METHODS

Evaluation of Antibiotic Sensitivity of *Chlamydia trachomatis* Using RT-PCR

O. Yu. Misyurina, E. V. Shipitsina*, T. M. Parfenova, V. N. Lazarev, A. M. Savicheva*, and V. M. Govorun

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 133, No. 3, pp. 357-360, March, 2002
Original article submitted July 1, 2001

The sensitivity of 11 clinical strains of *Chlamydia trachomatis* to azithromycin, ofloxacin, doxycycline, and erythromycin was evaluated. The minimum inhibiting concentrations of all antibiotics for 90% strains, determined by PCR with reverse transcription of *omp3B* gene RNA (GenBank U68443) corresponded to, and those with reverse transcription of *16S rRNA* gene RNA (GenBank X54451) far surpassed the minimum bactericidal concentrations for 90% strains determined by direct immunofluorescence with monoclonal antibodies to the major outer membrane protein.

Key Words: *Chlamydia trachomatis; RT-PCR; antibiotics; omp3B; 16S rRNA*

Chlamydia trachomatis is now the most prevalent sexually transmitted bacterial agent in countries with well-developed economy. Drug therapy (primarily, antibiotics) is now the main method of treatment of Chlamydia infection. The classical method for *in vitro* evaluation of antibiotic sensitivity consists in infection of cultured animal cell with Chlamydia in the presence of different concentrations of test antibiotics and detection of chlamydia by direct immunofluorescence (DIF) with monoclonal antibodies to the major outer membrane protein (MOMP) and lipopolysaccharide (LPS) [3]. However, *in vitro* studies showed that some antibiotics promote Chlamydia persistence and reduce the level of chlamydial MOMP and LPS [2,4]. Therefore, the routine method can provide false-negative results.

Reverse transcription PCR (RT-PCR) is now used for evaluation of antibiotic sensitivity of various bacteria, including Chlamydia. This method is based on detection of some Chlamydia mRNA in infected cell culture. As RNA rapidly degrades after death of microorganisms, only viable bacteria are detected. The minimum inhibitory concentrations (MIC) of antibiotics determined by RT-PCR far surpass those determined by DIF [3,6]. The data on the minimum bactericidal concentration (MBC) determined by repeated passages of Chlamydia in cell monolayer in the absence of antibiotic were not reported. The difference between MIC and MBC for some antibiotics is significant [10]. This can be explained by complexity of visual evaluation of morphologically altered Chlamydia.

We determined sensitivity of 11 clinical strains of *C. trachomatis* to four antibiotics and compared the results obtained by DIF and RT-PCR. Transcripts of *16S rRNA* gene expressed throughout the Chlamydia life cycle [7] *omp3B* gene encoding cysteine-rich membrane protein and expressed only at the late stage of the life cycle during the formation of elementary corpuscles [9] were used as molecular markers for detecting Chlamydia in the culture. Chlamydia can be cultured only on monolayer culture of animal cells, we analyzed also the presence of cell γ-actin gene mRNA

Institute of Physicochemical Medicine, Moscow; *D. O. Ott Institute of Obstetrics and Gynecology, Russian Academy of Medical Sciences, St. Petersburg

0007-4888/02/1333-0308$27.00

expressed by McCoy cells. This excluded possible false-negative results due to poor state of the monolayer or RNA degradation in the sample during testing.

MATERIALS AND METHODS

Clinical strains of *C. trachomatis* isolated from cervical and urethral samples were cultured in a McCoy cell monolayer in Eagle medium containing 0.5% glucose, 10% fetal calf serum, 25 μg/ml vancomycin, 25 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. Microscopic examination of chlamydial incorporations was carried out using fluorescein-labeled species-specific monoclonal antibodies to MOMP (Chlamyset antigen FA, Orion Diagnostica). For evaluation of antibiotic sensitivity, the monolayer was infected with an inoculum producing 20-30 incorporations per visual field at ×400 48 h after inoculation.

The following antibiotics were tested: ofloxacin, erythromycin (Roussel-Uclaf), doxycycline (Bios Firm), and azithromycin (Pfizer/Mack Pharm. Dev. Illertissen). MIC was determined as the lowest antibiotic concentration at which not a single typical incorporation was seen in the cells. In order to determine MBC, infected McCoy cell monolayer was washed from the antibiotic and fresh monolayer was repeatedly infected. Antibiotic concentration ensuring the absence of incorporations after repeated passage was taken for MBC.

Total RNA was extracted from infected cells using SV total RNA isolation system (Promega) according to the manufacturer's instruction. In order to remove DNA, each sample was additionally treated with DNAse I free from RNAse (Promega) under conditions recommended by the manufacturer.

cDNA was synthesized using MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. The following primers were used for amplification of *C. trachomatis* genes *16S rRNA* and *omp3B* cDNA and McCoy γ-actin gene cDNA, respectively: 16S1, direct — 5'-GGGGATCTTAGGACCTTTCG-3'; 16S2, reverse — 5'-CGCTAGCACCCTCCGTAT TA-3'; omp1, direct — 5'-TGTCGTATCGTTGACTG TTGC-3'; omp2, reverse — 5'GCCGTCAACTGGAA TTTCTC-3'; act1, direct — 5'-TGATCCACATCTGC TGGAAG-3'; act2, reverse — 5'-GCTCTTTTCCAG CCTTCCTT-3'. PCR was carried out in a reaction mixture with 8 pM of each primer, 25 mM Tris-HCl (pH 8.3), 5 mM magnesium chloride, a mixture of 4 deoxynucleoside triphosphates (0.2 mM each), and 1 Unit of Taq polymerase (Promega). 40 cycles of PCR were carried out as follows: 40 sec at 93°C, 30 sec at 55°C, and 1 min at 72°C.

After the end of the reaction 10 μl reaction mixture was analyzed by electrophoresis in 2% agarose gel and stained with ethidium bromide. RT-PCR with-

TABLE 1. *In vitro* Evaluation of Antibiotic Sensitivity of Clinical Strains of *C. trachomatis*

Antibiotic	MIC, μg/ml											
	MBC, μg/ml			DIF			*16S rRNA* RT-PCR			*omp3B* RT-PCR		
	50%	90%	range	50%	90%	range	50%	90%	range	50%	90%	range
Ofloxacin	2.0	256.0	2->512	2.00	8.00	2-16	>256	>512	4->512	2.0	256.0	2->512
Azithromycin	0.2	>3.2	0.2->3.2	0.1	0.2	<0.05->6.4	0.8	>3.2	0.1->6.4	0.2	>3.2	0.2->3.2
Doxycycline	>2.56	>2.56	0.32->2.56	0.32	1.28	0.32->2.56	>2.56	>2.56	0.32->2.56	>2.56	>2.56	0.32->2.56
Erythromycin	3.2	>12.8	0.4->12.8	0.4	3.2	0.4-3.2	3.2	>12.8	0.4->12.8	0.4	3.2	0.4-3.2

out reverse transcriptase was carried out in order to verify the absence of DNA; no PCR fragments were detected.

RESULTS

After RT-PCR with RNA isolated from cell culture infected with *C. trachomatis* the electrophoregram showed 194, 286, and 294 b.p. fragments, products of cDNA amplification of *omp3B*, *16S rRNA* genes, and cytoplasmatic γ-actin, respectively. γ-Actin RNA of McCoy cells was detected in RNA samples extracted from intact and infected *C. trachomatis* cultures. In further experiments only samples containing γ-actin mRNA were taken into account. The minimum concentration of the antibiotic, at which the electrophoregram showed no fragments after PCR with primers to *omp3B* or *16S rRNA* genes, was taken for MIC. Table 1 presents MIC and MBC for 50 and 90% isolated strains (MIC50% and MIC90%) determined by DIF and RT-PCR. MIC and MBC for ofloxacin, erythromycin, and azithromycin are close to the values obtained previously by DIF for at least 50% isolated strains [3,8]; MIC90% for erythromycin and ofloxacin is much higher. MBC90% values were notably higher than the values recommended in published reports for all test antibiotics [8]. The sensitivity to doxycycline was very low: its MBC was more than 2.56 μg/ml for more than 50% strains. In a previous study [8] only 8% of 50 tested clinical strains showed such resistance. MIC determined by *16S rRNA* RT-PCR far surpassed MIC and MBC evaluated by DIF. MIC evaluated by DIF and determination of *DnaK* gene transcription have been compared [3]. Significant difference was observed only in testing of antichlamydial activity of amoxicillin: MIC50%, evaluated by RT-PCR, was equal to 512 μg/ml. It seems that this discrepancy is due to higher sensitivity of *16S rRNA* RT-PCR detecting minimum number of incorporations.

Evaluation of MIC by detecting *omp3B* gene transcripts gave results close to MBC. However the term "MBC" is arbitrary when we speak about Chlamydia, as previous *in vitro* experiments showed, that even the maximum concentrations of doxycycline and azithromycin (up to 50 μg/ml) did not lead to complete death of clinical strains of *C. pneumoniae.* After 4 passages in cell culture without antibiotics the growth of all test strains did not cease [5], that is, viable bacteria remained in the culture even in the presence of the maximum concentrations of antibiotics. Nonetheless, comparison of the efficiency of antibiotics or of antibiotic sensitivity of different strains using this parameter is more objective than using MIC, because MBC characterizes the capacity of an isolated strain to rapid growth after antibiotic treatment. Expression of *omp3B* gene is initiated during the formation of elementary infectious corpuscles, and is therefore the marker of maturing of Chlamydia [9]. Exposure to a critical concentration of the antibiotic, determined as MBC, delays their formation; the same delay is observed, if the total RNA preparation contains no *omp3B* gene transcripts.

Hence, detection of *omp3B* gene transcripts by RT-PCR can be used for comparing antibiotic efficiency and sensitivity of *C. trachomatis* clinical strains to antibiotic treatment (similarly to MBC evaluation by DIF). However one should remember that there is no correlation between Chlamydia sensitivity in culture and efficiency of treatment [1]. One strain was isolated from a patient at first consultation; later she was effectively treated with doxycycline (*C. trachomatis* was not detected by PCR 2.5 months after the end of therapy). MBC of erythromycin, azithromycin, and doxycycline for this strain were higher than 12.8, 3.2, and 2.56 μg/ml, respectively; MBC for ofloxacin was 128 μg/ml.

Hence, RT-PCR can be used for comparing activity of antibiotics towards *C. trachomatis*, but the efficiency of antibiotic therapy of patients carrying resistant strains remains unclear.

REFERENCES

1. S. V. Sidorenko, *Antibiot. Khimioter.*, **46**, 3-9 (2001).
2. W. L. Beatty, G. I. Byrne, and R. P. Morrison, *Proc. Natl. Acad. Sci. USA*, **90**, 3998-4402 (1993).
3. N. A. Cross, D. J. Kellock, G. R. Kinghorn, *et al.*, *Antimicrob. Agents Chemother.*, **43**, 2311-2313 (1999).
4. U. Dreses-Werringloer, I. Padubrin, B. Jorgens-Saathoff, *et al.*, *Ibid.*, **44**, 3288-3297 (2000).
5. J. Gnarpe, K. Eriksson, and H. Gnarpe, *Ibid.*, **40**, 1843-1845 (1996).
6. M. A. Khan, C. W. Potter, and R. M. Sharrard, *J. Antimicrob. Chemother.*, **37**, 677-685 (1996).
7. S. A. Mathews, K. M. Volp, and P. Timms, *FEBS Lett.*, **458**, 354-358 (1999).
8. Z. Samra, S. Rosenberg, Y. Soffer, and M. Dan, *Diagn. Microbiol. Infect. Dis.*, **39**, 177-179 (2001).
9. E. I. Shaw, C. A. Dooley, E. R. Fischer, *et al.*, *Mol. Microbiol.*, **37**, 913-925 (2000).
10. J. Somani, V. B. Bhullar, A. Kimberly, *et al.*, *J. Infect. Dis.*, **181**, 1421-1427 (2000).
11. J. M. Woodcock, J. M. Andrews, F. J. Boswell, *et al.*, *Antimicrob. Agents Chemother.*, **41**, 101-106 (1997).