

Short notes

The value of systematic screening for *Trichinella* antibodies among individuals with eosinophilia in recognizing outbreak events: a seroprevalence study from Croatia

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ABSTRACT. Although trichinellosis represents one of the most significant global foodborne zoonotic diseases, human seroprevalence studies are scarce. Due to its endemicity in Croatia, the aims of this study were to establish the general prevalence of *Trichinella* infection in Croatian individuals presenting with eosinophilia during a 5-year period, and to assess the value of such screening endeavours for early detection/differentiation of outbreaks. A total of 1342 serum samples were collected from male and female subjects presenting with peripheral blood eosinophilia between 2013 and 2017, and tested for *Trichinella* IgG antibodies by employing commercial qualitative ELISA and *Trichinella* Excreted/Secreted (E/S) Western blot methods. The overall positivity was 3.65%, ranging from 0.49% to 1.50% between 2013 and 2016, but rising to 10.98% in 2017; such ten-fold increase in seroprevalence forecasted the epidemic situation in Croatia. Overall the prevalence of the infection rose with the increasing age and the rate was highest among those older than 40 years of age ($p=0.003$), without any significant sex-based differences ($p=0.438$). Considering the early appearance of eosinophilia in the infected individuals, this type of systematic screening can be seen as an additional epidemiological tool to unveil the trichinellosis outbreak in a timely manner.

Key words: *Trichinella* spp., trichinellosis, seroprevalence, eosinophilia, epidemiology

Introduction

Trichinellosis is a parasitic disease caused by nematodes of the genus *Trichinella*, and one of the most expansive zoonoses with world-wide distribution [1]. Due to the ease of transmissibility between animals and humans as a result of meat consumption there may be serious health issues, although the severity of human disease depends on a plethora of factors, such as parasitic burden, frequency of infected meat consumption, individual susceptibility, and the implicated species [2]. Regarding the latter, *Trichinella spiralis* (*T. spiralis*) is the species that continues to cause a majority of outbreaks [1,3].

At the end of last century, a substantial increase

in human trichinellosis has been demonstrated in the Eastern Europe and specifically Balkan countries (including Croatia) – primarily due to the political, social and economic challenges of an era [4–7]. The phenomenon can also be related with the upsurge of ecological farming practices and increase of animal species that are a part of wild life protection [8]. However, in Europe most outbreaks can be attributed to non-controlled outdoor/backyard pig husbandry [3,9].

Croatia is still considered endemic for *Trichinella* infections, especially the eastern parts of the country [10]. Although there was a sharp decline in the number of human cases after 2000 [8], the endemic pockets can act as potential reservoirs of trichinellosis outbreaks. There was even a

Table 1. *Trichinella* seropositivity trends in Croatia according to the number of tested individuals with eosinophilia in the 5-year period from 2013 to 2017

Year	Total number of tested (ELISA + WB)	Positive cases (ELISA + WB)	Negative	Indeterminate (ELISA positive or equivocal, WB negative)
2013	205	1	200	4
2014	225	3	218	4
2015	241	3	230	8
2016	334	5	318	11
2017	337	37	285	15
Overall	1342	49	1251	42

multidisciplinary working group in Croatia founded specifically for trichinellosis to minimize the threats and reduce all negative consequences of this disease, consisting of experts and researchers from different fields of work [7].

Despite all that effort, outbreaks are usually discovered when symptomatic cases start to emerge [11]; furthermore, seroprevalence studies on *Trichinella* in humans are scarce in general, as most research is focused on the analysis of animals and their product. This topic was thus far not explored in the Republic of Croatia, which is especially pertinent since there are no studies addressing the question whether systematic screening of individuals with eosinophilia may aid in early detection of this condition.

Therefore, the primary aim of this research was to establish the general prevalence of *Trichinella* antibodies in Croatian individuals presenting with eosinophilia during a 5-year period. Our aims were also to assess the value of such a serological screening for the early detection of outbreaks (most notably in countries endemic for this infections), as well as to additionally evaluate the need for confirmatory testing of positive serum samples when using classical screening methods (such as immunosorbent assay).

Table 2. Year-by-year breakdown of Croatian individuals with eosinophilia seropositive to *Trichinella* antibodies according to their respective age and sex

Age group	2013		2014		2015		2016		2017		All years	
	M	F	M	F	M	F	M	F	M	F	M	F
0–19	/	/	1	/	/	/	1	/	6	3	8	3
20–39	/	1	/	/	/	1	/	/	7	4	7	6
40–60+	/	/	1	1	2	/	4	/	9	8	16	9
Total	/	1	2	1	2	1	5	/	22	15	31	18

Materials and Methods

A total of 1342 serum samples were collected in a five-year period (i.e. between 2013 and 2017) from male and female Caucasian individuals residing in the Republic of Croatia. Established peripheral blood eosinophilia (defined as more than 500 eosinophils per mL of blood) was the primary inclusion criteria in this study. Serological analysis was performed at the Department of Parasitology of the Croatian National Institute of Public Health (CNIPH), Zagreb, Croatia.

Serum samples were first tested using a commercial qualitative enzyme-linked immunosorbent assay (ELISA) for the detection of IgG class antibodies against *Trichinella* spp. (NovaTec Immunodiagnostica GmbH, Germany), according to the manufacturer's recommendations. The manufacturer also stated a diagnostic sensitivity of 100% and diagnostic specificity of 94.81% of this test, and the testing kit was additionally validated in the laboratory.

Samples that were initially reactive were subsequently confirmed by employing *Trichinella* Excreted/Secreted (E/S) Western blot (WB) IgG test (LDBIO diagnostics, Lyon, France), a qualitative test of serologic *Trichinella* IgG diagnosis by

Table 3. Clustered seroprevalence to *Trichinella* spp. in Croatian patients with eosinophilia relative to age and sex

Age group (years)	Male	Female	Total
	no. positive/no. tested (%)	no. positive/no. tested (%)	no. positive/no. tested (%)
0–19	8/365 (2.19%)	3/227 (1.32%)	11/592 (1.86%)
20–39	7/157 (4.46%)	5/146 (3.42%)	12/303 (3.96%)
40–60+	16/255 (6.27%)	10/192 (5.21%)	26/447 (5.82%)
Total	31/777 (3.99%)	18/565 (3.19%)	49/1342 (3.65%)
<i>p</i> -value	0.036	0.076	0.003

immunoblot assay intended for confirmatory testing of positive or equivocal results obtained via classical screening methods. According to the manufacturer's instructions, a positive Western-blot was defined by the presence of three bands at 37, 41 and 50 kDa. Furthermore, all serum controls (R10) provided with the kit were systematically included in the immunoblot series. The manufacturer also stated a diagnostic sensitivity of 97.5% and diagnostic specificity of 96.3%, and the testing kit was additionally validated in the laboratory.

Data analysis was performed by using descriptive and quantitative statistical analysis, the latter by employing a chi-square test. Statistical significance was established at the *p*-value < 0.05 level (two-sided). Stata/IC 14.0 for Windows software (StataCorp LP, USA) has been used for analysis purposes.

Results

There was a total of 1342 individuals included in the study whose serum samples was interrogated for *Trichinella* seroprevalence, and in the aforementioned five-year period (2013–2017) the overall positivity was 3.65% (49/1342). In addition to 49 true positive samples, during the testing period a total of 42 serum samples were either positive or equivocal by ELISA testing, but then proved to be negative when WB was utilized for final assessment (Table 1).

The number of individuals with eosinophilia subjected to *Trichinella* testing steadily increased from 205 in 2013 to 337 in 2017 (Table 1). A total percentage of positive samples (as confirmed with Western blot) ranged from 0.49% to 1.50% between 2013 and 2016 (average 1.14%), but rose to 10.98% in 2017 (Table 1). This stark difference in trend was statistically significant ($\chi^2=69.08$, $p<0.001$), and such ten-fold increase in seroprevalence forecasted the epidemic situation in the Republic of Croatia.

A breakdown of those positive for *Trichinella* antibodies was statistically evaluated according to sex and respective age groups. During the whole testing period, a total of 3.99% seropositive men (31/777) and a total of 3.19% (18/565) seropositive women was noted (Table 2), but this sex-based difference was not statistically significant ($\chi^2=0.6009$, $p=0.438$). When considering just the epidemic year (or 2017), the difference between seropositive men and women was also not statistically significant (12.22% of seropositive men vs. 9.55% of seropositive women; $\chi^2=0.6108$, $p=0.434$); nonetheless, in the preceding year (or 2016) there were no women diagnosed with *Trichinella* infection, compared to five seropositive men (Table 2).

Our sample was divided to three age groups similar in size for comparison purposes: those between 0–19 years of age, those between 20–39 years of age, and those between 40–60 years of age and older. Overall there was a statistically significant difference between seropositivity among different age groups ($\chi^2=11.4505$, $p=0.003$); more specifically, prevalence of the infection rose with the increasing age (Table 3). The same tendency of rising prevalence with age was noted when men and women were observed separately, although the trend was statistically significant only for men ($\chi^2=6.6455$, $p=0.036$), and not for women ($\chi^2=5.1312$, $p=0.076$) (Table 3). It has to be noted that seropositive children up to 14 years of age were found only in the last year of testing (i.e. 2017), with an absolute predominance of male gender (66.67% of male vs. 33.33% of female children).

Discussion

Trichinellosis is a worldwide zoonosis reported in 55 countries worldwide, with an estimated ten million people currently at risk for this parasitic

infection [12,13]. One of its salient features is peripheral blood eosinophilia (sometimes termed hypereosinophilia) that arises as a consequence of T-helper 2 activation following stimulation from *Trichinella* antigens [14]. As eosinophilia appears early during trichinellosis (i.e. before general clinical signs and symptoms are evident), it can be seen as one of the forecasting signs of this infection [14].

But although this association with eosinophilia is well-established, *Trichinella* seroprevalence reports on human subjects with eosinophilia are scarce. This is the first serological study on the topic from Croatia, carried on 1342 individuals during a 5-year period that found overall positivity rate of 3.65%. More specifically, the average seropositivity rate between 2013 and 2016 was 1.14%, which increased by almost 10 times in 2017 (when *Trichinella* spp. was detected in 10.98% of those with peripheral blood eosinophilia), pointing towards the outbreak situation.

Considering the appearance of eosinophilia early in the course of the disease, this type of systematic screening can be therefore seen as an epidemiological tool to unveil the outbreak early when such drastic rise of prevalence is noted, and also to better appraise its extent once established. However, here it has to be noted that the presence of eosinophils in peripheral blood is not specific for this condition *per se*, since it can be seen in other helminth infections, allergic diseases, drug hypersensitivity reactions, malignancies and idiopathic hyper-eosinophilic syndrome [15].

Conversely, although hypereosinophilia is always present during the acute phase of the disease and it represents a key sign to diagnose acute trichinellosis, there are literature descriptions where it is absent. For example, a similar study from the US that covers a five-year period (2008–2012) describes a total of 84 confirmed trichinellosis cases that were reported to the US Centers for Disease Control and Prevention (CDC); however, only 45 (51.72%) of them had eosinophilia [16]. This means that, although eosinophilia can be considered a valid parameter when discussing surveillance and screening efforts for trichinellosis, it certainly cannot be the only criterion.

The aforementioned US study coincides with our study in several other findings. Considering all trichinellosis cases in the US study, 68% (57/84) of them occurred among men and 32% (27/84) among women [16]. In our study, when absolute numbers

are concerned, 63% of cases occurred among males (31/49), and 37% among females (18/49). Furthermore, the age distribution among male and female patients was similar to our study; more specifically, 81% (68/84) and 78% (38/49) of the cases were among those aged 20–60+ years of age in the US study and our study, respectively.

A very recent community-based study on Roma and non-Roma population of Slovakia that employed the same methodology as seen in our study aimed to estimate the seroprevalence of trichinellosis [17]. The authors found just two confirmed seropositive samples to *Trichinella* out of 823 tested ones (after ELISA test initially indicated three positive ones), both of them belonging to Roma women aged 35 and 44 [17]. This type of random, unguided screening shows a better yield of positive cases could be achieved with the use a predefined bundle of criteria, where peripheral eosinophilia would undoubtedly play a significant role.

But this Slovakian paper also highlighted one important finding in our study, which is possible indeterminate serological findings (i.e. ELISA positive or equivocal, and WB negative). WB has great specificity, but this technique is not amenable for routine diagnostic purposes as it is time-consuming, cumbersome, necessitates technical expertise and is rather expensive [18]. On the other hand, ELISA cannot be used as a sole diagnostic technique due to a significant number of indeterminate findings, as evidenced by our study. Thus, WB has to be used as an adjunct method to confirm positive results obtained with other serological tests. Moreover, an improved discrimination of trichinellosis from patients other helminth infections can be achieved with WB, although cross-reaction can also be observed in patients with toxocariasis, filariasis, and some other parasitic infections [18].

Our study has several limitations. Although *T. spiralis* is by far the most prevalent species in Croatia (and can be considered a putative parasitic agent in our study), the serological tests can only distinguish the infection on a genus level. In addition, as a screening approach *Trichinella*-specific IgG were sought in human sera, which are not only observed during current infection, but can also indicate that the infection with this parasitic agent had occurred sometime in the past. Furthermore, due to the unexcluded likelihood of infection by some other parasite, as well as other

potential causes of eosinophilia, it was impossible to ascertain that *Trichinella* was the actual (or sole) cause of eosinophilia in our positive patients. Finally, clinical course of our subjects with eosinophilia was not included in the analysis.

There is also a valid question regarding the applicability and economic aspects of this type of screening in larger countries. Making global recommendations for implementing such systems is not an easy task, as many practical aspects must be considered alongside scientific ones. In addition, working conditions in developing countries (particularly in tropical climates) often do not meet requirements for such systematic screening.

Nonetheless, we can conclude that this study has upheld the hypothesis that trichinellosis is a parasitic disease still pertinent to Croatia, and that ongoing, systematic screening of patients with eosinophilia can detect peaks of seropositivity that may point towards possible outbreak situation in a timely fashion. The approach is simple, yet highly valuable from both clinical and public health perspective. Its value should be hence advocated to health professionals and public at large, alongside other preventive measures to curb the burden of this parasitosis.

Conflict of interest: None.

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Received 18 February 2019
Accepted 25 April 2019