NEW BIOMEDICAL TECHNOLOGIES

Diagnosis of Urogenital Chlamydiasis by Dot Hybridization with Biotin-Labeled DNA Probe

A. V. Zhdanov, A. V. Kvasov, O. V. Burmenskaya, I. V. Pis'menskaya, L. Z. Faizullin, and G. T. Sukhikh

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 122, No. 9, pp. 329-333, September, 1996
Original article submitted January 19, 1996

The DNA fragment of *C. trachomatis* cryptic plasmid (1115 bp long) is cloned into pBR322 vector. The recombinant plasmid pCTp1 binds to purified chlamydial DNA and DNA isolated from epithelial cells of patients with clinical manifestations of chlamydiasis and does did not bind to DNA of *M. hominis*, *U. urealyticum*, *E. coli*, herpes simplex virus, and human DNA. The probe sensitivity is 64.5%, its specificity is 84.9%, and the specificities for positive and negative results are 79.2 and 85.7%, respectively, compared with the polymerase chain reaction. These parameters are 61,6, 81.4, 78.2, and 85.6%, respectively, compared with the immunofluorescent method.

Key Words: *biotin-labeled DNA probe; dot hybridization; C. trachomatis; diagnosis of chlamydiasis*

Chlamydia trachomatis is a widespread sexually-transmitted pathogenic microorganism. Chlamydial infection may take an asymptomatic, chronic, or acute course with numerous clinical manifestations involving the reproductive system, eyes, kidneys, liver, lymph vessels, and joints [1,2,9,10,15]. Chlamydia can persist for many years in the body causing no disease and exhibiting no antigenic activity. These specific features of chlamydial infection hamper its clinical and serologic diagnosis.

Diagnostic methods based on specificity of genetic material of a causative agent (including DNA hybridization) have found wide application in recent years. After the sensitivity and specificity of the method proposed for detection of chlamydia in 1984 [16] had been markedly improved, it is widely used in the diagnostics of chlamydial infections [8,11,19].

Research Center for Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences, Moscow

This paper describes the cloning of a DNA fragment of cryptic plasmid of *C. trachomatis* and the use of this fragment as a DNA probe for the diagnosis of chlamydial infection in clinical material from men and women with genital infections.

MATERIALS AND METHODS

Epithelial cells from the urethra (males) and cervical canal (females) were used in the study. Cells were collected with a Medscand or Abbott devices. Processing of clinical material, DNA isolation, and dot hybridization were carried out as described previously [3]. The cells were washed with normal saline and lysed with 1% SDS and 100 μg/ml proteinase K. DNA was precipitated with 2 volumes of 96% ethanol, dissolved in 10 volumes of SSC buffer (1.5 M NaCl and 0.15 M sodium citrate), degraded by boiling, and transferred onto a nitrocellulose filter. Hybridization with biotin-labeled probe was carried out in a 50% aqueous solution of formamide for 14-16 h at

0007-4888/96/0009-0939$15.00

TABLE 1. Nucleotide Sequence of Primers and Amplified Fragments of *C. trachomatis*

Primers	Amplified fragment
U1 5'-CACGAGCTGACGACAACCATGCA-3'	Fragment of gene coding for 16S RNA, 501 bp
L1 5'-AAAGGGCGTGTAGGCGGAAAG-3'	
U2 5'-ACAGCGGTCAAATAGAGCA-3'	Fragment of cryptic plasmid (1116 bp, A1 amplificate)
L2 5'-GAATCCAGAAATTCAATGCGT-3'	
U3 5'-GATCGGTTTTCTCTTCGGTA-3'	Fragment of cryptic plasmid (507 bp, A2 amplificate)
L3 5'-TCCATCGAGTTCTAGTTGCC-3'	

42°C. The filters were then treated with streptavidin conjugated to alkaline phosphatase. Spots stained much more intensely than the negative control spots were regarded as positive hybridization.

The amplification reaction and preparation of clinical specimens for it were performed as previously [4]. Cells were lysed with 6 M guanidine thiocyanate, DNA was adsorbed on microporous glass, eluted after several washings, and analyzed by the polymerase chain reaction (PCR). The reaction (30 cycles, volume 25 μl) was carried out in a BIS programmed thermostat (Vektor, Novosibirsk). Primers are listed in Table 1.

Samples for immunofluorescent detection of chlamydia were prepared according to the manufacturer's instructions using ChlamonoScreen and Chlamyscan kits (Moscow) and a Leica fluorescent microscope.

Latex diagnosticum was obtained from Rokhat company (Moscow). A smear of epithelial cells on a glass slide was dried, treated for 10 min with 1% nonimmune rabbit serum, and incubated for 5 min in a shaker with latex particles activated with anti-chlamydia monoclonal antibodies. The formation of white flaky precipitate indicated a positive reaction.

The Biocard Chlamydia diagnostic kit (Dialat, Moscow) is based on a chromogenic enzyme reaction. The material from the cervical canal or urethra was processed according to the manufacturer's instruction and incubated with a chromogen solution for 2-3 min. Crimson colored samples were regarded as positive.

The specificity and sensitivity of the probe was tested using human DNA, DNA *Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *E. coli*, type 2 herpes simplex virus (HSV-2), cytomegalovirus, and clinical samples. Biotinated A1 amplificate was used as an alternative probe in parallel with the recombinant plasmid. Nonspecific binding with analyzed DNA was evaluated in hybridization with biotinated pBR322 plasmid on a control filter. Filter treated as the test one with omission of hybridization served as the control of the specificity of the conjugate-sample binding.

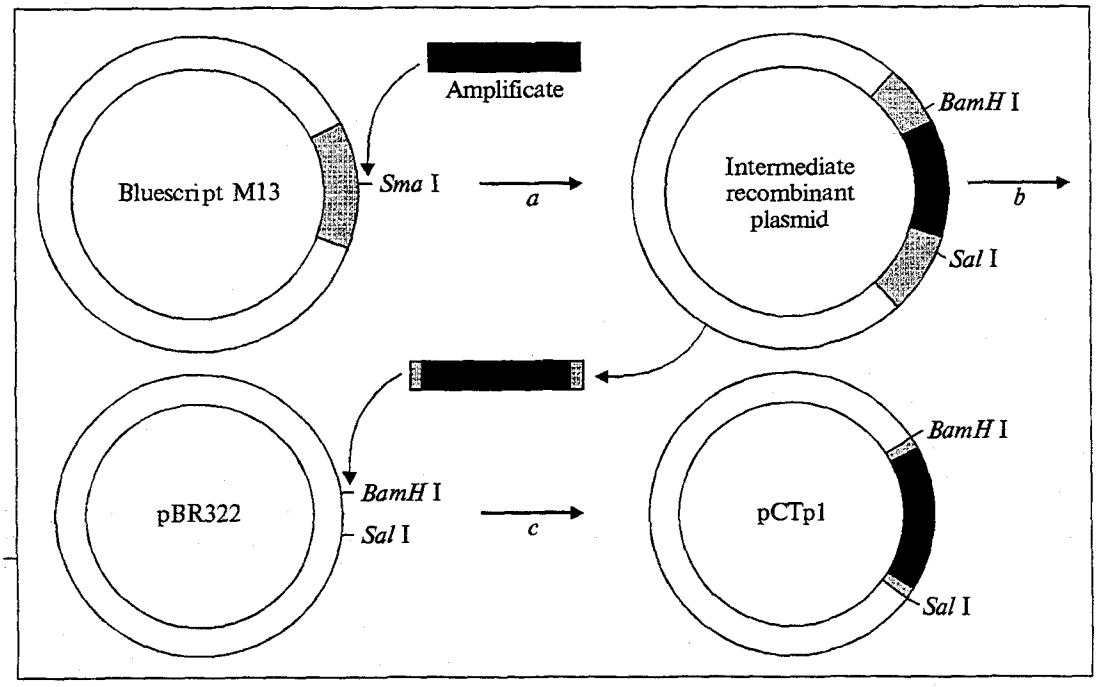

Fig. 1. Scheme of construction of pCTp1 recombinant plasmid. *a*) Cleavage of Bluescript M13 plasmid with *Sma* I restrictase and insertion of amplificate in plasmid vector by blunt end; *b*) cleavage of intermediate recombinant plasmid with *BamH* I and *Sal* I restrictases; *c*) cleavage of pBR322 plasmid *BamH* I and *Sal* I restrictase and grafting of the amplificate by cohesive ends.

RESULTS

The possibility of repeatedly copying the cryptic plasmid pCHL1 and the highly conservative nucleotide sequence of serologic variants of *C. trachomatis* make this plasmid a convenient target for the hybridization test and development of a DNA probe for the diagnosis of chlamydial infections [7,13].

After analyzing the known sequences, pCHL1 fragment of cryptic plasmid (1115 bp long, from 2751 to 3865 bp) was chosen as the hybridization probe and amplified by PCR with U2 and L2 primers.

A1 amplificate was purified from agarose gel by adsorption on NA-45 DEAE cellulose membrane, eluted, precipitated with ethanol [18], and inserted in a Bluescript M13 vector plasmid treated with *Sma* I restrictase. After cloning the recombinant plasmid, the insertion containing chlamydial DNA was cut out with *BamH* I and *Sal* I restrictases and inserted into pBR322 vector plasmid [17]. Figure 1 is a schematic drawing of the cloning procedure. The resultant recombinant plasmid pCTp1 was conjugated with biotin and used as a DNA probe for detecting *C. trachomatis.*

Restriction fragments of recombinant plasmid pCTp1 are shown in Fig. 2. Treatment by *BamH* I or *Sal* I restrictase leads to the formation of a linear fragment (tracks *2* and *3*) approximately 5200 bp long. Treatment by both restrictases yields fragments about 1200 and 4000 bp long, which corresponds to the size of the insertion and the vector (track *1*).

Analysis of specificity and sensitivity of the recombinant plasmid showed that it binds to both chlamydial DNA (sensitivity threshold 0.8 pg in a spot) and to A1 and A2 amplificates (0.16 pg) (Fig. 3, *a*). The plasmid did not bind to DNA of *M. hominis* and *U. urealyticum* (1.0 ng per spot), *E. coli* and HSV-2 (10 ng per spot), and human DNA (more than 1.0 μg per spot). Biotin-labeled amplificate A1 detected up to 1 pg of chlamydial DNA per spot (Fig. 3, *b*). Plasmid pBR322 did not bind to chlamydial or other negative control DNA (Fig. 3, *c*).

We compared the sensitivity and specificity of the DNA probe with other diagnostic methods: PCR by chromosomal and plasmid DNA, immunofluorescent Chlamyscan and ChlamonoScreen kits (Moscow), Rokhat latex diagnosticum (Moscow), and Biocard Chlamydia enzyme immunoassay kit (Table 2). The study was carried out using clinical material from men (smears from the urethra) and women (smears from the cervical canal) with urogenital disorders. The specificity and sensitivity of these methods were evaluated relative to PCR U1-L1, since it was reported to be not inferior to the culture method and is the most effective tool in the diagnostics of chlamydial infections [12,21].

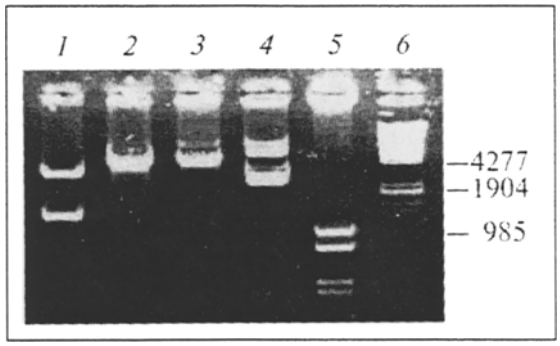

Fig. 2. Restriction fragments of pCTp1 recombinant plasmid. *1*) cleavage with *BamH* I and *Sal* I; *2*) cleavage with *BamH* I; *3*) cleavage with *Sal* I; *4*) native circular supercoiled plasmid; *5*) pTZ *Sau*3A molecular mass marker; *6*) λ *EcoR* I/*Hind III* molecular mass marker.

As expected, PCR most effectively detected chlamydia in clinical material: 32.3% with U1-L1 primers amplifying chromosomal DNA in the region of gene for 16S rRNA and 29.0% with U3-L3 primers amplifying a 500-nucleotide fragment of plasmid DNA. Positive results were obtained in 25% of cases by hybridization, 21.1% by immunofluorescence, 26.0% by a Rokhat kit, and 27.9% by a Biocard test system. The sensitivity and specificity of the DNA probe were 64.5 and 84.9%, PCR U3-L3 89.1 and 96.0%, immunofluorescence 61.6 and 81.4%, latex test 61.3 and 81.0%, and Biocard Chlamydia 73.7 and 82.1%, respectively.

The results of hybridization analysis coincided with PCR U3-L3 in 88.0% cases, PCR U1-L1 in 84.9%, immunofluorescence in 86.5%, latex test in 79.0%, and enzyme immunoassay in 80.0% cases. As expected, analysis based on PCR U3-L3, which was developed similarly to pCTp1 recombinant plasmid using pCHL1 cryptic plasmid, gives better coincidence with DNA hybridization than PCR U1-L1. In 5 cases, chlamydia were detected only by PCR U1-L1 but not by PCR U3-L3 or hybridization. This is probably due to the fact that these chlamydia carry no cryptic plasmid. Epidemic strains of *Chlamydia* containing no cryptic plasmid were described previously [5,6].

The sensitivity of amplification is $1\text{-}10^3$ physical corpuscles per sample. Theoretically, PCR detects solitary DNA copies, which may result in hyperdiagnosis. The sensitivity of hybridization diagnosis with radioactive phosphorus, biotin, or digoxigenin may be as high as 10^3 or 10^4 physical corpuscles per sample. It is 2-3 orders of magnitude more sensitive than enzyme immunoassay [14]. On the other hand, it was reported that the immunofluorescence method has a low specificity compared with other methods of diagnosis of chlamydial infection [20,22]. Being practically as sensitive as immunofluorescence, diag-

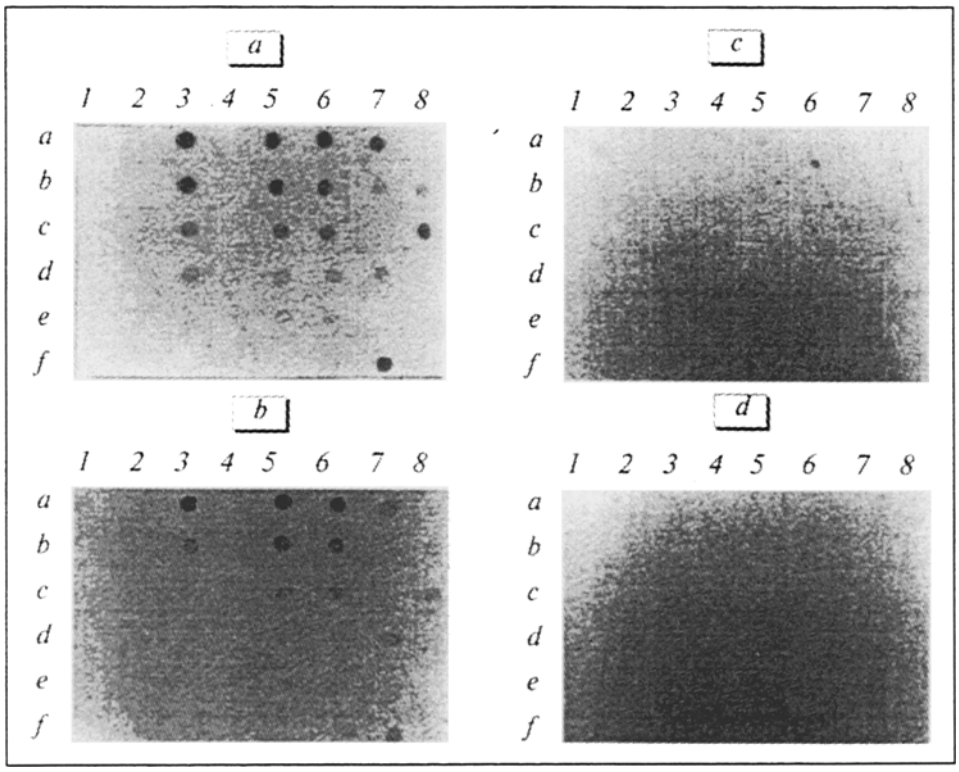

Fig. 3. Specificity and sensitivity of chlamydia detection with the use of pCTp1 probe. *a*) hybridization with biotinated probe pCTp1; *b*) hybridization with biotinated amplificate A1; *c*) hybridization with biotinated pBR322 plasmid; *d*) hybridization mixture contains no DNA probe. In each case, layered onto filters in 5-fold dilutions: line *1* (*a-f*): human DNA 1 μg-0.32 ng per spot; *2* (*a-c*): *M. hominis* DNA and (*d-f*) *U. urealyticum* DNA 1 ng-40 pg per spot; *3* (*a-f*): *C. trachomatis* DNA 0.5 ng-0.16 pg; *4* (*a-c*): HSV-2 DNA and (*d-f*) *E. coli* DNA 10-0.4 ng; *5* (*a-f*): A1 amplificate 0.1 ng-0.03 pg; *6* (*a-f*): A2 amplificate 0.1 ng-0.03 pg; *7* and *8*) clinical samples *1a, 1f, 2c* strongly positive signals, *1b, 1d, 2b* weakly positive signals, *1c, 1e, 2a, 2d, 2e* negative.

nosis with the use of DNA probes gives virtually no false positive results, which may be due to the fact that the specificity of hybridization is determined only by the nucleotide sequence of the probe. Recombinant plasmid pCTp1, which was obtained after analysis of known nucleotide sequences, bears a long DNA fragment specific for *C. trachomatis* and can be used for detecting chlamydia in clinical material.

REFERENCES

1. V. I. Kozlova and A. P. Pukhner, in: *Viral, Chlamydial, and Mycoplasmic Genital Diseases* [in Russian], Moscow (1995), pp. 238-261.
2. I. I. Mavrov, *Vestn. Dermatol.*, No. 11, 11-13 (1991).
3. G. T. Sukhikh, L. Z. Faizullin, N. M. Sinagatullina, and A. V. Zhdanov, *Use of Biotinated Probe for Detecting the Causative Agents of Chronic Infections in Obstetrics and Gynecology* [in Russian], Moscow (1994).
4. E. M. Khalilov, V. M. Govorun, M. Yu. Brodskii, *et al.*, *Vestn. Akush. Gin.*, No. 4, 22-29 (1994).
5. Q. An, D. Radcliff, R. Vassalo, *et al.*, *J. Clin. Microbiol.*, **30**, 2814-2821 (1992).
6. Q. An and D. M. Olive, *Mol. Cell. Probes*, **8**, 429-435 (1994).
7. M. Comanducci, S. Ricci, R. Cevenini, and G. Ratti, *Plasmid*, **23**, 148-154 (1990).
8. E. P. Diamandis, *Clin. Biochem.*, **23**, 437-443 (1990).
9. E. E. Edet, *Br. J. Clin. Pract.*, **47**, No. 1, 21-22 (1993).
10. M. A. Fisher, *W. V. Med. J.*, **89**, No. 8, 331-334 (1993).
11. P. C. Iwen, T. M. H. Blair, and M. D. Woods, *Am. J. Clin. Pathol.*, **95**, No. 4, 578-582 (1991).
12. G. Jaschek, C. A. Gaidos, and L. E. Welsh, *J. Clin. Microbiol.*, **31**, 1209-1212 (1993).
13. J. B. Mahoney, K. E. Luinstra, J. W. Sellors, and M. A. Chernesky, *Ibid.*, 1753-1758.
14. N. Miyashita and A. Matsumoto, *Ibid.*, **30**, 2911-2916 (1992).
15. J. Paevonen, *J. Infect.*, **25**, Suppl. 1, 39-45 (1992).
16. A. Pavla, H. Joussimies-Somer, P. Saikku, *et al.*, *FEMS Microbiol. Lett.*, **23**, 83-89 (1984).
17. J. Sambrook, E. F. Fritch, and T. Maniatis, *Molecular Cloning*, Vol. 1, Cold Spring Harbor Lab. Press (1989), pp. 1.53-1.59.
18. J. Sambrook, E. F. Fritch, and T. Maniatis, *Ibid.*, pp. 6.24-6.27.
19. D. Taylor-Robinson and B. J. Thomas, *Genitourin. Med.*, **67**, 256-266 (1991).
20. D. Taylor-Robinson, *J. Infect.*, **25**, Suppl. 1, 61-67 (1992).
21. W. H. Vogels, P. C. van-Voorst-Vader, and F. P. Schroder, *J. Clin. Microbiol.*, **31**, 1103-1107 (1993).
22. G. L. Woods and J. A. Bryan, *Arch. Pathol. Lab. Med.*, **118**, No. 5, 483-488 (1994).