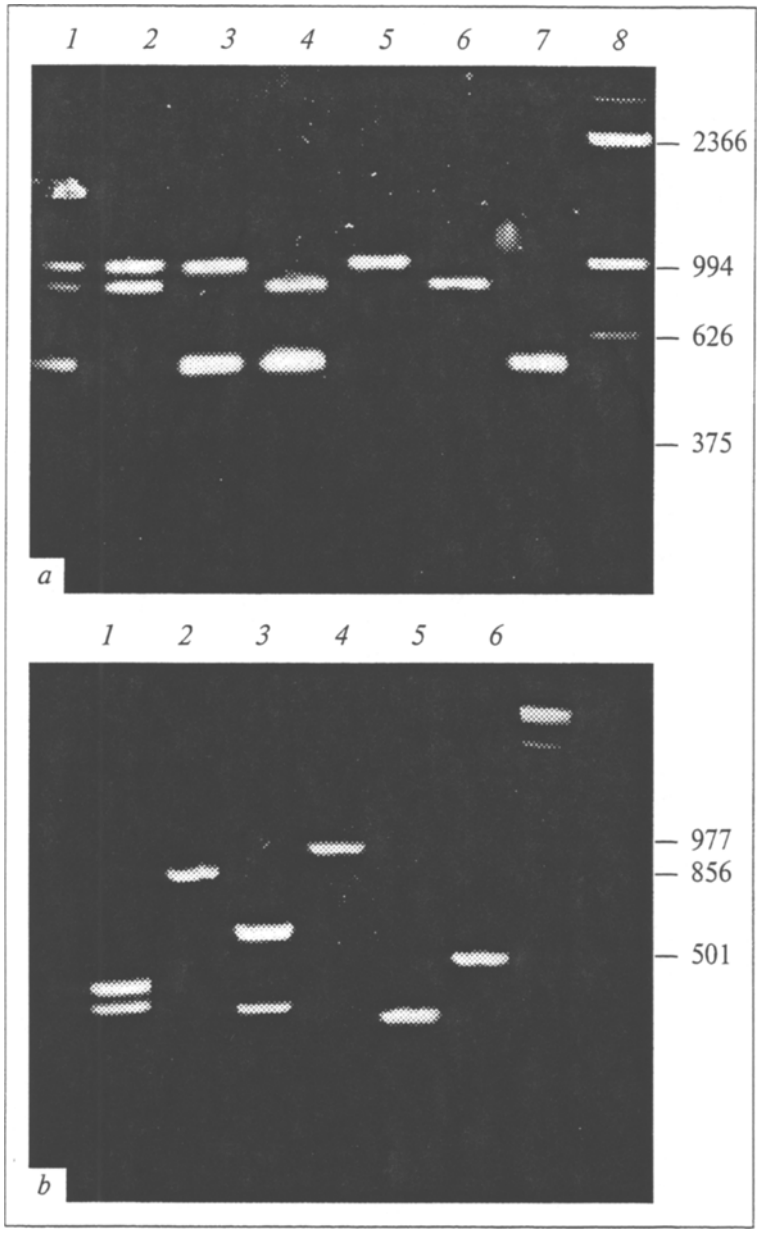

Fig. 1. Electrophoretic analysis of *C. trachomatis, M. hominis, and U. urealyticum* amplification products. *a*) amplification of control *C. trachomatis, M. hominis, and U. urealyticum* DNAs and their combinations in the R_1, R_2, R_3, R_4 primer system: *1*) *C. trachomatis*+*M. hominis*+*U. urealyticum*; *2*) *M. hominis*+*U. urealyticum*; *3*) *C. trachomatis*+*M. hominis*; *4*) *C. trachomatis*+*U. urealyticum*; *5*) *M. hominis*; *6*) *U. urealyticum*; *7*) *C. trachomatis*; *8*) PvuI–EcoRI–BamHI–StyI pBR322 DNA. *b*) cleavage of DNA amplification products by restriction endonucleases: *1*) *U. urealyticum* product by XbaI; *2*) unrestricted fragment; *3*) *M. hominis* product by EcoRI; *4*) unrestricted fragment; *5*) *C. trachomatis* product by ApaI; *6*) unrestricted fragment.

of interest in 100 μl of 0.25 M NaCl solution. The samples were each incubated for 10-15 min at room temperature with periodic agitation and then centrifuged in a microcentrifuge at 8000-12,000 rpm for 15-30 sec. The supernatant was discarded, 100 μl of a solution containing 4 M guanidine thiocyanate was added to the pellet, and the sample was shaken. The glass was sedimented by centrifugation for 15 sec and the supernatant discarded.

The washing procedure was then repeated three times using 700 μl of a water-alcohol solution containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.3), and 50% (v/v) ethanol. The samples were dried off for 5 min at 45-55°C without capping of the test tubes.

Finally, 50 μl of deionized water were added, and the tubes were agitated, incubated for 5 min at 45-55 °C, and centrifuged for 15 sec. The aqueous phase was collected and used as the test DNA sample for PCR.

DNA amplification was carried out in a volume of 50 μl. The concentration of each primer was 0.2 μM, that of deoxynucleotide triphosphates (Promega) 200 μM, and that of Taq-polymerase (Silex-M) 2.5 units per reaction. PCR was carried out in a programmable incubator "BIS" (*Vektor* Research and Manufacturing Company, Russia) set to the following parameters: 40 sec at 94°C for DNA denaturation, 30 sec at 58°C for annealing the primers, and 30 sec at 72°C for DNA synthesis; 35 amplification cycles.

Reaction products were separated by electrophoresis in 1.5% agarose gel at a field intensity of 10 V/cm [10]. The results were analyzed on an ultraviolet tran-

silluminator after the gels were stained with ethidium bromide (0.5 μ/ml).

RESULTS

We used a system of oligonucleotide primers selected during a comparison of mycoplasmal and chlamydial 16S RNA gene sequences published by the time of our study. Computer processing of the sequence data using an Oligo software package (Version 3.4) led us to choose the following four primers: 5'-CACGAGCT GACGACAACCATGCA-3' (R_1, "universal" primer); 5'-CGTTTGCGACGCTTTTGGATG-3' (R_2, primer for detecting *U. urealyticum*); 5'-GGTTAGCAATAA CCTAGCGGCGA-3' (R_3, primer for detecting *M. hominis*); and 5'-AAAGGGCGTGTAGGCGGAAAG-3' (R_4, primer for detecting *C. trachomatis*).

The amplification of specific regions in the 16S RNA gene of these three organisms resulted in the synthesis of fragments composed of 501 base pairs (bp) for chlamydia, 856 bp for ureaplasmas, and 977 bp for mycoplasmas (Fig. 1, *a*). PCR specificity was checked by treating the fragments with the following restriction endonucleases: the *M. hominis* 16S RNA gene fragment, with EcoRI; the *U. urealyticum* 16S RNA gene fragment, with XbaI; and the *C. trachomatis* 16S RNA gene fragment, with ApaI (Fig. 1, *b*). In addition, tests were run using DNAs isolated from other organisms by the method of Wenzel and Herrmann [13] (Table 1).

When serial dilutions of DNA from the microorganisms under study were amplified, the sensitivity of the system was found to depend on the combination of primers used in the amplification reaction. Thus, the use of R_1, R_2, and R_3 together in one reaction mixture resulted in the detection of *U. urealyticum* and *M. hominis* DNAs in an amount of 100 fg each per sample even at a 1:100 ratio of the DNA standards, but the addition of R_4 to the reaction medium lowered the detection limit to 0.5-1.0 pg. With the R_1+R_4 pair, the *C. trachomatis* DNA was detected in amounts of ≥250 fg per sample. These tests showed that 50-100 mycoplasmal and ureaplasmal cells can be detected with the combination of R_1, R_2, and R_3 and 200-300 chlamydial cells with the R_1+R_4 pair.

In testing the performance of our system of oligonucleotide primers on clinical specimens (scrapings from cervical or urethral epithelium), the main problem was to find a simple, reproducible method for specimen preparation. In serial analyses, we at first employed two previously described methods of DNA isolation from clinical specimens for amplification. One of these methods involves their treatment with proteinase K followed by precipitation of the DNA with ethanol, while the other calls for boiling of the specimens; both methods, however, have serious shortcomings [5,6,12].

TABLE 1. Results of Amplifying Genomic DNAs from Various Organisms Using the R_1, R_2, R_3, R_4 Primer System

Organism	Amount of DNA taken, ng	Result
Homo sapiens	200	-
Toxoplasma gondii	10	-
Escherichia coli	100	-
Salmonella	10	-
A. proteus	10	-
Yersinia pestis	1	-
Phage λ DNA	100	-
Herpes simplex virus I	1	-
Herpes simplex virus II	1	-
Cytomegalovirus	1	-
Mycoplasma pneumoniae	1	-
M. gallisepticum	1	-
Acholeplasma laidlawii	1	-

Note. When 100 ng of *M. pneumoniae* or *A. laidlawii* DNA were used, the fragments identified after amplification differed in electrophoretic mobility.

In the modification of Saito's method which we ended up using, DNA is extracted from mycoplasmal, ureaplasmal, or chlamydial cells with guanidine, after which the liberated nucleic acids are bound to particles of the macroporous glass. With this procedure, samples can be prepared for amplification under optimal conditions and the number of manipulations with each sample can be minimized; moreover, all manipulations can be carried out in a single test tube. The DNA yield in tests using the macroporous glass ranged between 70 and 80%, as indicated by the count of colony-forming units for *Acholeplasma laidlawii* cells (those of the *A. laidlawii* strain we used as the standard) - both in the case of a pure culture and after these cells were added to a clinical specimen free of the infectious agents under study.

In summary, our system of primers for the identification of *M. hominis*, *U. urealyticum*, and *C. trichomatis* can detect the DNAs of all three species in a single reaction with a sensitivity that in the case of three primers (R_1, R_2, and R_3) is not inferior to that achieved using a pair of primers to identify only one species. Although the addition of R_4 to the system reduces the sensitivity by one order of magnitude to 1 pg of chromosomal DNA for all three organisms, this level of sensitivity is sufficient for most practical purposes in a diagnostic laboratory. The system described is specific and can be used in diagnostic pro-

cedures to detect chlamydia, mycoplasmas, and ureaplasmas in a clinical setting.

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