Molecular and biological criteria of activity of the infectious process in *Chlamydia* infection

Molekularne i biologiczne kryteria aktywności procesu infekcyjnego w przebiegu zakażenia drobnoustrojami *Chlamydia*

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Abstract

Aim: The study aims to evaluate molecular and biological criteria of activity of the infectious process in various variants of *Chlamydia* infection.
Material and methods: Blood, and scrapings from the urethra and cervical canal served as the study material. Real-time polymerase chain reaction (PCR), direct reaction of immune fluorescence, immunoenzymatic analysis and light microscopy were applied in the study.
Results: Determination of *Chlamydia trachomatis* DNA concentration serves as an important additional diagnostic technique that enables one to evaluate activity rate of the infectious process according to its character, distribution, its combination with other causative agents of urogenital pathology, and the presence of complicated forms of the disease (sterility, *Chlamydia*-induced arthropathy).
Conclusions: The established ranges of fluctuation of *Chlamydia* DNA will enable us to add to the list of clinical and laboratory criteria for evaluation of the activity rate of the infectious process, to reconsider indications for administration of rational antibiotic therapy in categories of patients with different course and activity rate of the disease, to adequately schedule monitoring of DNA concentration within the course of therapy, and to predict the outcome (elimination, persistence) of the disease in various variants of clamidiosis.

Key words: clamidiosis, infectious process, activity, PCR.

Streszczenie

Cel: Celem pracy była analiza molekularnych i biologicznych kryteriów aktywności procesu infekcyjnego w przebiegu różnych odmian zakażenia drobnoustrojami z rodzaju *Chlamydia*.
Materiał i metody: Materiał badawczy stanowiły krew oraz wyskrobiny z cewki moczowej i kanału szyjki macicy. Przeprowadzono badania metodami: *real-time* PCR, immunofluorescencji bezpośredniej, immunoenzymatyczną oraz mikroskopii świetlnej.
Wyniki: Oznaczenie stężenia DNA *Chlamydia trachomatis* stanowi przydatny, dodatkowy rodzaj techniki badawczej, pozwalający na ocenę aktywności procesu infekcyjnego w zależności od jego charakteru, szerzenia się oraz zestawienia z innymi czynnikami sprawczymi różnych rodzajów patologii układu moczowo-płciowego, a także obecności powikłań w przebiegu choroby (niepłodność, artropatia indukowana zakażeniem *Chlamydia*).
Wnioski: Stwierdzone zakresy wahań DNA Chlamydii pozwalają na: uzupełnienie listy klinicznych i laboratoryjnych kryteriów aktywności procesu infekcyjnego, zweryfikowanie wskazań do odpowiedniej antybiotykoterapii u chorych prezentujących rozmaity przebieg i nasilenie choroby, adekwatne zaplanowanie monitorowania stężenia DNA w trakcie procesu leczniczego oraz ocenę rokowań (eliminacja zakażenia, jego utrzymywanie się) w przebiegu różnych odmian chlamydiozy.

Słowa kluczowe: chlamydioza, proces zakaźny, aktywność, PCR.

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Introduction

Application of molecular and genetic methods along with amplification technologies in diagnosis of sexually-transmitted infections (STI) enables one to qualitatively evaluate the presence of the DNA fragment as well as to define the relative concentration of DNA of the causative agent in the study material [1-3].

It is assumed that the development of the disease and intensity of its clinical picture tend to greatly depend upon the "massive" nature of the contamination of the organism when a pathologically significant concentration of causative agents is present in the urogenital tract [4]. Thus, molecular and genetic monitoring enables one to control the course and the outcome of the infectious process of some infections as well as to achieve more efficient results in therapy and prevention of STI [5].

Currently the qualitative analysis of concentration of specific DNA is made on the basis of real-time PCR [6]. The essence of this method deals with detection of accumulation of a fluorescent reaction mixture [7]. In this case the intensity of the signal is proportional to the concentration of the final product of PCR [8]. Synchronization of registration and amplification serves as the most important particular feature of this method [9]. It enables one to evaluate the kinetics of the process, which is known to depend upon the initial quantity of the hereditary material under study [10]. Today the method of quantitative definition of PCR products right at the moment of amplification is one of the most popular ones both in clinical gene diagnosis and research [5, 10].

Aim

This study aims to define molecular and biological criteria of the activity rate of the infectious process in various cases of *Chlamydia* infection.

Material and methods

Complex clinical and microbiological evaluation was conducted with the involvement of 154 patients (86 females and 68 males) aged 17 to 44 who applied for consultation at the Department for Prevention of STI attached to the Grodno Regional Dispensary for Skin and Venereal Diseases, Belarus. The diagnosis of urogenital clamidiosis (UGC) was made on the basis of anamnesis, subjective and objective clinical signs as well as the results of laboratory studies in compliance with ICD-10. All patients under study were divided into three groups depending on the peculiar features of the course, localization and character of urogenital tract pathology (A56.0, A56.1). Group I comprised patients with acute forms of *Chlamydia* infection (16 males) who suffered from acute inflammation of the urethra (urethritis). Group II consisted of 102 patients (40 males and 62 females) with a chronic course of *Chlamydia* infection. They had both clinical and laboratory signs of exacerbation of the disease in the form of urethritis (12), adnexitis (22), cervical erosion (36), endocervicitis (4) and combined damage of the urethra and prostate (28). Group III comprised 36 subjects with chronic persistent course of the disease without clinical or laboratory manifestations of the inflammatory process. This group was represented by 24 females suffering from sterility (N97.1) and by 12 males with *Chlamydia*-induced arthropathy (M02.3 – Reiter's syndrome).

To establish association between DNA concentration and the prevailing localization of the inflammatory process in the patients under study (predominant damage of organs and systems) in each study group, separate nosological forms of UGC were chosen. Patients with *Chlamydia*-induced arthropathy and sterility, some of whom also had various manifestations of the pathological process in the urogenital tract, were also included in the study. The criteria for acute clamidiosis were as follows: the initially diagnosed clinically manifested syndrome of under two-month-old urogenital tract damage (complaints, objective signs of inflamed urethra, changes in laboratory findings and diagnostic tests) with further complete clinical and laboratory recovery and post-therapy elimination of the causative agent. The study group of chronic clamidiosis comprised patients with the confirmed fact of an over two-month-old infection with the previously diagnosed pathology and with the clinically diagnosed signs of an inflamed urogenital tract. The criteria of persistent course of clamidiosis included over six-month isolation of the causative agent in patients suffering from sterility or *Chlamydia*-induced arthropathy with no signs of active inflammation in the urogenital tract.

The following methods were used to aetiologically verify the UGC: exposure of class A, G and M antibodies to *Chlamydia trachomatis* (*C. trachomatis*) antigens in blood serum by the method of immunoenzymatic analysis (IEA), and isolation of *Chlamydia* in scrapings from the cervical canal and urethra by means of direct reaction of immune fluorescence (DIF). In case of positive IEA and DIF analysis examined material underwent PCR studies (quantitative evaluation). Aetiological decoding of mixed infection was done using the routine methods applied in ureaplasmosis and mycoplasmosis, i.e. DIF reaction and culture studies (inoculating onto the IST medium with further evaluation of resistance to antibiotics) as well as in cases of candidosis and gardnerellosis, i.e. bacterioscopic evaluation of smears stained using Gram's method and in cases of trichomoniasis, i.e. bacterioscopic study of smears stained with 1% solution of methylene blue.

Real-time PCR was used to conduct quantitative analysis of the specific fragment of *C. trachomatis* DNA. That enabled us to do the quantitative measurement of the nucleic acids of the infectious agent in the biological test. Oligonucleotide tests labelled with fluorescent TaqMan agent and complementary to the PCR product

area were used in the study. TaqMan PCR is based on the use of 5´-exonuclease activity of polymerase. The reaction mixture was added to DNA tests labelled at the 5´ end with fluorescent 6-carboxyfluorescein [FAM], and at the 3´ end with the phosphate group and the extinguisher of fluorescence of 6-carboxytetramethylrhodamine [TAMRA]. The tests were complimentary to the area of the amplified zone. The extinguisher absorbed the emanation produced by the fluorescent marker while a phosphate group blocked polymerase in the 3´ position. Upon annealing of the primers the oligonucleotide labelled test was quantitatively bound with the complimentary zone of DNA. At the stage of elongation polymerase tended to synthesize the complimentary chain of DNA and having reached the area annealed with the test it began to split the test at the expense of 5´-exonuclease activity. As the result of this the fluorescent marker would separate from the extinguisher and its fluorescence could be detected. Thus, increase in fluorescence was directly proportional to the quantity of the generated PCR product.

To conduct the quantitative study of specific fragments of *C. trachomatis ompA* gene, coding the main surface protein of *C. trachomatis* (MOMP), real-time PCR was used with application of TaqMan tests and species-specific primers:

C. trachomatis 1 – 5´-TTCGTGATTATAGCAGCTAG-3´ (forward primer),

C. trachomatis 2 – 5´-GCGCTCATTTCTAGAGATAG-3´ (reverse primer).

Test 5´ (FAM)GACATGGCGATAAATCTTGCGGTTACTCAACAGCGTA GAGTTGGT(TAMRA)3´.

PCR was used to synthesize DNA in the «RotorGene» 300 (Australia) amplifier. Standardization of the study was made against the NAGK one-copy human gene, succession of primers, probes and the condition of amplification for its quantitative evaluation [3].

Definition of the relative concentration of substrate was made on the basis of the analysis of the kinetic PCR curve, which was based on comparison of reaction kinetics in the standard samples and the ones under study. Cultivations of the control samples (positive control) with the known concentration of the genome DNA were used in the study. To build a standard curve we performed amplification of a 10-fold titrated *C. trachomatis* DNA standard, containing human DNA in a concentration of 10.0-1.0-0.1-0.01 and 0.001 µg/ml, within the range of 10^3 to 10^6 copies/ml. Each sample of DNA standards was pro-amplified in duplicate along with NTC, i.e. a complicated DNA amplification mixture in which the DNA standard target was substituted by water. The standard curve was represented in the form of a CT series (determination of the cycle in which the threshold line and the amplification curve intersects) of standard cultivations (Figure 1).

Samples with a high level of DNA tended to have a low CT level, while a low level of DNA would have a high level

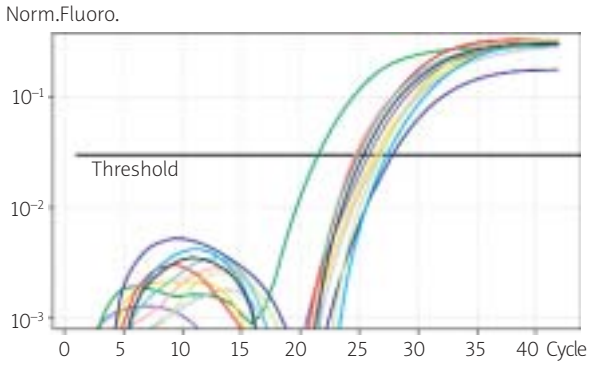

Figure 1. Fluorescence curves for evaluation of the threshold cycle of amplification of the specific fragment of *C. trachomatis* DNA

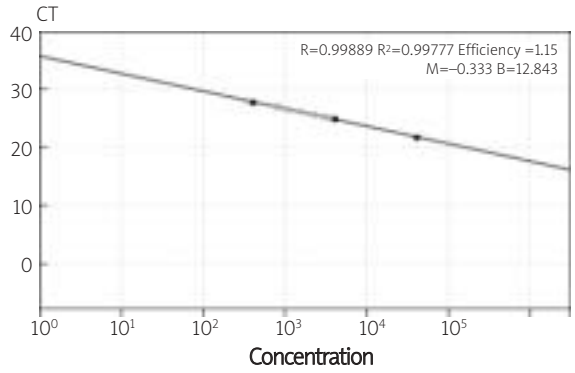

Figure 2. Standard curve for calculation of the number of copies of a specific fragment of *C. trachomatis* DNA

of CT. Calculation of DNA concentration was based on the character of the slope of the standard curve. The value of the DNA concentration of causative agents in the biological material was calculated using the formula $10^{(-M*CT+B)}$, in which M stands for the slope and B stands for the section of standard curve (Figure 2).

The correlation between the threshold CT cycle and the log10 concentration of copies of the DNA fragment, coding the main surface *C. trachomatis* protein (MOMP), was R^2>0.99777 ml. The value of concentration of the causative agent was automatically computed using the formula: concentration = $10^{(-0.333*CT+12.843)}$. The presence of specific fragments of C. trachomatis in clinical samples was quantitatively analyzed in compliance with Figure 1 and Figure 2.

Results and discussion

The results obtained proved clamidiosis to be a mono-infection that was diagnosed in 48 (31.2%) patients. In 106 (68.8%) patients various variants of mixed *Chlamydia* infection prevailed, the latter being presented by the following combinations: *Chlamydia* + ureaplasmas (38 cases, 35.8%), *Chlamydia* + trichomonades (20 cases, 18.9%), *Chlamydia* + mycoplasmas, *Chlamydia* + *Candida*

and *Chlamydia* + *Gardnerella* (16 cases for each combination, 15.1%).

It is worth mentioning that the quantitative values of relative concentrations of *C. trachomatis* DNA, defined in the biological material, significantly varied, i.e. from 0.5×10^3 to 9.9×10^6 copies of DNA per ml (Table 1).

This phenomenon presupposes the presence of significant differences in DNA concentrations among the patients with various variants of chlamidiosis from the point of view of aetiology (mono-infection, mixed infection), course (acute, chronic) and localization of the inflammatory process (localized, local or diffused, poly-organ), as well as fixing the maximal and minimal DNA concentrations, exceeding the range of which would require individual evaluation.

In compliance with the above differences, all the patients under study were divided into groups depending on concentration of DNA, i.e. according to a low DNA level (up to 10 000 copies/ml), an average one (from 11 000 to 200 000 copies) or a high DNA concentration (over 201 000 copies/ml). The mean value of *Chlamydia* DNA concentration was 145 381.1±21 202.5 copies/ml in males and 42 934.4±4856.0 copies/ml in females.

Analysis of all the clinical and laboratory findings obtained from all the patients under study enabled us to define the maximum intervals (fluctuations) of concentration in various variants of clamidiosis, i.e. voltage of the values of relative *C. trachomatis* DNA concentration in acute forms of *Chlamydia* infection ranged from 318 381.0 to 584 700.9 copies/ml, in chronic and persistent forms from 13 300.3 to 114 850.7 and from 1740.6 to 27 420.4 copies/ml, correspondingly. Table 2 presents the data on DNA concentrations in the patients under study.

Table 2 shows that 75% of patients with acute clamidiosis demonstrated DNA concentrations of 401 000 copies/ml and higher, which represented high values of DNA concentration. In 95% of cases with chronic clamidiosis DNA concentration predominantly ranged from 51 000 to 100 000 copies/ml (mean values) and 88.9% of patients with persistent forms of chlamidiosis displayed the lowest levels of DNA concentration.

Examination of female and male patients of any prevailing localization of the inflammatory process showed that the mean value of *Chlamydia* DNA concentration in acute forms of clamidiosis was 443 888.2±22 999.4 copies/ml in males, 68 010.7±3618.9 copies/ml in males with chronic clamidiosis, in persistent forms of the disease it was 2540.0±320.0 copies/ml; in chronic and persistent forms in females it equalled 57 211.4±5783.5 and 6052.1±1034.7 copies/ml, correspondingly, which was significantly different from similar data in the group of patients with chronic clamidiosis ($p<0.05$).

According to the clinical manifestations of the UGC the patients belonged to the following groups: those with acute *C. urethritis*, chronic *C. urethritis*, prostatitis-induced urethritis, and with *Chlamydia*-induced arthropathy. In cases of acute urethritis DNA concentrations ranged from 318 381.0 to 584 700.9 copies/ml; in acute forms from 13 096.9 to 94 000.5 copies/ml; in chronic prostatitis-complicated cases from 57 600.4 to 87 850.6 copies/ml; in patients with *Chlamydia*-induced arthropathy from 1400.0 to 3750.0 copies/ml. The mean values of DNA concentration in males are specified in Figure 3.

Figure 3 shows that mean values of DNA concentration of *Chlamydia* in cases of acute *C. urethritis* were 443 888.2± 22 999.4 copies/ml, in chronic cases 52 187.6±10 234.0

Table 1. Range of concentrations of *C. trachomatis* DNA according to the variant (mono- or mixed infection) and the course of the infection

Variant of infection		Course	DNA concentration [copies/ml]	
Mono-infection	*Chlamydia*	Acute	$9.5 \times 10^4 – 7.2 \times 10^5$	95 000-720 000
		Chronic	$1.2\text{-}9.4 \times 10^4$	12 000-94 000
		Persistent	$0.5 \times 10^3 – 1.1 \times 10^4$	500-11 000
Mixed	*Chlamydia* + *Mycoplasma* + *Ureaplasma*	Acute	$1.1 \times 10^5 – 1.0 \times 10^6$	110 000-1 000 000
		Chronic	$2.1 \times 10^4 – 1.0 \times 10^5$	21 000-100 000
		Persistent	$0.8 \times 10^3 – 2.0 \times 10^4$	800-20 000
	Chlamydia + *Trichomonas*	Acute	$1.5 \times 10^5 – 9.9 \times 10^6$	150 000-9 900 000
		Chronic	$2.9 \times 10^4 – 1.4 \times 10^5$	29 000-140 000
		Persistent	$1.0\text{-}2.8 \times 10^4$	10 000-28 000
	Chlamydia + *Gardnerella* + *Candida*	Acute	$9.6 \times 10^4 – 8.0 \times 10^5$	96 000-800 000
		Chronic	$1.3\text{-}9.5 \times 10^4$	13 000-95 000
		Persistent	$0.6 \times 10^3 – 1.2 \times 10^4$	600-12 000

copies/ml, in patients with urethritis 74 792.0±1769.7 copies/ml, in subjects with *Chlamydia*-induced arthropathy 2540.0±320.0 copies/ml. Statistical analysis confirmed the differences in the study groups ($p<0.05$).

The key manifestations of *Chlamydia* infection in females were sterility, adnexitis, cervical erosion, and endocervicitis. In sterile female patients without clinical signs of exacerbation of gynaecological pathology DNA concentration indices ranged from 1874.6 to 17 600.4 copies/ml; in combination with adnexitis the concentration ranged from 14 240.6 to 29 420.4 copies/ml; in combination with cervical erosion from 21 300.5 to 22 740.5 copies/ml; in cases of "pure" adnexitis from 17 600.4 to 134 000.4 copies/ml; in females with cervical erosion from 13 300.3 to 88 300.5 copies/ml; and in endocervicitis from 107 420.1 to 114 850.7 copies/ml. The mean values of DNA concentration in females are specified in Figure 4.

Figure 4 shows that the mean value of DNA concentration in cases of sterility with no signs of gynaecological pathology was 6052.1±1034.7 copies/ml, which was significantly lower than DNA concentrations in cases of combination of sterility with adnexitis and clinical signs of inflammation (21 830.5±4382.0) as well as with cervical erosion (22 020.5±415.7). DNA concentration in cases of adnexitis equalled 97 892.7±10 645.2 copies/ml, in cases of cervical erosion 36 409.2±5150.0 copies/ml, in patients with endocervicitis 111 135.4±2145.0 copies/ml, which corresponded to the highest concentration values in this group of females, which differentiated them from the rest of the patients with UGC under study ($p<0.05$).

Evaluation of DNA concentrations in cases of sterility demonstrated that in the presence of gynaecological pathology the indices ranged from 14 240.6 to 29 420.4 copies/ml; in cases of absence of this pathology they ranged from 1874.6 to 17 600.4 copies/ml. In combination of sterility with mixed infection the range of DNA concentrations varied from 1740.6 to 25 420.4 copies/ml; in mono-infection of clamidiosis from 1930.3 to 5750.4 copies/ml. Mean values of DNA concentrations in sterile females are specified in Table 3.

Table 2. Indices of concentration of DNA in UGC

DNA [copies/ml]	UGC					
	initial (n=16)		chronic (n=102)		chronic persistent (n=36)	
	abs.	%	abs.	%	abs.	%
up to 10 000	0	0.0	0	0.0	32	88.9
11 000-50 000	0	0.0	32	31.4	4	11.1
51 000-100 000	0	0.0	54	52.9	0	0.0
101 000-200 000	0	0.0	16	15.7	0	0.0
201 000-300 000	0	0.0	0	0.0	0	0.0
301 000-400 000	4	25.0	0	0.0	0	0.0
401 000-500 000	6	37.5	0	0.0	0	0.0
over 501 000	6	37.5	0	0.0	0	0.0
Total	**16**	**100.0**	**102**	**100.0**	**36**	**100.0**

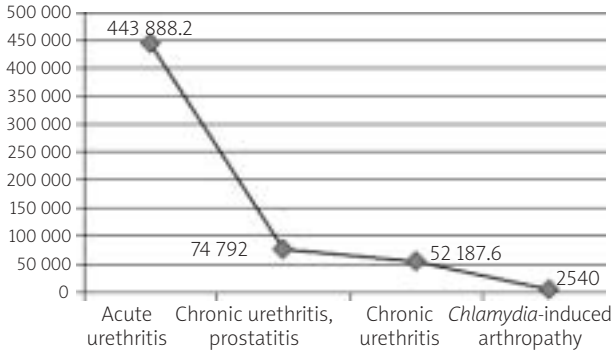

Figure 3. Concentration indices of DNA in UGC males according to their pathology

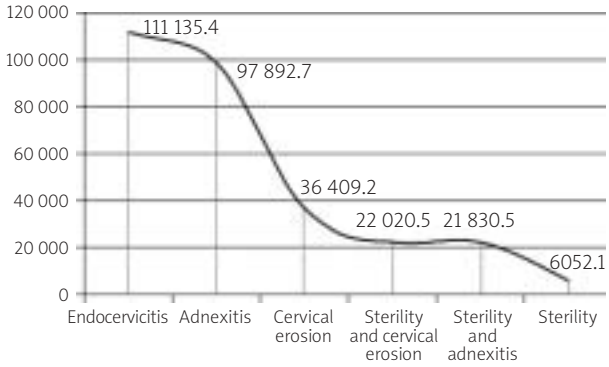

Figure 4. Indices of concentration of DNA in UGC in females according of their pathology

Table 3. DNA concentration indices in sterile females according to their pathology as well as mono- and mixed clamidiosis infection

Diagnosis, variant of infection	DNA [copies/ml]	
	n=32	M±m
Sterility without clinical symptoms of exacerbation of gynaecological pathology	24	6052.1±1034.7
Sterility with clinical signs of exacerbation of chronic gynaecological pathology	8	21 925.5±2037.9
Sterility in the background of mixed infection	28	10 819.7±1700.1
Sterility in the background of mono-infection	4	4425.4±765.0

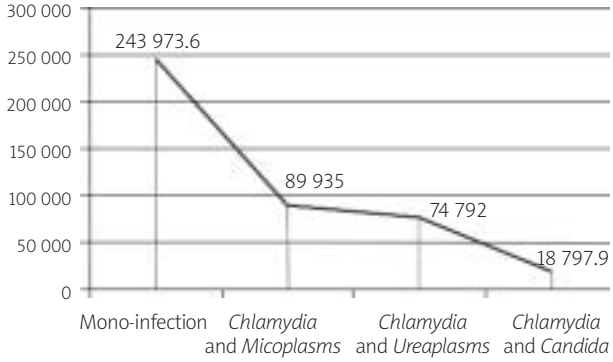

Figure 5. DNA concentration indices in males with UGC in mono- and mixed infections

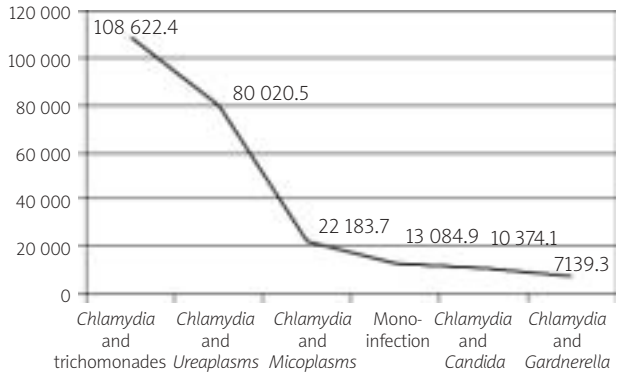

Figure 6. DNA concentration indices in females with UGC in mono- and mixed infections

Table 3 shows that indices of DNA concentrations in sterile females with gynaecological pathology tended to be higher than in cases with no such pathology ($p<0.05$). Also, the DNA index in cases of mixed infection was higher than that in mono-infection ($p<0.05$) in spite of the fact that females with no gynaecological pathology dominated in that group. That index was obviously influenced by other causative agents of sexually transmitted infections that accompanied clamidiosis.

In this aspect the study aimed to make comparative evaluation of *C. trachomatis* DNA indices in males with mono- and mixed infections. In 30 males *C. trachomatis* was isolated as the only aetiological agent; in 38 patients other causative agents were revealed, i.e. in 28 patients ureaplasmas, in 6 cases *Candida*, in 4 patients mycoplasmas. The range of DNA indices fluctuation in cases of equalled 2830.5-584 700.9 copies/ml; in cases of mixed infection it was 13 096.9-94 000.5 copies/ml. In cases of combination of UGC with candidosis that index ranged from 13 096.9 to 22 148.5 copies/ml; in combination with mycoplasmas and ureaplasmas it varied from 85 870.6 to 94 000.5 and from 57 600.4-87 850.6 copies/ml. Mean values of DNA concentrations in males are specified in Figure 5.

Figure 5 shows that higher DNA concentration was noted in cases of mono-infection, which in compliance with the above specified criteria exceeded the mean values of DNA concentration; a lower one was registered in cases of combination of clamidiosis and candidosis, which represented the medium range of DNA concentration.

Chlamydia mono-infection was diagnosed in 18 females and a mixed one in 68 subjects. Mean values of DNA concentration in females are specified in Figure 6.

Figure 6 shows that DNA concentration in cases of mono-infection represented the low range, whose indices differed from those in males with mono-infection. In 20 cases *C. trachomatis* was combined with trichomonades (DNA ranged from 25 420.4 to 13 400.4 copies/ml); in 16 cases with *Gardnerella* (DNA ranged from 1740.6 to 15 970.5 copies/ml), in 12 subjects with mycoplasmas (DNA ranged from 15 740.5 to 28 300.5 copies/ml); in 10 cases with ureaplasmas and *Candida* (DNA ranged from 67 600.4 to 98 300.5 and from 5148.5 to 18 000.5 copies/ml, respectively). Much lower concentrations of DNA were registered in combination of *Chlamydia* with *Gardnerella* and *Candida*, which was in line with the *Candida* indices in males. Maximal DNA concentration was registered in cases of combination of *Chlamydia* with trichomonades (108 622.4±6701.8 copies/ml); it was slightly lower in combination with ureaplasmas and mycoplasmas (80 020.5±3944.6 and 22 183.7±1395.1 copies/ml, respectively).

Conclusions

Determination of *C. trachomatis* DNA concentration serves as an important additional diagnostic technique that enables one to evaluate the activity rate of the infectious process according to its character, spread, its combination with other causative agents of urogenital

pathology, and the presence of complicated forms of the disease (sterility, *Chlamydia*-induced arthropathy). Differences in indices of DNA concentration among patients with the same clinical diagnosis may be associated with different immunopathological mechanisms of formation of chronic and complicated forms of *Chlamydia* infection, which is important for further studies of causes of persistence of *Chlamydia* and inefficacy of administered therapy.

Molecular and biological criteria that support the presence of activity of the infectious process of *Chlamydia* infection of the urogenital tract are as follows:

- acute and chronic course of *Chlamydia* mono-infection, the presence of clinical manifestations of the disease, *C. trachomatis* DNA concentration exceeding 12 000 copies/ml;
- acute course of *Chlamydia* infection and chronic course of *Chlamydia* mixed infection, the presence of clinical manifestations of the disease, combination of *C. trachomatis*, urea- and mycoplasmas (mixed clamidiosis), DNA concentration over 13 000 copies/ml.

Molecular and biological criteria that support absence of the infectious process in *Chlamydia* infection (persistence of *C. trachomatis*) of the urogenital tract are as follows:

- chronic course of *Chlamydia* mono-infection, absence of clinical manifestations of the disease, concentration of *C. trachomatis* DNA below 11 000 copies/ml;
- chronic course of *Chlamydia* mixed infection, absence of clinical manifestations of the disease, combination of *C. trachomatis* with *Candida* and *Gardnerella* at DNA concentrations below 20 000 copies/ml, with trichomonades at DNA concentration below 28 000 copies/ml.

The established ranges of fluctuation of *Chlamydia* DNA will enable us to add to the list of clinical and laboratory criteria for evaluation of the activity rate of the infectious process, to reconsider indications for administration of rational antibiotic therapy in categories of patients with different course and activity rate of the disease, to adequately schedule monitoring of DNA concentration within the course of therapy, and to predict the outcome (elimination, persistence) of the disease in various variants of clamidiosis.

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