

Research Article

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## Trichinellosis in Serbia has become a rare event – one outbreak with pulmonary complications

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**Abstract:** An outbreak of trichinellosis occurred in Stari Banovci, a settlement in the municipality of Stara Pazova, Srem, Republic of Serbia, in March–April 2019. A total of 28 persons were exposed and trichinellosis was confirmed in 24 of them. This outbreak involved members of eight families, their relatives and friends. The infection, due to *Trichinella spiralis* (Owen, 1835), was associated with consumption of pork sausages procured in a local butcher's shop. The trace-back study revealed that the meat originated from swine that was raised on a small farm belonging to the owner of the shop, who did not have permission from the Veterinary Directorate for slaughtering animals and who put on the market sausages prepared from uninspected meat. Trichinellosis was accompanied by typical clinical symptoms. However, the unusual occurrence of high percentage of pulmonary complications was noticed. The description of this outbreak indicates that medical practitioners should initiate treatment immediately in cases of high suspicion of trichinellosis, even if the serology is negative, to prevent the complications of the disease. In spite of significant achievements in the control of *Trichinella* infection among domestic pigs and humans in the last 10 years, it is obvious that such cases of breeding animals under inappropriate conditions, slaughtering them without approval and placing uninspected pork on the market represent a source of sporadic outbreaks in Serbia.

**Keywords:** *Trichinella spiralis*, outbreak, uninspected pork

Trichinellosis is a human disease caused by infection with nematodes of the genus *Trichinella* Railliet, 1895. Disease is caused after consumption of meat containing infective larvae of species of *Trichinella* and can be divided into two phases. The enteral phase of acute infection is characterised by diarrhoea, nausea, vomiting and abdominal pain. The parenteral phase involves periorbital oedema, fever, weakness and muscle pain. Complications are seen not only in severe cases, but also in moderate form of disease, particularly in elderly persons, as well as in persons treated improperly or too late (Dupouy-Camet and Bruschi 2007). Major complications are cardiovascular (myocarditis and tachycardia), neurological (meningitis or encephalopathy) and respiratory (dyspnea, pneumonia, and obstructive bronchitis) (Dupouy-Camet et al. 2002).

Trichinellosis is a mandatory reportable disease in Serbia. Data show that sporadic cases or outbreaks have occurred every year. The number of infected persons continuously decreased in this millennium from the period 2001–2005 (1,565 cases) over the period 2006–2010 (692 cases) (Sofronic-Milosavljevic et al. 2013), and period

2011–2015 (450 cases) to the period 2016–2019 (246 cases, current data of NRLT, INEP). Nevertheless, it may be concluded that, while trichinellosis was a serious public health issue in Serbia, continued joint efforts to educate the population both by human and veterinary medical services (one health approach) have enabled progress in this field.

The epidemiological investigations on trichinellosis between 2006 and 2018 revealed that undercooked pork or meat products (traditional homemade sausages and smoked meat) containing larvae of *Trichinella* spp. were the source of 93% of the trichinellosis outbreaks in Serbia (Sofronic-Milosavljevic et al. 2013, data from NRLT, INEP). Unlike humans, animals tend not to exhibit clinical signs of trichinellosis. Thus, *Trichinella* spp. infection in pigs can only be diagnosed by direct methods that detect the presence of larvae in the muscles. Insufficient awareness and knowledge of small pig farm owners, who avoid controlling the meat for the presence of *Trichinella* spp., are the reason why trichinellosis persists from year to year. Also, backyard pig owners give meat products to friends and relatives or sell it on the market, thus spreading

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trichinellosis in Serbia, as well as in other countries as exported trichinellosis (Milne et al. 2001).

This study presents results from clinical and epidemiological investigation, and laboratory testing of 28 patients involved in an outbreak of trichinellosis with an unusual percentage of pulmonary complications. The infection occurred after consumption of sausages sold in a registered butcher's shop and prepared from uninspected pork containing larvae of *Trichinella spiralis* (Owen, 1835).

## MATERIAL AND METHODS

### Trace-back study and outbreak investigation

On March 8, 2019, the Institute of Public Health of Vojvodina informed the Republic Veterinary Inspection of the Ministry of Agriculture, Forestry and Water Management, Republic of Serbia, about a possible infection with *Trichinella* spp. in Stari Banovci, Srem, Serbia. The Veterinary Inspection conducted an emergency (extraordinary) inspection.

### Clinical and epidemiological investigation

Diagnosis and defining of suspected, probable and confirmed cases was based on the ECDC Case Definition (European Commission 2018). All suspected patients were examined at the Hospital for Infectious and Tropical Diseases, Belgrade, Serbia (HITD). All of them provided information that they had consumed dry fermented sausages bought in a single butcher's shop in Stari Banovci, Serbia. Consumption of these meat products began in mid-February 2019 by at least 28 persons. Clinical forms of trichinellosis were defined as mild, moderately severe, or severe based on classification proposed by Dupouy-Camet et al. (2002). All patients were monitored during treatment until symptoms had disappeared.

### Laboratory testing

Paired blood samples from six hospitalised persons were taken on the first day of hospitalisation (sample 1) and after 15 days (sample 2), which corresponded to the second and fourth week after consumption of infected meat products. Serum samples were sent for immunological analysis to the National Reference Laboratory for Trichinellosis (NRLT), Institute for the Application of Nuclear Energy – INEP, Belgrade, Serbia. Serodiagnosis was performed by indirect immunofluorescence antibody assay-IFA (FITC *Trichinella spiralis* Antibody Detection Kit, INEP, Serbia), in-house indirect ELISA and Western blot (Wb) as the confirmatory test. Tests were carried out as described previously (Radovic et al. 2012, Ilic et al. 2014, Gnjatovic et al. 2019). Serum samples with anti-*Trichinella* antibody titres  $\geq 1 : 40$ , which produced a bright, apple-green signal on the cuticle and inside the stichocytes of the parasite muscle larvae, were considered seropositive in IFA.

For i-ELISA the index of positivity (IP%) was calculated according to the equation:  $IP\% = [(OD \text{ mean duplicate sample} - OD \text{ mean duplicate blank}) / (OD \text{ mean duplicate highest positive control} - OD \text{ mean duplicate blanks})] \times 100$ . Serum samples with  $IP \geq 26\%$  were considered seropositive. The optimal cut-off value determined for IP was 20%. Results around the cut-off value (20–25%) are defined as a grey zone. Wb was used as a confirmatory test in cases of disagreement between the results obtained for IFA and i-ELISA. Interaction of excretory-secretory products

of muscle larvae with the monoclonal antibody (MoAb) 7C2C5 and with *Trichinella* positive human serum served as control for a *Trichinella*-positive reaction. The MoAb 7C2C5 recognises an epitope on 45, 49 and 53 kDa proteins, which is unique for muscle larvae antigens of nematodes of the genus *Trichinella* (see Gamble and Graham 1984).

Sera were considered positive if, among others, they reacted with the three above mentioned proteins. The positive control was a *Trichinella*-positive serum acquired from a blood bank in NRLT INEP. Serum without parasite-specific antibodies was used as a negative control. For all outpatients, an immunological analysis was performed in the Hospital Laboratory by i-ELISA (NovaLisa *T. spiralis* IgG-ELISA, R-Biopharm AG, Germany) and the findings were presented as positive or negative.

### Parasitological examination

Samples of dry fermented sausages were analysed for the presence of *Trichinella* spp. muscle larvae in the NRLT INEP with magnetic stirrer method (European Union 2015). Larvae were collected after digestion and the worm burden was expressed as larvae per gram (LPG) of tissue. *Trichinella* larvae were identified at the species level by multiplex PCR analysis using the test accredited by the European Union Reference Laboratory for Parasites (Istituto Superiore di Sanità, Rome, Italy) (Zarlenga et al. 1999, Pozio and La Rosa 2003) in INEP with slight modifications as described by Cvetkovic et al. (2011). Single larvae from two encapsulated and one non-encapsulated reference strains were used as controls (*T. spiralis*, ISS3; *Trichinella britovi* Pozio, La Rosa, Murrell et Lichtenfelds, 1992, ISS2; and *Trichinella pseudospiralis* Garkavi, 1972, ISS13).

## RESULTS

### Trace-back study and outbreak investigation

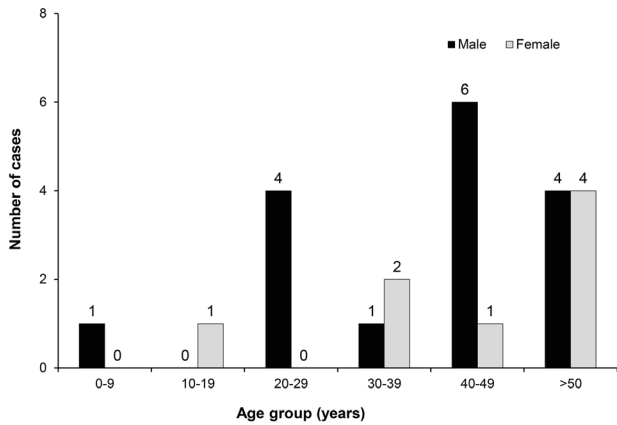
During the investigation it was found that pork, which was a possible source of the disease, originated from the small family farm belonging to the owner of a butcher's shop in Stari Banovci (44.987804N, 20.256701E). The owner did not have a permit from the Veterinary Directorate for slaughtering activities in the facility. He prepared dry fermented sausages from uninspected meat and sold them in the shop. About 80 kilograms of these meat products were confiscated, withdrawn from the shop, samples were provided to NRLT INEP for parasitological analyses, and infected products were disposed in the manner prescribed by law and relevant regulations.

This outbreak of trichinellosis began on 8 March 2019 and lasted until 24 April 2019 when the last case was detected. As the parasitological investigations revealed, the source of this outbreak was pork sausages made from a swine infected with *Trichinella spiralis*. The worm burden in pork sausages was 5 LPG.

### Clinical and epidemiological investigation

It was established that about 28 persons had consumed sausages made from pork, suspected as the source of infection. Trichinellosis was confirmed in 24 persons at the HITD, Belgrade, Serbia, among whom six (25%) were hospitalised ('inpatients'). The others who underwent clin-

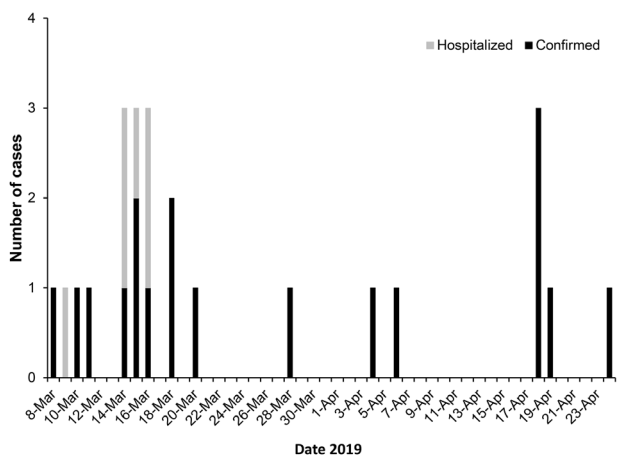
ical observation and analyses were treated as outpatients. All hospitalised persons (n = 6) were adult males between 20 and 49 years old (mean 36 years). Among the outpatients there were six females, ten males and two children (5 and 11 years old, boy and girl, respectively) (Fig. 1). All patients were members of eight families with their relatives and friends.



**Fig. 1.** Age and gender distribution of trichinellosis cases in the outbreak in Stari Banovci, Serbia, March–April 2019 (n = 24).

The first patient was detected on 8 March, while the last case was recorded on 24 April 2019 (Fig. 2). The outbreak lasted 48 days and no deaths occurred. The incubation period between ingestion of infected meat products and appearance of clinical signs ranged from 7 to 15 days.

All patients reported eating the suspected meat products and manifested some signs and symptoms related to trichinellosis. The frequencies of the main clinical symptoms myalgia (100%), fever (83%), periorbital oedema (50%) and diarrhea (50%) among hospitalised persons were paralleled those noticed in ambulatory treated persons, where these features occurred in 83%, 67%, 33% and 33% of patients, respectively. Cough was present in 33% of inpa-



**Fig. 2.** Cases of trichinellosis reported in the outbreak presented by date of disease onset, Stari Banovci, Serbia, March–April 2019 (n = 24).

tients and 17% of outpatients. One outpatient complained of dyspnea and hoarseness (Table 1). Auscultation of the lungs in five patients (two inpatients and three outpatients) revealed decreased breath sounds with basal inspiratory crackles. X-rays showed radiological changes characteristic for suspected focal bronchopneumonia which represented pulmonary complications in these patients (almost 21% of the total number of patients).

**Table 1.** Characteristics of trichinellosis cases associated with consumption of pork products (n = 24), Stari Banovci, Serbia, March–April 2019

Clinical characteristic (Sign/Symptom)	<i>Trichinella</i> positive inpatients n = 6		<i>Trichinella</i> positive outpatients n = 18	
	n	%	n	%
Myalgia	6	100	15	83
Fever > 38°C	5	83	12	67
Periorbital oedema	3	50	6	33
Diarrhea	3	50	6	33
Cough	2	33	3	17
Weakness	1	17	1	6
Profuse sweating	1	17	1	6
Dispnea	0	0	1	6
Hoarseness	0	0	1	6
Laboratory results	n	%	n	%
Elevated eosinophil percentage (≥ 5%)	6	100	16	89
C-reactive protein (> 5 mg/L)	6	100	18	100
Elevated creatine phosphokinase (> 200 U/L)	6	100	18	100
Lactate dehydrogenase (> 227 U/L)	6	100	18	100
Treatment	n	%	n	%
Albendazole	6	100	16	89
Mebendazole	0	0	2	11
Glucocorticoids	2	33	3	17
Complications	n	%	n	%
Pulmonary disease	2	33	3	17
Outcome	n	%	n	%
Complete recovery	6	100	18	100

Abbreviations: n – number of cases among all cases.

At the day of admission of inpatients or during the first examination of outpatients, the laboratory analysis of serum samples from all inpatients (100%) and most outpatients (89%) showed marked eosinophilia (Table 1). Also, raised levels of muscle enzymes, such as creatine phosphokinase (CPK) and lactate dehydrogenase (LDH), as well as C-reactive protein, were present in all cases (Table 1).

**Laboratory testing**

Clinically suspected *Trichinella* infection was confirmed by serology of the first blood sample in all six hospitalised patients. Western blot analyses were necessary to confirm the diagnosis in five cases, due to discrepancy of the results obtained by IFA and i-ELISA (Table 2). Patient 24 and 25 were positive in IFA, but were in the grey zone in i-ELISA, whereas Patients 20, 22 and 26 were negative in IFA, but in a grey zone or positive in i-ELISA. In the second blood samples, both IFA and i-ELISA tests revealed the presence of anti-*Trichinella* antibodies in four patients. In two cases, Patient 22 and 24, the Wb was used as trichinellosis confirmatory test, since the i-ELISA results were in the grey zone, whereas IFA was positive in one case and in the other negative. The immunological findings for cir-

**Table 2.** Clinical and laboratory data of confirmed and suspected trichinellosis patients (n=28), Stari Banovci, Serbia, March-April 2019

	No	Gender	Age	Symptoms	Complications	Hospitalisation	Days in hospital	IFA S1/S2	i-ELISA S1/S2	WbS1/S2
Family 1	1	F	43	D,O,M	None	No	0		+	
	2	M	43	F,D,M,C	Pulmonary	No	0		+	
Family 2	3	M	67	F,M	None	No	0		+	
	4	F	70	F,D,M	None	No	0		+	
	5	F	59	D,M	None	No	0		+	
Family 3	6	M	69	F,D,W,S, M,Dis,H,C	Pulmonary	No	0		+	
	7	M	41	M	None	No	0		-	
	8	M	11	nda	None	No	0		+	
	9	F	5	nda	None	No	0		+	
Family 4	10	M	72	M	None	No	0		+	
	11	F	69	F,D,O,M,C	Pulmonary	No	0		+	
	12	M	47	M	None	No	0		+	
	13	M	20	D,M,C	None	Yes	3	+/+	+/+	
Family 5	14	M	53	F,M	None	No	0		+	
	15	M	19	F,O,M	None	No	0		+	
	16	M	65	F,M	None	No	0		+	
Family 6	17	F	65	F,O,M	None	No	0		+	
	18	F	38	F,O,M	None	No	0		+	
	19	M	43	M	None	No	0		-	
Family 7	20	M	48	F,D,O,M	None	Yes	5	-/+	+/+	+/nd
	21	M	20	F,D,O,M	None	No	0		+	
Family 8	22	M	36	F,M	None	Yes	6	-/-	gz/ gz	+/+
	23	M	22	F,O	None	No	0		+	
	24	M	44	F,D,M	Pulmonary	Yes	4	+/+	gz/gz	+/+
	25	M	22	F,O,M	None	Yes	4	+/+	gz/+	+/nd
	26	M	49	F,O,W,S M,C	Pulmonary	Yes	6	-/+	+/+	+/nd
	27	M	69	M	None	No	0		-	
	28	F	33	M	None	No	0		-	

**Abbreviations:** IFA – Indirect Immunofluorescence Assay; i-ELISA – Indirect Enzyme Linked Immunosorbent Assay; Wb – Western blot; S1/S2-Sample 1 at admission/Sample 2 two weeks later; M – male; F – female; F – fever; D – diarrhea; O – oedema; M – mialgia; C – cough; W – weakness; Dis – dispnea; S – sweating; H – hoarseness; - - negative; + - positive; gz – grey zone; nd – not done; nda – no data available because children were monitored and treated by a pediatrician.

culating anti-*Trichinella* antibodies are shown in Table 2. Serum samples for all 18 outpatients were found positive in ELISA test, specified in Material and Methods.

The patients received oral albendazole 400mg twice daily for 14 days followed by analgesic ibuprofen (400 mg/8h). Two children were treated with mebendazole oral suspension for 14 days due to serologically confirmed trichinellosis. Five patients who developed pulmonary disease associated with trichinellosis, received prednisone to prevent worsening of the symptoms, after which the respiratory symptoms regressed. All patients were followed clinically during and after treatment and all recovered fully.

### Outbreak control measures

In accordance with its authorisations, the Veterinary Inspection submitted an infringement application to the competent prosecutor's office for criminal offences referred to Article 256 of the Criminal Code of the Republic of Serbia, i.e., for placing harmful foodstuffs on the market. An application was also sent to the competent Misdemeanor Court to consider the performance of an unregistered activity in accordance with the Law on Inspection Supervision.

### DISCUSSION

Pork is still the predominant source of trichinellosis outbreaks in Serbia. The risk of *Trichinella* infection comes from the decisions of irresponsible owners who do not provide meat samples for parasitological testing. The ex-

isting Veterinary Law in Serbia obliges examination of meat from domestic pigs intended to be placed on the market and hunted wild boars for the presence of *Trichinella* larvae. Since 2006, artificial digestion has been validated as the reference method in Europe for detection of *Trichinella* muscle larvae (Forbes and Gajadhar 1999, Gamble 1999, Gamble et al. 2000, Rossi and Pozio 2008, European Union 2015). The main reason why farmers and hunters do not provide meat samples for veterinary inspection from all slaughtered animals, but only from randomly selected ones, is the cost of the test and a lack of knowledge about trichinellosis.

Usually, common signs and symptoms of trichinellosis appear one to four weeks after exposure and almost always include the classical triad of fever, periorbital oedema and muscular pain (Kociecka 2000). Symptoms in patients during this outbreak were consistent with the typical pattern reported in the literature. The clinical form of trichinellosis was moderate in five cases, whereas most of the infected persons had a mild form of the disease. Two inpatients and three outpatients developed pulmonary trichinellosis where symptoms included a cough and pulmonary infiltrates. This is in line with literature data describing that clinical pulmonary features consist of pneumonia, obstructive bronchitis or Löffler-type infiltrates or ventilation failures (Compton et al. 1993).

The fact that almost one fifths of patients developed pneumonia sets this outbreak apart from the previous ones.



Respiratory complications like pneumonia are uncommon while dyspnea is relatively common and is caused by parasitic invasion of the diaphragm and consequent inflammation of the respiratory muscles (Dupouy-Camet and Bruschi 2007). The severity of trichinellosis depends on the number of ingested living larvae on one side and host factors (gender, age, immune status) on the other (Bruschi and Murrell 2002). Serious form of trichinellosis with neurological or cardiovascular complications can lead to death particularly in elderly patients (Dupouy-Camet and Bruschi 2007). Among the five patients with pulmonary trichinellosis there were one female and four males. Three of them were middle-aged (43, 44 and 49 years old) and two were 69 years old.

The clinical course of trichinellosis may also depend on the species of *Trichinella* involved. There are indications that infection caused by *T. spiralis* could be more severe than that with *Trichinella britovi* (see Gottstein et al. 2009). The main differences between *T. spiralis* and *T. britovi* infection were a longer duration of parasite-specific IgG, increased creatine kinase levels and a more severe intestinal symptomatology in *T. spiralis*-infected persons (Pozio et al. 1993). Trichinellosis induced by *T. britovi* was reported as milder disease with a long incubation period (Capo and Despommier 1996). At a *T. britovi* outbreak in Italy patients had a mild form of the disease and short-term symptoms with no need for hospitalisation (Fichi et al. 2015). However, investigation of a large *T. britovi* outbreak in 2016 in Serbia showed that out of 111 individuals involved, 17.1% were hospitalised (Pavic et al. 2020).

A comparison of data related to the outbreak caused by *T. britovi* and the *T. spiralis* outbreak reported in the present paper indicates that *T. britovi* may cause more severe clinical picture. While *T. spiralis* infection resulted in no severe cases, 21% moderate and 79% mild disease forms, *T. britovi* outbreak was characterised by 8% of severe cases, 41% moderate and 51% mild (Pavic et al. 2020). There was no significant difference in the appearance of the most common clinical symptoms and signs (myalgia, fever, periorbital oedema) or laboratory findings between patients infected with *T. britovi* and *T. spiralis* in those two outbreaks, but they did differ in the frequency of cardiac or pulmonary complications. At the *T. britovi* outbreak 8% of patients had cardiac (Pavic et al. 2020), while at *T. spiralis* outbreak presented here 21% of patients had pulmonary complications.

The infectious dose is another thing that significantly affects the course of the infection, and usually the higher the ingested dose, the more severe the disease. Parasitological analysis showed that larval burden in *T. britovi* outbreak was 0.18 LPG in dry meat and 0.87 LPG in sausages, while the worm burden in pork sausage was 5 LPG in *T. spiralis* outbreak. However, since the aforementioned outbreaks were caused by different species of *Trichinella*, and there is no way to estimate the number of ingested larvae, we cannot compare the effects of the worm burden on the severity of the disease.

The number of ingested larvae determines also the length of the incubation period (Capo and Despommier

1996). In our study, incubation period ranged from 7 to 15 days. Two hospitalised patients with pulmonary complications had the shortest incubation of 7 days, which could indicate that the ingested larvae load in those two patients was high. The interval between ingestion of infected meat and the appearance of clinical signs was similar to that reported in other *T. spiralis* outbreaks. The incubation period noted in cases from Vietnam was 7–14 days (Taylor et al. 2009), whereas the first symptoms appeared after an average period of 15 days in an outbreak in Belgium (Messiaen et al. 2016).

Seroconversion after infection with *Trichinella* spp. usually occurs between the second and fifth week and is inversely correlated with the infective dose (Dupouy-Camet and Bruschi 2007). Serum samples from our six inpatients were first tested by IFA and ELISA in NRLT INEP. Since the examination of sera with these two methods did not give unambiguous results (which could be seen in Table 2), Wb as a confirmatory test was performed. All serum samples collected at the day of hospital admission showed reactivity with *Trichinella*-specific antigens at 45, 49, 53 kDa in Wb (Ilic et al. 2014), which indicated that seroconversion occurred approximately 15 days post infection, in all six inpatients.

Discrepancies in the results obtained with IFA and ELISA assays originate from differences in the sensitivity of the tests, and this sensitivity depends, among other things, on the choice of antigens and secondary antibodies used to detect anti-*Trichinella* specific antibodies in patient's sera. Although generally used to confirm other serological tests, Wb is not applicable for routine diagnosis as a screening tool (Gamble et al. 2004). Confirmation of diagnosis allows early administration of antiparasitic treatment, which is crucial to prevent or reduce any trichinellosis symptoms. The main problem in late treatment is the poor susceptibility of migrating and encapsulated muscle larvae to anthelmintic drugs (Gottstein et al. 2009).

Five patients in the presented outbreak had pulmonary complications of trichinellosis. All of them refused hospitalisation at the first examination, despite advice from a physician. Their condition worsened over the next few days and they returned for another medical check. Two of them accepted hospitalisation while the other three accepted the prescribed therapy only. It should be emphasised that this outbreak had an unusual frequency of pulmonary complication, which was probably the consequence of delayed anthelmintic treatment. It is therefore of great importance, in cases of highly suspected trichinellosis, to start the treatment immediately, based solely on clinical signs and symptoms and epidemiological data, even if the serology is negative. Sometimes early testing or delayed seroconversion could be the reasons why some of the patients were not serologically confirmed.

To prevent the spread of the infection, medical practitioners should alert the public health and veterinary authorities, because effective communication is required to identify the source of the infection. A project (with the help of the EU) is underway that should strengthen the capacities of reference laboratories and improve communication between medical

and veterinary services. We can say that trichinellosis in Serbia is successfully kept under control and only individual cases occur annually. In 2020, there were only 20 cases of trichinellosis. Unfortunately, despite the success achieved in reducing the number of patients with trichinellosis and the number of outbreaks in Serbia, intentional breaking of the regulations (placing uninspected pork and meat products on the market) led to the trichinellosis outbreak described here. Lack of awareness of risks endangering human health takes an important place among the other risk factors contributing to this zoonotic infection.

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