*Journal of Helminthology*

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# Seroprevalence and microscopy detection rates of strongyloidiasis in Croatian patients with eosinophilia

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## Abstract

Infection with the parasitic nematode *Strongyloides stercoralis* is characteristic for tropical and subtropical regions of the world, but autochthonous cases have been reported in European countries as well. Here we present the first nation-wide survey of *S. stercoralis* seroprevalence in Croatian individuals presenting with eosinophilia, and evaluate the fraction of positive microscopy rates in stool specimens of seropositive individuals. In our sample of 1407 patients tested between 2018 and 2021, the overall prevalence of strongyloidiasis was 9.31%, with significantly higher rates in those older than 60 years of age ($P = 0.005$). Of those, one-quarter (25.95%) were also positive following microscopy examination of faeces after using the merthiolate–iodine–formaldehyde concentration method. Our findings reinforce the notion of endemic strongyloidiasis transmission in Croatia, particularly in older individuals, and highlight the need to consider the presence of *S. stercoralis* in patients with eosinophilia.

**Short Communication**

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**Cite this article:** Sviben M, Meštrović T, Topić MB, Sternak SL, Dida GO (2023). Seroprevalence and microscopy detection rates of strongyloidiasis in Croatian patients with eosinophilia. *Journal of Helminthology* **97**, e10, 1–5. https://doi.org/10.1017/S0022149X22000888

Received: 28 April 2022
Revised: 26 August 2022
Accepted: 15 December 2022

**Keywords:**
*Strongyloides stercoralis*; strongyloidiasis; seroprevalence; microscopy; eosinophilia; epidemiology; parasitic diseases; Croatia

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## Introduction

Strongyloidiasis represents a neglected, but pervasive parasitic helminth disease caused by the nematode *Strongyloides stercoralis* (Krolewiecki & Nutman, 2019). Although it is most commonly observed in tropical and subtropical regions of the world due to the impact of warmer temperatures and humidity on the growth/survival of geohelminths, both sporadic and endemic transmission can be observed in certain temperate countries as well, such as the United States, Japan, France, Italy and Spain (Kitchen *et al.*, 2000; Román-Sánchez *et al.*, 2003; Hirata *et al.*, 2007; Abrescia *et al.*, 2009; Masucci *et al.*, 2011). Southern Europe is particularly viewed as a pool of autochthonous cases among individuals who never travelled to tropical/subtropical areas (Duvignaud *et al.*, 2016).

A recent study suggested that the transmission of infection can still be observed in central Croatia, with certain arguments in favour of disease endemicity (such as the diagnosis of acute infections in an immunocompetent autochthonous population lacking any significant travel history) (Balen Topić *et al.*, 2021). Nonetheless, the aforementioned study is based on 65 patients with strongyloidiasis, thus we still lack epidemiological data on a larger number of patients with certain hallmark signs of infestation – such as eosinophilia. More specifically, can *S. stercoralis* infection (infestation) be viewed as an important cause of autochthonous eosinophilia in countries such as Croatia, but also other neighbouring countries?

However, the issue of accurate diagnosis still remains a significant challenge, resulting in a general underestimation of strongyloidiasis prevalence (Requena-Méndez *et al.*, 2013). A direct examination of the stool smear has a tremendously low sensitivity for *S. stercoralis*, primarily due to intermittent excretion of larvae in the faeces. The sensitivity of other stool-based tests, such as agar plate culture, the Baermann method and polymerase chain reaction (PCR) assay, is comparatively better, but still of limited utility for low-burden infections; in addition, molecular methods come with important cost considerations (Requena-Méndez *et al.*, 2013). In addition, the PCR can be used for occasional testing on other body fluids (Buonfrate *et al.*, 2018). Therefore, serology testing is currently considered the most effective diagnostic method for screening purposes in either the general population or among at-risk subjects (such as migrants from endemic areas).

Consequently, the primary aim of our nation-wide study was to establish the general prevalence of *S. stercoralis* antibodies in Croatian individuals presenting with eosinophilia during a

recent four-year period, especially since such country-level prevalence studies of strongyloidiasis in Europe are very scarce. Our aim was likewise to compare its prevalence in different age groups, as well as to appraise the proportion of positive microscopy rates in concentrated stool specimens of seropositive individuals for informing further diagnostic considerations.

## Materials and methods

This retrospective study aimed to analyse serology and stool microscopy data (based on a specific stool concentration method, as described below) of 1407 patients whose samples were collected in a four-year period between January 2018 and December 2021. The study included both male and female Caucasian individuals residing in the Republic of Croatia, resulting in a nation-wide coverage. Their immune status (i.e. whether they were immunocompromised or immunocompetent) has not been determined. The diagnostic appraisal has been performed at the Diagnostic Parasitology Section of the Department of Parasitology and Mycology, Microbiology Service, Croatian National Institute of Public Health, Zagreb, Croatia.

Established peripheral blood eosinophilia was the primary inclusion criterion in the study, which has been defined as more than 500 eosinophils per microlitre of blood ($>0.5 \times 10^9$ per L). A country-wide convenience sampling approach has been utilized, which means all individuals with eosinophilia who provided their sample for serological appraisal to detect *S. stercoralis* have been included in this study. Additionally, those patients that were seropositive were additionally contacted to submit their stool for further analysis.

For serology testing, a commercial qualitative immunoenzymatic determination of specific immunoglobulin G (IgG) antibodies based on the enzyme-linked immunosorbent assay (ELISA) technique (Bioactiva Diagnostica GmbH, Bad Homburg vor der Höhe, Germany) has been utilized, per the manufacturer's recommendations. The manufacturer has also stated a diagnostic sensitivity of 89.47% (95% confidence interval (CI) 75.2%–97.06%) and diagnostic specificity of 94.12% (95% CI 83.76%–98.77%) of this assay, while the testing kit was additionally validated in the laboratory against a faecal-based reference standard. In those with a positive serological test result, a microscopy examination of faeces (three samples) concentrated by the merthiolate–iodine–formaldehyde concentration (MIFC) method has been pursued.

All data analyses were performed by using descriptive and quantitative statistical methods, the latter with the use of a Chi-square test for comparing percentage positive rates. Statistical significance was established at the $P$-value < 0.05 level (two-tailed). All analyses were done in R version 4.0.5 (htttp://www.r-project.org).

## Results and discussion

The overall seroprevalence of strongyloidiasis in our sample of patients with eosinophilia was 9.31% (131 positive samples out of 1407 tested). In seropositive individuals, the total number of positive microscopy findings following stool analysis by the MIFC method was 34 out of 131 (or 25.95% when presented as a fraction). There were no statistically significant differences in either seropositivity ($\chi^2 = 6.42$; $P = 0.093$) or percentage positive microscopy rates ($\chi^2 = 3.09$; $P = 0.379$) when different study years are compared (table 1).

Among 1407 individuals whose samples were included in the study, there were 736 male and 671 female patients. No statistically significant differences in seropositivity by gender have been demonstrated, and the percentage rates were quite comparable at 9.24% for women and 9.38% for men ($\chi^2 = 0.008$; $P = 0.931$). Moreover, even though we observed a higher percentage of positive microscopy rates following the MIFC method in seropositive individuals when men were compared to women (30.43% vs. 20.97%, respectively), this difference was also not statistically significant ($\chi^2 = 1.52$; $P = 0.217$).

The age of our study participants ranged from one to 96 years (mean: 43.7, median: 43, mode: 34, interquartile range: 39). When analysing different age groups, we observed a notably higher seroprevalence rate in those older than 60 years of age (13.10%) when compared to those between 31 and 60 years of age (8.75%) and those 30 years and younger (6.57%) ($\chi^2 = 10.7$; $P = 0.005$). Further fragmentation of age groups also showed statistically significant differences, with most serologically positive results observed in those older than 70 years of age (table 2), highlighting in turn the positive correlation of seropositivity and age.

On the other hand, there were no statistically significant differences in regards to age for MIFC microscopy positivity rates ($\chi^2 = 0.901$; $P = 0.924$) (table 2). Of note, the positive MIFC microscopy rate was higher in those older than 50 years of age in comparison to those younger than 50 years of age (28.17% vs. 23.33%), which are two study sub-populations of similar size when the number of analysed samples is concerned.

**Table 1.** Percentage positivity rates for *Strongyloides stercoralis* with the use of serology and confirmatory microscopy of samples concentrated by the merthiolate–iodine–formaldehyde concentration method in accordance with study years (note: microscopy was pursued only in seropositive individuals) ($P$-value < 0.05 was considered as statistically significant; Chi-square test).

| | Serology | | Microscopy | |
|---|---|---|---|---|
| Year | Number positive/number tested (percentage) | Number negative/number tested (percentage) | Number positive/number tested (percentage) | Number negative/number tested (percentage) |
| 2018 | 36/359 (10.03%) | 323/359 (89.97%) | 10/36 (27.78%) | 26/36 (72.22%) |
| 2019 | 29/346 (8.38%) | 317/346 (91.62%) | 10/29 (34.48%) | 19/29 (65.52%) |
| 2020 | 20/311 (6.43%) | 291/311 (93.57%) | 6/20 (30.00%) | 14/20 (70.00%) |
| 2021 | 46/391 (11.76%) | 345/391 (88.24%) | 8/46 (17.39%) | 38/46 (82.61%) |
| total | 131/1407 (9.31%) | 1276/1407 (90.69%) | 34/131 (25.95%) | 97/131 (74.05%) |
| $P$-value | $P = 0.093$ ($\chi^2 = 6.42$) | | $P = 0.379$ ($\chi^2 = 3.09$) | |

https://doi.org/10.1017/S0022149X22000888 Published online by Cambridge University Press

**Table 2.** Percentage positivity rates for *Strongyloides stercoralis* with the use of serology and confirmatory microscopy of samples concentrated by the merthiolate–iodine–formaldehyde concentration method in accordance with age groups (note: microscopy was pursued only in seropositive individuals) (*$P$-value < 0.05 was considered as statistically significant; Chi-square test).

| | Serology | | Microscopy | |
|---|---|---|---|---|
| Age category | Number positive/number tested (percentage) | Number negative/number tested (percentage) | Number positive/number tested (percentage) | Number negative/number tested (percentage) |
| 0–18 | 15/243 (6.17%) | 228/243 (93.83%) | 3/15 (20.00%) | 12/15 (80.00%) |
| 19–30 | 13/183 (7.10%) | 170/183 (92.90%) | 4/13 (30.77%) | 9/13 (69.23%) |
| 30–50 | 32/421 (7.60%) | 389/421 (92.40%) | 7/32 (21.88%) | 25/32 (78.12%) |
| 50–70 | 42/343 (12.24%) | 301/343 (87.76%) | 12/42 (28.57%) | 30/42 (71.43%) |
| 70–96 | 29/217 (13.36%) | 188/217 (86.64%) | 8/29 (27.59%) | 21/29 (72.41%) |
| total | 131/1407(9.31%) | 1276/1407(90.69%) | 34/131(25.95%) | 97/131(74.05%) |
| $P$-value | $P = 0.011^*$ ($\chi^2 = 13.1$) | | $P = 0.924$ ($\chi^2 = 0.901$) | |

To our knowledge, this is a first study from Croatia that aimed to assess the frequency of strongyloidiasis in Croatian patients with eosinophilia. Our results reveal substantial seroprevalence rates in both women and men and a rather high infection burden in older individuals, but also additionally highlight how the MIFC method is largely insufficient to capture all *S. stercoralis* infections. The fraction of positive microscopy findings (following the use of the MIFC method) in seropositive individuals in our study (i.e. 25.95%) is highly comparable to a recent study by Balen Topić *et al.* (2021) from Croatia where it was shown how the MIFC method revealed only 26.2% of cases that were positive when more sensitive diagnostic techniques have been used.

The latter is important as the MIFC concentration procedure is still predominantly utilized as a standard diagnostic tool in most microbiology laboratories in Croatia and other neighbouring countries; however, it is actually insufficiently sensitive, taking into account the importance and increasing recognition of this disease. Studies have shown how the Baermann concentration technique and agar plate culture have a much better diagnostic yield, but the number of stool samples, faecal amount and/or faecal dilution also affect sensitivity and detection power (Hailu *et al.*, 2022). Importantly, a meta-analysis by Campo Polanco *et al.* (2014) that evaluated conventional parasitological methods for the diagnosis of *S. stercoralis* found the highest sensitivity for the agar plate method (89%), followed by the Baermann technique (72%), formalin–ether concentration technique (which is akin to the MIFC method) (48%) and direct wet smear (21%).

Furthermore, the use of PCR may prevent misidentification of morphologically kindred helminth species (Becker *et al.*, 2015), and sometimes even the analysis of aspirates and biopsy samples from upper gastrointestinal endoscopy and/or bronchoscopy is endorsed (Balen Topić *et al.*, 2021). For both epidemiological studies and quotidian clinical practice, serology is considered a diagnostic mainstay; however, if the accuracy of serology is evaluated against a faecal-based reference standard, discordant results (i.e. serology-positive and faecal-negative results) would make classification endeavours impossible. This issue has been bypassed in several ways, as described by Buonfrate *et al.* (2015).

That said, there is no official single reference standard for diagnosing strongyloidiasis, thus a combination of methods is the most feasible approach. This is particularly pertinent when corticosteroid therapy is prescribed, as previously undetected and/or subclinical *Strongyloides* infestation can develop into hyperinfection syndrome during treatment and subsequently disseminate, leading to very high mortality rates (Ahmed *et al.*, 2019). Another important group where serological evaluation should be routinely sought are transplantation candidates (Toledo *et al.*, 2019), although reduced sensitivity of serology in immunocompromised individuals should be taken into account. A study by Winnicki *et al.* (2018) suggests that kidney transplantation programmes in Central Europe should entail recipient and donor screening as *Strongyloides* may be underestimated.

In other patient groups, especially those that are not critically ill, a stepwise approach can be a good way to increase the diagnostic yield – with MIFC as the first step, and then (if the result is negative), serology or even molecular diagnostics may be pursued in some instances (if available) (Balen Topić *et al.*, 2021). Underlying patient conditions also have to be taken into account. A study from the United Kingdom showed a significant association of strongyloidiasis with diabetes and eosinophilia (McGuire *et al.*, 2019). Moreover, a steadfast parasitological diagnosis of *S. stercoralis* infection in alcoholic patients necessitates repeated examination by at least two parasitological methods – including agar plate culture as a result of its higher sensitivity (Silva *et al.*, 2019).

Our study is a direct contribution to a very scarce body of country-level epidemiological data on *S. stercoralis* in Europe, which significantly influences the accuracy of various estimation endeavours – the most recent one showing *S. stercoralis* prevalence of 2.8% and 26.1 million infected individuals in the World Health Organization European region (Buonfrate *et al.*, 2020). In a study from San Marino there was a 4.8% seroprevalence rate and one additional case was detected with the use of gastric biopsy (Cappella *et al.*, 2019). A recent systematic review of endemic cases in Spain showed a significant concentration of infected individuals in the province of Valencia, with a high predominance of male patients; this is in contrast with our study where there was no statistically significant difference when sexes have been compared (Barroso *et al.*, 2019).

Co-infections and occupational information are also relevant pieces of the puzzle, as studies have shown a higher exposure to *S. stercoralis* larvae among patients with leptospirosis and those working in agriculture, respectively (Varzegar *et al.*, 2021). Some recent studies highlight the need to implement stringent screening due to high seroprevalence rates in migrant populations

https://doi.org/10.1017/S0022149X22000888 Published online by Cambridge University Press

coming to Europe, highlighting serology as an optimal approach (Requena-Méndez *et al.*, 2020). Furthermore, as a result of the characteristic auto-infective cycle of the parasite, *S. stercoralis* can persist even though other helminth infections may have disappeared in this region, as was described in Italy (Buonfrate *et al.*, 2016). These are undoubtedly important epidemiological concerns that have to be taken into account.

Global age-related findings imply that children are generally not at a higher risk for *S. stercoralis* infection; nonetheless, behavioural factors might have a significant influence here. Based on the study conducted in Spain, Bustamante *et al.* (2021) recently argued how strongyloidiasis should always be considered as a differential diagnosis in children presenting with eosinophilia. In our study there were 1.36% patients below 18 years of age with eosinophilia that were seropositive, and 20% of them had a positive microscopy finding following the use of the MIFC method, which adds another layer of evidence that there is a genuine autochthonous transmission.

The link to eosinophilia also warrants a separate discussion, as the literature shows that it might be a much more frequent finding in comparison to other chronic intestinal parasitic infections (Salvador *et al.*, 2014; Krolewiecki & Nutman, 2019). In that regard, eosinophilia can indeed be viewed as a potentially valuable marker for screening asymptomatic individuals suspected to be infected with *S. stercoralis*. Nevertheless, it has to be emphasized that eosinophilia in chronic strongyloidiasis might be intermittent and some reports on strongyloidiasis have documented eosinophilia in 57–63% of cases (Requena-Méndez *et al.*, 2013); furthermore, it is not a reliable predictor of hyperinfection and it may not be present during the treatment with immuno-suppressant therapy (Greaves *et al.*, 2013).

Our study has several important limitations. First, we did not exclude the possibility of infection by some other parasite, as well as other potential causes of eosinophilia; hence, it was impossible to ascertain that *S. stercoralis* was in fact the actual (or sole) cause of eosinophilia in our positive patients (also taking into account the fact that eosinophilia can be an insensitive marker of infection). Second, as specimens have been received through a reference laboratory, we only had basic patient demographic and clinical information at our disposal, without details regarding their medical history, clinical presentation, the course of eosinophilia or additional laboratory results – hampering other potentially valuable analyses within the epidemiological context. Third, the serological method used prevented us from separating the acute or latent phase of strongyloidiasis in our studied population, and there is a possibility of false-positive reactions as a result of cross-reactivity with other parasites (e.g. *Ascaris lumbricoides* or *Toxocara* spp.). Likewise, detailed analytical specificity studies were not conducted, even though serological cross-reactivity among the anti-*Strongyloides* IgG assays has been observed in individuals who previously had filarial infections. The use of a more sensitive stool detection method could result in different percentage positive rates for confirmatory microscopy. We are also aware that sensitivity can be lower in immunocompromised individuals; however, a minimal influence on the overall results is expected here, taking into account a high number of screened individuals. There are also several types of selection biases pertinent for our study: eosinophilia as a principal inclusion criterion (even though this is absent in a significant proportion of individuals with chronic strongyloidiasis); stool testing conducted only on serologically positive individuals (considering the reported sensitivity of the serological test of approximately 90%); as well as the use of MIFC as a stool test with a reported sensitivity of approximately 48% compared to a composite reference standard.

Notwithstanding the aforementioned limitations, by providing important, country-level data for the first time, we can conclude that this study reinforces the notion of endemicity and strongyloidiasis transmission in Croatia. Although the disease incidence is generally low, patients on immunosuppressive drugs and organ donors from Croatia should be included in the screening programme for *S. stercoralis* infection – with the caveat that the sensitivity may be reduced in such circumstances, potentially necessitating additional methodologies. As the occurrence of this parasite in the stool can be intermittent and in low concentrations (particularly in chronically infected asymptomatic patients), corroborated by only a quarter of positive microscopy findings in our seropositive patients, serology can be viewed as the preferred diagnostic approach, emphasizing the need for combining different methods whenever possible.

**Conflict of interest.** None.

**Financial support.** This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

**Ethical standards.** The authors declare that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation, as well as with the Helsinki Declaration of 1975, as revised in 2008.

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