

SEROLOGICAL DIAGNOSIS OF TRICHINELLOSIS IN PATIENTS IN SOUTHERN AND EASTERN SERBIA IN THE PERIOD FROM 2007 TO 2018

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Abstract

Introduction. The Nišava District has had low prevalence and incidence rates of trichinellosis for a long period. The aims of this study were to present the frequency of occurrence of trichinellosis in Nišava District and other districts in Southern and Eastern Serbia during 2007-2018, to investigate the demographic characteristics in the region and to compare the tests used for serological diagnosis of trichinellosis.

Materials and Methods. This was a retrospective epidemiological study in which we analyzed the results of serological analyses of 139 serum samples taken from individual patients suspected to be infected with *Trichinella spp.* Indirect immunofluorescence (IIF) and immunoenzyme (ELISA) methods were used for serological diagnosis of trichinellosis.

Results and Conclusions. Of 139 examined sera, 36 (25.9%) were seropositive. Most seropositive findings were encountered in 2012 and 2014 (in both these years, 12 sera were positive, i.e. 33.3% of the total 36 positive sera), when three minor outbreaks of trichinellosis were reported. Seropositivity was equally distributed according to patient sex and according to the district where they reported with illness. Positive serological findings were statistically significantly more common in hospitalized patients than in out-patients. Comparison of the findings with both IIF and ELISA test established the performance level of the tests, with the ELISA test being less sensitive than the IIF test.

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Immunodiagnostic procedures for the diagnosis of trichinellosis must be improved in view of the sporadic cases and minor outbreaks of the disease in the studied districts of Eastern and Southeastern Serbia.

Key Words: trichinellosis, incidence, serodiagnosis, epidemiology, *Trichinella*

INTRODUCTION

The nematodes belonging to the *Trichinella* genus cause the human parasitic zoonotic disease, trichinellosis. In many parts of the world, trichinellosis is an endemic disease and a major public health problem (Maizels et al., 2012). It ranks seventh among the ten food-borne parasites and endangers the health of a million people worldwide (FAO/WHO, 2019).

In Eastern European countries, trichinellosis is one of the most important food-borne diseases. In Serbia, it usually occurs sporadically, but endemic regions nevertheless exist (Srem, the river valleys of Danube, Drina, Kolubara), while individual smaller outbreaks also occur (Čuperlović et al., 2005). Nišava District is not a region with any significant prevalence of trichinellosis. A study of seroincidence of trichinellosis in the period from 1988 to 2004 reported low incidence and prevalence rates of anti-*Trichinella* seropositivity (Mišić et al., 2006).

Human infection with *Trichinella* species is the result of ingestion of insufficiently cooked meat of domestic or wild animals containing the infectious larvae. Most human infections are associated with consumption of pork, horse meat or wild game meat or their products, depending on cultural and socioeconomic factors, animal housing/feeding conditions, veterinary control of the infection in animals and food preparation and consumption habits (Pozio, 2007). The most widespread species and most pathogenic to humans is *Trichinella spiralis*. In Serbia, this pathogen is present in domestic animals (usually pigs), as well as in synanthropic and sylvatic animals (Pozio, 2007).

In addition to the clinical picture trichinellosis produces, laboratory biochemical and immunodiagnostic tests are essential in the diagnosis of this disease, especially for sporadic cases and household outbreaks of the disease (Muñoz-Carrillo et al., 2018).

The aim of this study was to present the incidence of trichinellosis in Nišava District and other regions in the Southern and Eastern Serbia, based on seropositive findings of anti-*Trichinella* antibodies, to investigate the demographic characteristics of patients and to compare the tests used for serological diagnosis of human trichinellosis.

MATERIALS AND METHODS

This retrospective epidemiological study encompassed a period of twelve years – from 01.01.2007 to 31.12.2018 – in which we analyzed the results of serological analysis of 139 serum samples taken from individual patients suspected to be infected with *Trichinella* spp. Results were retrieved from the database of the Centre for Microbiology,

Institute of Public Health Niš (IOPHN). Serum samples from patients suspected to be infected with *Trichinella* spp. were sent to IOPHN not only by doctors from Nišava District, but from other Southeastern Serbian districts as well. We sourced data on the occurrence of trichinellosis in Serbia from annual reports published by The Institute of Public Health of Serbia (IOPHOS) Milan Jovanović Batut.

In IOPHN's Laboratory for Immunological Diagnosis of Parasitic and Fungal Infections, specific antibodies against *Trichinella spiralis* antigens were detected using the indirect immunofluorescence test (IIFT, INEP, Belgrade) and the immunoenzyme test (ELISA) (NovaLisa *Trichinella spiralis* IgG, ELISA, NovaTec Immunodiagnostica GmbH, Germany). Serological analyses were performed in accordance with the test manufacturer's guidelines. In accordance with these guidelines, IIFT values were considered as positive if $\geq 1:40$, i.e. if the absorption values obtained with the ELISA test exceeded 10% related to the cut-off value, or negative if the absorption values were lower than 10%. All the values between the above ones were considered as equivocal, and required repeated testing with a new sample 2-4 weeks later. The results of ELISA testing were calculated in accordance with the manufacturer's guidelines and expressed in NovaTec units (NTUs): positive values were those >11 NTU, negative those <11 NTU, and those 9-11 NTU were considered as equivocal (gray zone).

Statistical data processing

The data were presented as mean, standard deviation, median, minimum and maximum values, or as frequencies and relative numbers. The Mann-Whitney U test was used to compare continuous variables between two groups of subjects; chi-squared or Fisher's exact test were used to compare groups. The null hypothesis was tested at $p < 0.05$. Data were analyzed using SPSS for Windows version 16.0.

The non-standardized incidence rate of patients seropositive for trichinellosis in the population of Nišava District was calculated per 100,000 inhabitants. The number of inhabitants was taken from the vital statistics for Nišava District in the observed period.

RESULTS

In the period from 2007 to 2018, we examined 139 serum samples from individual patients suspected to be infected with *T. spiralis*. The largest number of examinations was performed in 2014 (33/23.7%), and the smallest in 2007 and 2009, with 2 examinations in each year (1.4%). Of 139 serum samples, 36 (25.9%) were seropositive using the ELISA and/or IIFT. The greatest numbers of positive findings were reported in 2012 and 2014 (in both years, 12 were positive, i.e. 33.3% of all 36 positive sera). Using the serological tests, there were no seropositive patients in 2007, 2016 and 2017, while in 2009, 2010, 2015 and 2018 only one seropositive finding was reported in each year. The highest ratio of tested/seropositive findings was seen in 2012, when 12/21 (57.1%) sera were seropositive (Table 1).

Table 1. Annual total number (n) and percentage of the total number of suspect sera examined for the presence of specific anti-*Trichinella* antibodies, and number (n) and percentage of total seropositive sera, 2007-2018

Year	Tested sera (n)	Tested sera (%)	Seropositive sera (n)	Seropositive sera (%)
2007	2	1.4	0	0.0
2008	8	5.8	3	8.3
2009	2	1.4	1	2.8
2010	4	2.9	1	2.8
2011	10	7.2	2	5.6
2012	21	15.1	12	33.3
2013	8	5.8	3	8.3
2014	33	23.7	12	33.3
2015	15	10.8	1	2.8
2016	7	5.0	0	0.0
2017	17	12.2	0	0.0
2019	12	8.6	1	2.8
Total	139	100.00	36	100.00

In the studied period, viewed by months, we established two disease occurrence peaks: in March (winter period) (n=11, 30.6%) and September (autumn period) (n=9, 25.0%) (Table 2).

Table 2. Structure of *Trichinella*-seropositive findings by month, 2007-2018

Month	Positive findings		
	number of positive/ total number	% ¹	% ²
January	1/10	10.0	2.8
February	0/14	0.0	0.0
March	11/30	36.7	30.6
April	2/7	28.6	5.6
May	3/6	50.0	8.3
June	1/4	25.0	2.8
July	1/6	16.7	2.8
August	3/8	37.5	8.3
September	9/17	52.9	25.0
October	1/11	9.1	2.8
November	4/19	21.1	11.1
December	0/7	0.0	0.0
Total	36/139	25.9	100.0

¹% in relation to the number of tested sera per month, ²% in relation to the total number of positive findings

Seropositivity was not affected by patient sex ($p=0.544$), although men were slightly more frequently infected than women (Table 3). District had no effect on seropositivity ($p=0.064$) (Table 3). In two districts (Nišava and Bor), men were more frequently affected than women, again without statistical significance ($p=0.755$). However, more seropositive patients were identified in Nišava District (66.7%) than in Bor District (30.6%), while one (1) patient was in Zaječar District (2.8%). In patients from other districts, there were no seropositive findings for the presence of anti-*Trichinella* antibodies. Seropositivity was detected statistically significantly more often in hospitalized patients compared to those who were examined in out-patient settings ($p=0.009$) (Table 3). Patients with positive sera were statistically significantly older (49.5 ± 18.1 years old) than those with negative serological findings (39.0 ± 22.7 years old) ($p=0.011$) (Table 3). Infected patients in Nišava District were older than those in Bor District, although the difference was not statistically significant ($p=0.845$).

Table 3. Demographic and clinical characteristics of the studied population

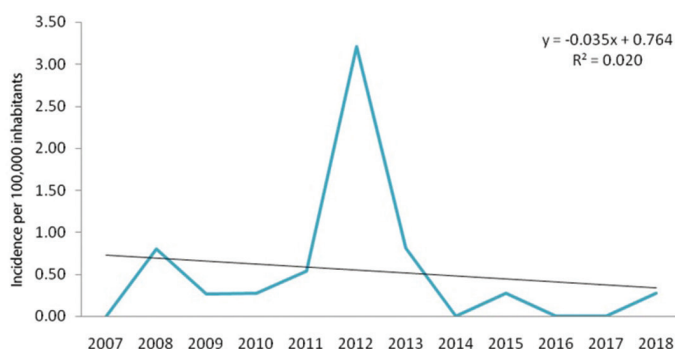
	Total		Positive finding		Negative finding		p ¹
Characteristics	N	%	N	%	N	%	
Gender							
Male	77	55.4	22	61.1	55	53.4	0.544
Female	62	44.6	14	38.9	48	46.6	
Age	41.7±22.1		49.5±18.1		39.0±22.7		0.011 ²
District							
Nišava	88	64.2	24	66.7	64	62.1	0.064
Zaječar	16	11.7	1	2.8	15	14.6	
Bor	27	19.7	11	30.6	16	15.5	
Toplica	2	1.5	0	0.0	2	1.9	
Pirot	3	2.2	0	0.0	3	2.9	
Kosovska Mitrovica	1	0.7	0	0.0	1	1.0	
Jablanica	2	1.4	0	0.0	2	1.9	
Treatment							
Out-patient	78	56.1	13	36.1	65	63.1	0.009
Hospital	61	43.9	23	63.9	38	36.9	
ELISA	27/136	19.9					
IIFT	34/120	28.3					

¹Chi-squared test, ²Mann-Whitney test

The highest incidence of anti-*Trichinella* seropositivity in Nišava District was established in 2012 (3.21 cases per 100,000 inhabitants), while for the whole studied period in this district, it was 6.43 cases per 100,000 inhabitants. In four of the studied years (2007, 2014, 2016 and 2017) the incidence was 0.0 (Table 4), and the overall incidence trend in Nišava District is decreasing (Figure 1).

Table 4. Incidence of seropositive patients per 100,000 inhabitants of Nišava District, 2007-2018

Year	Incidence of anti- <i>Trichinella</i> seropositivity per 100,000 inhabitants
2007	0.00
2008	0.80
2009	0.27
2010	0.27
2011	0.54
2012	3.21
2013	0.81
2014	0.00
2015	0.27
2016	0.00
2017	0.00
2018	0.27
Total	6.43

**Figure 1.** Incidence of anti-*Trichinella* seropositive findings in Nišava District, 2007-2018

IIFT was conducted on 120 patients' sera (86.3%), with 34 (28.3%) being positive. ELISA was conducted on 136 patients' sera, with 27 (19.9%) being positive (Table 3). Comparing the findings of ELISA and IIF tests, 106 coincided, while 11 findings did not.

Based on these data, the performance of the ELISA test was calculated: sensitivity of 70.6%; specificity of 97.1%; positive predicted value (PPV) of 88.9%; negative predicted value (NPV) of 90.8%, and; diagnostic effectiveness of 90.4%. The performance of the IIF test was as follows: sensitivity of 85.3%; specificity of 95.2%; PPV of 85.3%; NPV of 95.2%, and; diagnostic effectiveness of 92.8%.

DISCUSSION

A study of serological incidence from 2006 demonstrated that Nišava District had had low incidence and prevalence rates of anti-*Trichinella* seropositivity near the end of the 20th century and at the beginning of 21st century (Mišić et al., 2006). During 2005 and 2006, due to the lack of tests, serological diagnosis of trichinellosis was not performed in Nišava District, so this period was excluded from the current analysis. According to IOPHOS annual reports, there were 339 reported cases of trichinellosis in Serbia in 2005 (4.52 cases per 100,000 inhabitants), while in 2006 there were 188 reported cases (2.53 cases per 100,000 inhabitants), with 13 reported outbreaks of the disease.

The retrospective analysis of serological findings which we performed for patients suspected to be infected with *Trichinella* spp. involved a twelve-year period. Demographic characteristics had not changed compared to earlier investigations in the region (Mišić et al., 2006). Altogether, 139 examined sera samples were from Nišava and other districts. The greatest number of suspect trichinellosis patients (n=33) occurred in 2012, while in 2007 and 2009, only two suspect patients occurred in each of these years. Of the 139 examined sera, 25.9% were seropositive using anti-*Trichinella* ELISA and/or IIFT.

In Serbia, trichinellosis showed a decreasing trend of incidence in the period 2002-2014. In 2012, there were 46 cases of trichinellosis, an incidence rate of 0.64 cases per 100,000 inhabitants, and in 2014 there were 86 cases of trichinellosis, an incidence rate of 1.2 cases per 100,000 inhabitants, which was markedly higher than in EU/EEA countries (EFSA, 2015).

Most positive findings were seen in 2012 and 2014, when there were three reported trichinellosis outbreaks: in 2012 in Nišava District, and in 2014 in Bor and Zaječar Districts. The highest incidence of anti-*Trichinella* seropositivity per 100,000 inhabitants in Nišava District was seen in 2012 (3.21), while in 2014 there were no suspected trichinellosis cases in this district. In 2012, there were seven outbreaks of trichinellosis in Serbia, and patients seropositive for *T. spiralis* antigens were seen in two outbreaks of the disease, in Bor (Kladovo Municipality) and Zaječar (Negotin and Zaječar Municipality) Districts, after patients consumed infected pork meat or smoked pork sausages. In both cases, infected animals originated from small farms and privately-owned butcher shops. In the first outbreak, veterinary examination did not detect *Trichinella*-infected pork, but in the second outbreak, veterinary examination conducted after the outbreak was confirmed established the meat product contained *Trichinella* spp. larvae.

During the studied period, none of the sera submitted in 2007, 2016 or 2017 were seropositive, while in 2009, 2010, 2015 and 2018, one seropositive serum sample was detected in each year. The highest ratio of tested/seropositive findings was seen in 2012, during the outbreak of trichinellosis in the Nišava District.

The two peaks of trichinellosis incidence we detected, in winter (March) and autumn (September), coincided with the times of two disease outbreaks.

Men were slightly more frequently seropositive than women in our study as a whole and in two key districts (Nišava and Bor), but the differences were not statistically significant. However, patients who were seropositive were older than those who were seronegative (49 *vs.* 39 years old). The results obtained related to sex and age did not differ from the data for Serbia as a whole, or from international data for that matter (Murrell & Pozio, 2011).

Positive serological findings were more frequently found in hospitalized patients than in out-patients. In the first outbreak, a large number of patients had more severe clinical forms of trichinellosis with complications, and so required hospital treatment of the disease (Popović-Dragonjić & Kocić, 2018).

In Nišava District in four of the observed years (2007, 2014, 2016 and 2017), none of the submitted patient sera were seropositive. In 2012 in Nišava District, the incidence of anti-*Trichinella* seropositive patients was 3.21 cases per 100,000 inhabitants. In that year, 21 patients were examined for the presence of anti-*Trichinella* antibodies, of which 13 were found to be affected with trichinellosis during a household outbreak in Subotinac village, Aleksinac Municipality, Nišava District. All of them had positive serological findings for the presence of anti-*Trichinella* antibodies with both tests employed (ELISA and IIF).

In this outbreak, the infection occurred on January 15 and 22, after consumption of traditional home-made smoked pork products (produced without any veterinary control). The most common clinical manifestations of the infection were muscular pain, upper eyelid edema, difficulty chewing, accompanied by marked elevation of creatine phosphokinase and lactate dehydrogenase levels. During the outbreak, eight patients were hospitalized, and 61.5% were diagnosed with myocarditis. The outbreak, however, ended without any fatalities (Popović-Dragonjić & Kocić, 2018).

When human trichinellosis is in its episodic or epidemic form, the diagnosis is much easier to make than in sporadic cases or smaller outbreaks, since these latter patients usually have clinical manifestations that are similar to those of many bowel diseases. In addition to clinical manifestations and epidemiological investigation, laboratory parameters are included in the diagnostic algorithm for trichinellosis, i.e. eosinophilia, muscle enzyme levels, direct parasite detection methods – detection and identification of L1 *T. spiralis* in muscle tissue (plate compression, polymerase chain reaction (PCR), artificial or enzymatic digestion and histology) – and indirect parasite detection methods – detection of serum antibodies against *T. spiralis* antigens (Gottstein & Nöckler, 2009).

Detection of antibodies against *T. spiralis* antigens is valid proof of contact of the affected person with the parasite, and the techniques developed for that purpose are IIF (Chavez et al., 2017), ELISA, Western blot (Wb) (Yera et al., 2003) and double microimmunodiffusion (Moreno et al., 2012).

Immunoglobulin G (IgG) antibodies can be detected 15-60 days after the infection (Dupouy-Camet & Bruschi, 2007) and they can be detected in the patient's blood for more than 30 years after infection (Fröscher et al., 1988). The titer of anti-*Trichinella* specific antibodies does not correlate with disease severity or clinical course in the acute stage trichinellosis (Boszon et al., 1981). The ELISA test, due to its high sensitivity, is most commonly used to detect IgG against *Trichinella* (Dupouy-Camet & Bruschi, 2007, Dupouy-Camet et al., 2002). After the ELISA test, seropositive sera are submitted to specialized laboratories that use the Wb confirmatory test to confirm previous test results (Gamble et al., 2004., Pozio et al., 2003, Robert et al., 1996).

In routine diagnosis in the current study, 34/120 (28.3%) of sera were positive by the IIF test. In comparison, 27/136 (19.9%) of sera were positive by the ELISA test. Altogether, 106 findings agreed between the two tests, while 11 findings did not agree. The commercial ELISA test showed lower sensitivity than the IIF test (70.6% and 85.3%, respectively), while other performance indicators for the two test methods were similar. During two outbreaks of trichinellosis in 2014, confirmatory Wb testing was completed by the Serbian National Reference Laboratory for Trichinellosis for seropositive patients and those with equivocal serological findings.

CONCLUSION

Nišava District remains a region with low prevalence of trichinellosis and low incidence of anti-*Trichinella* seropositivity. In order to improve the immunodiagnostic algorithm for trichinellosis, it is necessary to improve serodiagnostic procedures and to employ confirmatory Wb testing, since sporadic cases of trichinellosis and smaller outbreaks of the disease still occur in Nišava District and regions endemic for trichinellosis in Southern and Eastern Serbia.

Authors contributions

NMT drafted the manuscript. AI performed statistic data analysis

Competing interests

The authors declare that they have no competing interests.

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SEROLOŠKA DIJAGNOZA TRIHINELOZE KOD LJUDI U JUŽNOJ I ISTOČNOJ SRBIJI U PERIODU OD 2007. DO 2018. GODINE

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Kratak sadržaj

Uvod. Nišavski okrug već dugo ima nisku prevalenciju i incidenciju trihineloze. Cilj studije je prikazivanje učestalosti trihineloze u ispitivanom periodu na području Nišavskog i drugih okruga u regionu Južna i Istočna Srbija, ispitivanje demografskih karakteristika i komparacija korišćenih testova u serološkoj dijagnostici trihineloze.

Materijal i metode. U retrospektivnoj epidemiološkoj studiji analizirani su rezultati seroloških nalaza 139 uzoraka seruma osoba suspektnih na infekciju izazvanu vrstom *Trichinella* spp. Indirektna imunofluorescencija (IIF) i imunoenzimski test (ELISA) korišćeni su za serološku dijagnostiku trihineloze. Statistička obrada podataka vršena je u programskom paketu SPSS 16.00.

Rezultati i zaključak. Od ukupnog broja pregledanih uzoraka ustanovljeno je 36 (25.9%) seropozitivnih osoba. Najveći broj seropozitivnih nalaza je utvrđen 2012. i 2014. godine (12/33.3%) kada su zabeležene tri manje epidemije trihineloze. Seropozitivan nalaz je podjednako zastupljen u odnosu na pol, a takođe i distribucija seropozitivnih nalaza je ujednačena po okruzima. Pozitivan serološki nalaz se statistički značajno češće registruje kod pacijenata koji su hospitalizovani u odnosu na ambulantno pregledane. Upoređivanjem nalaza dobijenih IIF i ELISA testom utvrđene su performanse oba testa i ELISA test je manje osetljiv nego IIF test. Dobijeni rezultati pokazuju da je ispitivano područje sa niskom prevalencijom i incidencijom trihineloze. Neophodno je poboljšanje imunodijagnostičkih procedura u dijagnostici trihineloze s obzirom na sporadične slučajeve oboljevanja i manje periodične epidemije trihineloze u ispitivanom okrugu, ali i u drugim okruzima jugoistočne Srbije.

Ključne reči: trihineloz, incidencija, serodijagnoza, epidemiologija, jugoistočna Srbija