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Prevalence of *Toxoplasma gondii* among different groups of patients in Bulgaria and algorithm for diagnosis of toxoplasmosis, applied in the National Reference Laboratory for Diagnosis of Parasitic Diseases, Bulgaria

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Abstract

Background: Toxoplasmosis is one of the most common parasitic infections in Bulgaria. The diagnostic algorithm includes serological and molecular tests.

Objectives: Aim of this study was to establish the prevalence of toxoplasmosis in examined persons and to discuss the algorithm for diagnosis of toxoplasmosis, applied in the National Reference Laboratory for Diagnosis of Parasitic Diseases, Bulgaria.

Material and Methods: Data from the annual reports of the National Reference Laboratory for Diagnosis of Parasitic Diseases were used. The classes of specific IgG, IgM and IgA antibodies, and IgG avidity were identified by ELISA.

Results: For the period from 2011 to 2020 a total of 2969 individuals from different regions of the country were tested for toxoplasmosis in NRL, and it was found an average seropositivity of 27%. With evidence of recent infection were 18.74% of the pregnant women, 4.5% of the patients with fever of unknown origin, 3.3% of the patients with ocular involvement and 13.9% of the tested for prophylaxis. In the group of HIV infected persons we detected only latent form of toxoplasmosis in 29.9% of the examined patients.

Conclusion: For the ten-year period of the study with data on acute toxoplasmosis were 11.2% of those studied for clinical indications, and with latent infection were 27% of examined persons. We believe that the algorithm we have adopted for diagnosis is suitable for the management of the infection and helps for timely treatment in clinically manifested cases and pregnant women with data for recent infection.

Keywords: Toxoplasmosis; Prevalence; Diagnosis; Recent Infection

1. Introduction

Toxoplasmosis is a cosmopolitan protozoan infection caused by the intracellular parasite *Toxoplasma gondii*. Diagnosis of the disease is based on the humoral immune response. Tests for presence of several classes of antibodies may be used

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to assess whether the infection, is in acute or latent stage. In Bulgaria, more than 10,000 people are tested for toxoplasmosis every year, both by clinical indications or for prophylaxis. According to the existing legislation, only cases of congenital toxoplasmosis are subject to a mandatory notification and registration. However, special attention is paid to the pregnant women and immunocompromised patients, in whom this infection can lead to a serious consequences [1]. The laboratory of parasitology at the National Center for Infectious and Parasitic Diseases (NCIPD) is commissioned by the Ministry of Health (MoH) as National Reference Laboratory (NRL) for diagnosis of parasitic diseases. Serological methods are used in the laboratory to diagnose toxoplasmosis and to define the humoral immune response in immunocompetent or immunocompromised patients. Molecular biological methods for detection of parasitic DNA in tissue samples are used as a confirmatory diagnostic tests. Depending on results the clinicians recommend treatment and follow up tests over a period of time.

Aim of the present study was to analyze the seroprevalence of toxoplasmosis in the country for a ten year period (2011 – 2020) and to describe the algorithm for diagnosis of this parasitic disease implemented at the NRL.

2. Patients and methods

2.1. Patients

By clinical and prophylactic indications were examined for toxoplasmosis people from all over the country. The largest groups were those of the pregnant women, women with pending in vitro procedure, persons with unclear febrile condition, lymphadenitis, ocular pathology and patients with compromised immunity.

2.2. Materials

For study of the humoral immune response to toxoplasmosis received in the laboratory were blood samples obtained by venipuncture with a vacuum system. After collection of the serum, serum samples were stored until their examination at - 20 °C.

2.3. Methods

2.3.1. Serological methods

The algorithm for serological diagnosis, developed in the laboratory, includes monitoring the level of specific anti-*toxoplasma* antibodies from classes IgG, IgM and IgA and the avidity of IgG. Tests based on the ELISA solid-phase method (PLATELIA TOXO IgG, IgM and IgA, BIO-RAD, France) were used, as the detection of IgG was performed by indirect method and the level of antibodies was reported in IU / ml. For test of IgM and IgA double sandwich ELISA method was used and the level of specific antibodies was compared to a calibrator (IgM) or cut-off (IgA). According to the algorithm, all new samples were initially tested for presence of IgG and IgM antibodies. When the results were negative toxoplasma infection was excluded, but the pregnant women were provided with medical advice how to avoid possible infection and were also asked to come back, if it is convenient to them, for control tests (just for IgM), every 8-12 weeks in order to timely diagnose possible seroconversion during pregnancy. Accordingly, to the immunocompromised patients a hygiene regimen and periodic follow-up tests for possible seroconversion were also recommended. When test results displayed presence of specific IgG antibodies and a lack of IgM, the infection was considered as latent, but pregnant women needed to be retested after three weeks for possible seroconversion and development of acute toxoplasmosis.

Positive IgG and IgM values were considered as an indicator for possible recent infection. In these cases, because the persistence of specific IgM antibodies in the serum of a freshly infected person may last for a year or more and to approximate the time of infection, the levels of anti-*Toxoplasma* IgA were examined. The kinetics of IgA in toxoplasmosis shows that the level of this class of specific antibodies increases about 7 days after infection and persists for about 6 months, after which it slowly decreases to a negative value. In addition, the avidity of anti-*Toxoplasma* IgG antibodies (PLATELIA TOXO IgG avidity, BIO-RAD, France) was examined to more accurately determine the duration of infection. If the IgG avidity values are high (avidity index (AI) above 0.5 according to the manufacturer's instructions, the infection is considered to have occurred about 5 to 6 months ago).

2.3.2. Molecular methods (PCR)

Total genomic DNA was isolated from the tissue materials using a commercial column based PureLink Genomic DNA Mini Kit (Invitrogen, USA). From each sample at least three isolations were made and procedure was performed according to manufacturer instructions. Incubation of the tissue samples with proteinase K (20 mg/ml) was held overnight and immediately after the extraction amplification was applied. PCR using the 35-fold-repetitive B1 gene as

a target for amplification Primers and conditions for PCR were according to a method described by Burg et al. (1989) [2]. Primer pair (Bretagne et al., 1993) TOXOB22/TOXOB23 that detected B1 gene of *T. gondii* were with sequences as follows: TOXOB22 (sense) 5'-AAC GGG CGA GTA GCA CCT GAG A -3' and TOXOB23 (antisence) 5'- TGG GTC TAC GTC GAT GGC ATG ACA ACT-3' [3]. The PCR mix included 1.5 mM MgCl2; 200 µM of each dNTP; 1.5 µM of each primer, 0.625 U Taq polymerase and DNA matrix 1.0 µl in total volume of the reaction 25 µl. Amplification was carried out in a thermocycler (GeneAmp PCR System 2700, Applied Biosystems) under the following conditions: 1 cycle of pre-incubation at 95°C for 5 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; and a final extension for 15 min at 72°C. After PCR amplification products (the expected size is 115 bp) were analyzed by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide or GelRed® Nucleic Acid Stain 10,000X Water and the reaction was read under UV and photo documented (Syngen, Synoptic group).

3. Results

Table 1 Results of serological tests for toxoplasmosis by type of test indication and by year

Type of examined persons	Serological results	Year										Total
		2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	
Pregnant women	IgG-/ IgM- /IgA (-)	63	79	93	38	64	52	61	36	15	22	523
	IgG+/IgM-/IgA-	29	54	36	39	24	25	12	14	8	11	252
	IgG+/IgM+ /IgA-	8	12	9	11	25	11	15	17	4	13	125
	IgG+/IgM+ /IgA+	9	4	0	7	7	6	11	2	3	3	52
Total		109	149	138	87	120	94	99	69	30	49	944
Suspected for ocular toxoplasmosis	Low IgG avidity	3	3	2	2	5	7	9	7	3	5	46
	High IgG avidity	3	5	7	3	10	7	8	12	5	13	73
	IgG-/IgM-/IgA-	16	20	15	13	13	8	7	4	3	2	101
	IgG+/IgM-/IgA-	14	7	14	6	5	6	4	4	1	5	66
	IgG+/IgM+ /IgA-	0	0	2	0	0	0	0	1	0	1	4
	IgG+/IgM+ /IgA+	0	1	0	0	0	1	0	0	0	0	2
	IgG -/IgM-/IgA+	2	1	0	1	0	0	0	0	0	0	4
	IgG+/IgM-/IgA+	2	0	0	1	0	0	0	0	0	1	4
Total		34	29	31	21	18	15	11	9	4	9	181
Patients with temperature of unknown origin	IgG-/IgM-/IgA-	6	12	7	6	4	7	4	2	1	4	53
	IgG+/IgM-/IgA-	4	3	8	2	0	0	0	1	0	0	18
	IgG+/IgM+ /IgA-	0	0	1	1	0	0	0	0	0	0	2
	IgG+/IgM+ /IgA+	0	0	0	0	1	0	0	0	0	0	1
Total		10	15	16	9	5	7	4	3	1	4	74
HIV infected persons	IgG-/IgM-/IgA-	72	93	18	3	3	0	2	2	2	2	197
	IgG+/IgM-/IgA-	33	37	6	2	1	0	0	2	3	0	84
Total		105	130	24	5	4	0	2	4	5	2	281
Persons tested for prophylactic indications	IgG-/IgM-/IgA-	32	28	33	44	28	43	11	2	7	17	245
	IgG+/IgM-/IgA-	34	19	22	10	10	14	7	5	4	8	133
	IgG+/IgM+ /IgA-	6	3	8	5	3	5	1	2	3	1	37
	IgG+/IgM+ /IgA+	6	4	0	5	3	1	3	2	0	0	24
Total		78	54	63	64	44	63	22	11	14	26	439

Through the use of the described algorithm at the NRL for the period 2011-2020 for toxoplasmosis were studied a total of 2969 people by clinical and prophylactic indications. Of the groups most commonly diagnosed with toxoplasmosis, 944 were pregnant, 215 had lymphadenitis, 181 had ocular pathology, 74 had fever, 286 had HIV and AIDS, and 439 had been tested prophylactically (Table 1). The remaining 830 patients tested for toxoplasmosis were referred to the laboratory with other primary diagnoses (urticaria, leukemia, brain tumors, etc.). Except for the group of patients with HIV and AIDS, who were referred for examination by specialized hospitals in all other patients studied in the laboratory, there were no evidence for immune deficiency.

Table 2 Results of PCR analyzes of various clinical materials for the period 2011 – 2020

Year	Patient	Age	Primary diagnosis	Clinical material	Result from PCR - test
2011	Pregnant woman	25	16 gestational weeks	amniotic fluid	negative
	Pregnant woman	40	22 gestational weeks	amniotic fluid	negative
2012	dead fetus	0	congenital infection	brain	negative
	dead fetus	0	congenital infection	brain	negative
	new-born baby	7 days	congenital infection	umbilical cord and blood sample	negative
	dead fetus	0	congenital infection	brain	negative
2013	Pregnant woman	40	18 gestational weeks	amniotic fluid	negative
2014	dead fetus	0	congenital infection	brain and lung sample	negative
2015	no patients studied				
2016	man	28	acute iridocyclitis	intraocular fluid	negative
	woman	61	pan uveitis	intraocular fluid	negative
	woman	29	Uveitis posterior oc. sin.	intraocular fluid	negative
	dead fetus	0	Dandy-Walker syndrome	brain	negative
	dead fetus	0	Dandy-Walker syndrome	brain	negative
2017	baby	1 month	Suspected congenital toxoplasmosis	blood sample	negative
	man	85	Melanoma malignant	brain sample	negative
	baby	22 days	Hydrocephaly interna	cerebrospinal fluid	negative
	woman	48	Lymphadenitis reg. coli sin.	blood sample	negative
2018	HIV-infected man	33	Suspected cerebral toxoplasmosis	cerebrospinal fluid	negative
	new-born baby	7 days	Suspected congenital toxoplasmosis	umbilical cord	negative
	new-born baby	23 days	Suspected congenital toxoplasmosis	brain sample	negative
2019	HIV-infected woman	27	Suspected cerebral toxoplasmosis	cerebrospinal fluid	negative
	woman	73	Generalized lymphadenopathy	lymph node	negative
	woman	41	Uveitis posterior oc. sin.	vitreous body	negative
	baby	1 year	acute iridocyclitis	vitreous body	negative
2020	man	71	Suspected cerebral toxoplasmosis	cerebrospinal fluid	negative
	man	42	Generalized lymphadenopathy	cerebrospinal fluid	negative
	woman	24	Uveitis oc. dex.	vitreous body aspirate	positive
	HIV-infected man	43	Suspected cerebral toxoplasmosis	cerebrospinal fluid	negative
	new-born baby	2 months	Suspected congenital toxoplasmosis	cerebrospinal fluid	positive

As a result of testing pregnant women aged 16 to 43, and in different stage of pregnancy for the period 2011-2020, we found that 18.74% of pregnant women respond to the criteria for presence of recent *Toxoplasma* infection. In 4.87% of them, low IgG avidity was found, and in 7.7% values were for high avidity, despite the presence of specific IgM and / or IgA antibodies. The patients suspected for ocular form of toxoplasmosis (uveitis, chorioretinitis, iridocyclitis) ranged in age from 2 to 79 years, of which 74 were males and 107 females. In 56% (43 men and 58 women), the tests were negative, only with specific IgG antibodies were 36.6% (23 men and 43 women aged 8 to 74 years), with evidence of acute infection were 3.3%, and four of them were positive for specific IgG and IgM antibodies, and two for IgG, IgM and IgA antibodies. In 8 of the studied patients were detected only IgG and IgA, as 3 (37.5%) of them were male and 5 (62.5%) were female. Febrile conditions of unknown origin often are associated with toxoplasmosis. At the NRL for the period 2011-2020 were studied 24 men and 50 women, all over the age of 18. With negative tests were 72% of them. Positive for IgG were 24.3% (5 men and 13 women). Presence of specific IgG and IgM, as well as IgG, IgM and IgA anti-*Toxoplasma* antibodies were detected in two males. Patients with HIV and AIDS tested for toxoplasmosis during the study period were 225 men and 61 women, and the materials for the study at the NRL were obtained from clinics in the country specialized for treatment of such patients. Latent toxoplasmosis (presence of IgG antibodies only) was detected in 64 males and 20 females, and in 50 (60%) of them the levels were equal to or greater than 200 IU / ml (37 males and 13 females.). By prophylactic indications, including suspected toxoplasmosis, forthcoming in vitro, recent spontaneous abortion, pending transplantation, etc. 84 men and 354 women were studied. Of them 56% were negative, 30% had only specific IgG antibodies, and 13.9% had evidence of recent infection. Of these, IgG and IgM were detected in 8.4% (2 men and 35 women) and IgG, IgM and IgA antibodies in 5.5% (2 men and 22 women). Data from the PCR analyzes performed at the NRL are presented in Table 2.

Only in 6.7% of the studied patients was detected DNA of *T. gondii* in the examined clinical materials.

4. Discussion

Toxoplasmosis, caused by the obligate intracellular protozoan *Toxoplasma gondii*, is an important zoonotic disease of medical and veterinary importance worldwide. The diagnosis and genetic characteristics of *T. gondii* infection are crucial for the surveillance, prevention and control of toxoplasmosis. Key to effective treatment of toxoplasmosis is the timely and accurate diagnosis of the disease [4]. The detection of acute *Toxoplasma gondii* infection in pregnant women due to the risk of congenital toxoplasmosis is of particular global interest [5]. The results of the NRL tests dysplayed that over 80% of pregnant women are negative for toxoplasmosis or have a latent form that does not require treatment. Interpreting the results of serological tests in pregnant women and assessing the need of etiological treatment must take into consideration the gestational age of the fetus and the findings of ultrasound monitoring of its development. Serological study from the 90s of 20 pregnant women at the NRL found presence of IgG and IgM anti-*Toxoplasma* antibodies in 5 of them (25%) and data of latent infection with presence of IgG only in 15 (75%) [6]. another study for toxoplasmosis of 301 pregnant women was performed in 2014-2016, and the results were close to those obtained in the present study [7]. A total of 67 (22.24%) of the surveyed pregnant women had evidence of recent infection, as 6.63% were positive for IgG, IgM and IgA specific antibodies, 15.61% for IgG and IgM, with data for latent infection and presence of IgG antibodies alone were 26.93%, and over 50% were negative for toxoplasmosis. A comparison of the results of the studies shows that in the 1990s, latent infection and only IgG antibodies were found in pregnant women, and mainly IgG and IgM antibodies in those with suspected recent infections. For the period between 2011-2020 of the pregnant women studied at the NRL, 6% were with presence of specific IgG, IgM and IgA antibodies and low avidity, which allows the time of infection to be placed at about 4 to 6 months ago According to the stage of pregnancy, most of the studied women were in their second trimester - 411, of which 79 (19.2%) were positive for IgG and IgM or IgG, IgG, IgM and IgA specific antibodies. During the first trimester of pregnancy, 361 pregnant women were tested for toxoplasmosis, of which 60 (16.6%) had serological evidence of recent infection. The relative share of pregnant women in the third trimester with acute toxoplasmosis was 36 (21%) in a total of 172 suspected patients. The results of the study of pregnant women according to the described algorithm confirm the data of other authors that the infection with *T. gondii* increases with the progress of pregnancy [8]. In an immunocompetent host, the primary infection is usually oligosymptomatic, self-limiting, has a favorable prognosis and does not require specific treatment. Less than 10% of infected patients are symptomatic, with lymphadenopathy being the most common clinical sign [9]. The number of patients with lymphadenitis identified in the ten-year period of our study was 215. Of them 132 (62%) were negative. Positive for IgG only were 37 (17%). Positive for IgG and IgM were 35 (13%) and for IgG, IgM and IgA were 11 (5%). Women diagnosed with lymphadenitis and acute toxoplasmosis is higher than the number of men (34 to 19), and such results have been reported by other authors who point out that women are more exposed to a risk from infection due to the fact that they cook and care for cats more often [10]. Detection in Bulgaria of the virulent genotype I of *Toxoplasma gondii* is probably the reason for more frequent detection of patients with evidence of acute disease and lymphadenitis. Among the children and adolescents under 19, a total of 29, of whom 12 boys and 17 girls, recent toxoplasmosis was not diagnosed. Presence of specific IgG antibodies was found in 2 girls aged 7 and 9, and in a boy aged 10. Ocular

toxoplasmosis has been demonstrated as progressive and recurrent necrotizing retinitis, with vision-threatening complications such as retinal detachment, choroidal neovascularization, and glaucoma that may occur at any time during the clinical course. Seropositivity for *T. gondii* infection is relatively high worldwide and the presence of antibodies against *T. gondii* is only useful for confirming previous parasite exposure. However, this serology findings cannot confirm the diagnosis of ocular toxoplasmosis. The diagnosis of ocular toxoplasmosis is based on ophthalmological examinations and various clinical manifestations that are consistent with retinal infection with *T. gondii*. When this clinical diagnosis cannot be definitively established by fundoscopic examination, detection of elevated titers of antibodies against *T. gondii* in ocular fluids or amplification of *T. gondii* DNA has been successfully used to confirm the diagnosis [11]. Despite the fact that serological diagnosis based on the detection of specific anti-*Toxoplasma* antibodies in serum is not leading in the diagnosis of recently acquired ocular toxoplasmosis, 3.3% of the patients tested at the NRL correlated with newly discovered clinical symptoms and serological evidence of current infection. In individuals with only IgG and IgA, we assume reactivation of latent infection due to anamnestic evidence of past ocular pathology and current evidence of active inflammatory foci in the retina. Unfortunately, in Bulgaria ocular fluid is rarely used as a study material in patients with suspected ocular toxoplasmosis. Intraocular production of anti-*Toxoplasma* IgA antibodies is significantly more reliable evidence of disease reactivation, and there is relatively little evidence in the literature between the presence of specific IgG and IgA antibodies in serum samples from ocular toxoplasmosis patients and whether the infection is recent or reactivated [12]. The presence of intra ocular anti-*T. gondii* IgA antibodies not only during postnatally acquired but also during recurrent disease could be the result of the unique properties of the local environment of the antibody-producing plasma cells. It is well known that TGF-ß is an important factor in directing B cells to produce IgA antibodies and that the intraocular compartment contains relatively high levels of this cytokine, which may explain intraocular anti-*T. gondii* IgA antibody production in ocular toxoplasmosis.

Fever of unknown origin (FUO) and weakness in the upper legs are among the symptoms of acute toxoplasmosis, which can occur in immune-competent individuals [13]. According to Ho-Yen (1992), fever as one of the nonspecific signs was reported in 70% of cases of acute toxoplasmosis in an outbreak among 99 students [14]. The results of our study displayed that the presence of pyrexia may be associated with toxoplasmosis in 2.7% of the subjects with FUO. Human latent toxoplasmosis occurs in about half of the world's population, although most cases are asymptomatic. With the onset of the human immunodeficiency virus (HIV) pandemic, toxoplasmic encephalitis (TE) has become one of the most common opportunistic infections and the most common cause of focal brain lesions complicating the course of AIDS [15]. For the period studied by us, no data were found for HIV carriers with acute toxoplasmosis, but in 60% of the studied patients the titer of specific IgG anti-*Toxoplasma* antibodies was high. The high titer of anti-*Toxoplasma* IgG may be a sign of both primary infection and reactivation of a latent process due to advanced immune suppression. According to some authors, the titer of specific IgG antibodies above ≥150 international units / ml in the enzyme-linked immunosorbent assay (ELISA) is a prognostic indicator for the development of toxoplasmic encephalitis [9]. For a period of 10 years, clinical manifestations of cerebral toxoplasmosis were found in two of the HIV patients infected with *T. gondii*, and the diagnosis was confirmed by imaging studies (MRI and CAT) [16]. Specific anti-*Toxoplasma* IgM and IgA in these patients have not been demonstrated.

The subjects examined for prophylaxis lacked clinical symptoms, although 13.9% had serological evidence of current infection. We believe that in these cases, it is an infection with apathogenic strains of the parasite, in which the infection is asymptomatic and the diagnosis can be made after serological tests.

5. Conclusion

Laboratory "Diagnosis of Parasitic Diseases" at the NCIPD received a status of National Reference Laboratory by decree of the Ministry of Health in 2001. Over the years, it has established itself as a laboratory performing reference and confirmatory diagnostics in unclear and complicated cases. Carrying out serological tests for toxoplasmosis is one of the important activities of the laboratory, moreover, the study of the avidity of specific IgG antibodies is carried out only in the reference laboratory, as well as most PCR for *Toxoplasma gondii* DNA studies of autopsy material from fetuses, amniotic fluid, lymph node aspirations, and vitreous humor. For the ten-year period of the study with evidence of recent toxoplasmosis were 11.2% of those studied for clinical indications, and with latent infection were 20% of the surveyed, which shows that in Bulgaria this parasitic disease is widespread. Although the latent form prevails, acute toxoplasmosis is often diagnosed, especially in pregnant women, which poses a risk for the fetus. We believe that the diagnostic algorithm we have adopted is suitable for management of the infection and helps for timely treatment of the clinically manifested cases, despite the fact that in the health network of the country there are limitations on the use of molecular diagnostic methods.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

Statement of ethical approval

The study was reviewed and approved by the institutional review board (IRB) 00006384 and informed consent was obtained from the patients.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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