First detection of *Echinococcus multilocularis* in Bosnia and Herzegovina

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ABSTRACT

*Echinococcus multilocularis* has been spreading through Central Eastern Europe but has not yet been reported in Bosnia and Herzegovina (B&H). Recently, this parasite is confirmed in Croatia suggesting the movement of the parasite’s distribution limit further south. Given that there is no surveillance or monitoring system for echinococcosis in B&H, our study was designed as a pilot study of *E. multilocularis*. A total of 57 red foxes originating from 24 localities all over the country were collected during the routine rabies monitoring, autopsied and examined for the presence of echinococcosis. Based on intestinal scraping technique and microscopy, *E. multilocularis* adult worms have been detected in one (1/57, 1.75%) red fox. To verify this finding and to differentiate *Echinococcus* spp., DNA extracted from adult worms was subjected to species-specific PCR targeting part of the mitochondrial 12S ribosomal RNA gene. *E. multilocularis* PCR-positive samples were further confirmed by NGS sequencing of a 203 bp amplified fragment of 12S rRNA, which has been deposited in GenBank (Accession no.: OP047920). This finding represents the first detection of *E. multilocularis* in B&H, strongly suggesting its presence in the country. The confirmation of the parasite in the same locality where migrants/refugees temporarily stay on their route to Western Europe highlights the need for a One Health approach in addressing all future questions. Moreover, the first detection of *E. multilocularis* in B&H warrants the need for the implementation of an appropriate state surveillance program.

1. Introduction

Human alveolar echinococcosis (AE) is a zoonotic disease caused by the metacestode stage of the tapeworm *Echinococcus multilocularis* and represents one of the most pathogenic zoonoses in the northern hemisphere (Kern et al., 2003). Transmission of *E. multilocularis* to humans is by ingestion of parasite eggs, either through direct contact with the definitive host or indirectly through the consumption of contaminated food or water (Torgerson et al., 2010). Considering the circulation of the parasite among the red fox population as an indicator of the epidemiological threat to humans, the monitoring of *E. multilocularis* in these animals is imperative (Oksanen et al., 2016). The presence of this parasite in red foxes was confirmed in 22 European countries (Oksanen et al., 2016). On the other hand, studies from Finland, Ireland, the United Kingdom and Norway reported the absence of *E. multilocularis* in foxes except in Arctic foxes (*Vulpes lagopus*) from the Arctic Archipelago of Svalbard in Norway (Oksanen et al., 2016). Considering the distribution of the parasite in Southeastern Central Europe, the scientific information is mainly scarce and fragmented, and there is a need for further investigation (Deplazes et al., 2017). In Slovenia, *E. multilocularis* was previously detected in the population of red foxes (Rataj et al., 2010), while the same parasite was likely present in its intermediate hosts, i.e., cattle and piglets (Brigelj and Krystufek, 1984). Accordingly, the cases of human alveolar echinococcosis were serologically confirmed in the same study areas (Logar et al., 2007). The relatively high prevalences of this parasite were detected in the population of red foxes (17.9%) and golden jackals (14.3%) in Serbia (Lalovečić et al., 2016). However, this detection was localized in the northern part of the

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country, and the absence of *E. multilocularis* in the southern part of the country could be an artefact of a lack of investigation (Deplazes et al., 2017). *E. multilocularis* was detected in the red fox population in Romania with the positive animals found in regions bordering neighboring countries, suggesting the potential moving of the parasite beyond the state borders. Moreover, human cases of alveolar echinococcosis are reported in the same study (Deplazes et al., 2017). Recently, an *E. multilocularis* infection in red fox is confirmed in Croatia (Beck et al., 2017), thus moving the parasite’s southern distribution limit in Southeastern Central Europe further south closer to B&H. Although the *E. multilocularis* infection is reported in neighboring countries, surveillance of *Echinococcus* spp. tapeworms is lacking in B&H. Hence, there are several open questions, including the presence, prevalence, and geographic distribution of *Echinococcus* spp. tapeworms throughout B&H. Considering that the surveillance and control systems of this parasitic disease do not exist in B&H, the primary goal of this study was to address the issue of the *E. multilocularis* presence in the red fox population, as an early indicator of a public health threat.

2. Materials and methods

We examined a total of 57 red foxes originating from 24 localities of B&H in the period December 2021 to February 2022 (Fig. 1).

All animals were shot during an official survey concerning the efficacy of anti-rabies vaccination. After the autopsy, intestines were excised and opened with gut scissors and visually inspected for the presence of parasites. The small intestines of red foxes were organized to form three portions of roughly equal length (anterior, middle and posterior). Afterwards, the routine intestine scraping technique (IST) was performed, according to the previously described protocol for the detection of *E. multilocularis* adult worms in the small intestines of red foxes (Tackmann et al., 2006). In short, gross intestinal contents were carefully removed and at least four samples per portion were taken at regular intervals of 5–10 cm, depending on the total length of the small intestine, by scraping the mucosa with a glass microscope slide. Material adhering to the slides was transferred to plastic Petri dishes and examined stereomicroscopically at 100 x magnification.

To confirm morphological identification, PCR-based molecular techniques and NGS were performed. Parasitic DNA was extracted directly from three pools each consisting of two adult parasites using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) as described elsewhere (Beiromvand et al., 2011). DNA was stored at –20 °C until further analyses.

Molecular detection and confirmation of the parasite were performed using PCR-based molecular methods, adapted from Stieger et al. (2002), Stefanic et al. (2004) and Trachsel et al. (2007). All pools were analyzed using PCRs that were either genus-specific (Stefanic et al., 2004) or species-specific (Stieger et al., 2002; Trachsel et al., 2007), enabling the differentiation between *E. granulosus* and *E. multilocularis* by targeting mitochondrial small subunit ribosomal RNA (12S rRNA) gene. All tested pools were positive only for *E. multilocularis* (Table 1).

Amplicons originated from three positive *E. multilocularis* species-

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**Table 1**

| Target species          | PCR results | Gene | Primer designation | Reference         |
|-------------------------|-------------|------|-------------------|-------------------|
| *E. multilocularis*      | Positive (all pools) | 12S rRNA | EM-H15/EM-H17     | Stieger et al. (2002) |
| *E. granulosus*          | Negative (all pools) | 12S rRNA | Cest4/Cest5       | Trachsel et al. (2007) |
| *E. granulosus* (sheep strain) | Negative (all pools) | 12S rRNA | Eg1f/Eg1r        | Stefanic et al. (2004) |

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Fig. 1. Study area localities (dots) and collection sites for red foxes were included in the study. The red dot shows the location with a determined *E. multilocularis* positive red fox. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
specific PCRs, amplified with EM-H15 and EM-H17 primers (Stieger et al., 2002), were prepared for sequencing using the PCR Barcoding Kit (Oxford Nanopore Technologies, Oxford, United Kingdom) according to the manufacturer’s instructions and subsequently loaded on a MinION Mk1C instrument (Oxford Nanopore Technologies, Oxford, United Kingdom). Raw FAST5 files were basecalled and demultiplexed using the MinKNOW software (Oxford Nanopore Technologies, Oxford, United Kingdom), which was also used for primer trimming. Three 12S rRNA gene sequences were assembled using CLC Genomics Workbench 22.0.2 (Qiagen, Hilden, Germany).

3. Results

Based on the characteristics of scolex and strobila E. multilocularis adult parasites (Fig. 2) were found in one red fox (1/57, 1.75%).

All three sequences (203 bp each) were used in a BLAST search in GenBank, which confirmed 100% identity to each other and other E. multilocularis 12S rRNA gene sequences publicly available in GenBank (such as MN444822.1, MN444821.1, MN444820.1, JN175268.1, KY094610.1, MN444819.1, MN444818.1, MN444817.1, MN444816.1, MW558108.1). As three obtained sequences were found to be identical, one of them was published in GenBank under the accession number OP047920 (available upon article publication).

4. Discussion and conclusions

Echinococcus spp. tapeworms’ infections in humans or animals in B&H are underreported, partially due to neglecting the One Health aspect of the disease. Namely, the public health authorities in B&H reported 34 human cases and 1 death from echinococcosis in the period 2017–2021, with the respective disease incidence ranging between 0.05 per 100,000 inhabitants in 2021 and 0.4 per 100,000 inhabitants in 2018 (IPF F&H, 2022; iPH RS, 2019). However, information about the causative agent (E. granulosus sensu lato or E. multilocularis) is absent from the official reports. The apparent lack of interest in the public health threat posed by echinococcosis is most likely caused by the absence of scientific publications regarding the AE cases in B&H. This is supported by the recently described case of AE in Croatia (Dusik et al., 2020). It was unusually difficult to diagnose AE since it was assumed that cystic echinococcosis is the only type of human echinococcosis in the country. This phenomenon should not be neglected in B&H, especially after the first detection of E. multilocularis in the state territory. On the other hand, there have been only incidental reports of the presence of Echinococcus spp. tapeworms in domestic animals that mainly remained unpublished. However, the molecular detection of E. multilocularis in red fox points out that the public health risk in B&H is present and, given the continued expansion of animal and human infections in Europe, could even be increased.

As noted by Beck et al. (2017), the confirmation of E. multilocularis in Croatia in two infected foxes in the Dinaric Mountains that continuously extend from Slovenia to Albania, raises new questions about the parasite’s southern distribution range. This finding and observation indicated that the spreading of this parasite in the red fox population could likely occur in B&H which is proved by our study. However, there are potential risk factors associated with the occurrence and persistence of human AE (Conraths et al., 2017). Some of the categories of potential risk factors included pet-related factors, but also vocational and socio-economic factors and human habits. Pet-related risk factors showed high statistical significance in the occurrence of human AE (Conraths et al., 2017) and should not be neglected in future investigations of echinococcosis in B&H. Although consuming unwashed fruits is not recognized as a statistically significant risk factor for acquiring AE (Conraths et al., 2017), this phenomenon showed the increased odds ratio for acquiring AE. Nevertheless, our finding emphasizes the fact that infected animals, i.e., red foxes, can commonly contaminate soil, vegetables and fruits which can serve as a source of infection for consumers who unintentionally get infected (Alić et al., 2017). In addition, echinococcosis might be also considered a disease in certain occupations such as veterinarians, slaughterhouse workers, farmers, and hunters who are more predisposed to the infection. This is in accordance with the results of a meta-analysis (Conraths et al., 2017) which confirmed that specific activities such as hunting/handling foxes inadvertently increase the probability of ingesting the parasite’s eggs. In addition, according to the same study, being a farmer brings the most evident risk of acquiring AE. Moreover, outdoor activities such as hiking and camping as well as rural or nomadic lifestyles pose the risk of contact with infected material of animal origin. Regarding the specific lifestyles migrant population should be considered as a group of people with substantially increased risk of E. multilocularis infection in B&H. This is supported by our finding that E. multilocularis positive animal was confirmed in a locality where migrant people temporarily stay and spend their daily time in improvised shelters on the soil. Our finding calls for attention to routine screening for echinococcosis of immigrants from the Middle and Central East. Richter et al. (2019) have reported cystic echinococcosis in apparently healthy unaccompanied minor refugees originating from countries in the Middle and Central East (Richter et al., 2019). Our results indicate that the source of infection for these people does not necessarily have to be in their countries of origin, but they can acquire the infection in places where they temporarily stay. The timely recognition of such places may be a significant step in the control of echinococcosis in Europe. To fulfill the existing scientific gap and in particular to focus on public health threats associated with Echinococcus spp. tapeworms there is an urge for implementing the surveillance program in B&H as laid down in EU Commission Delegated Regulation 2018/772. Appropriate surveillance of the parasite would potentially lead to an increase in the number of reported animal infections, but on the other hand, it would reduce the potential human cases or other adverse public health outcomes.

Ethical statement

This study was conducted under the Law on Animal Protection and the welfare of BH (“Official Gazette BH” issue number 316/09) and Law on Hunting of the Federation of Bosnia and Herzegovina (“Official Gazette BH” issue number 4/06). All animals were shot during routine official surveillance concerning the efficacy of anti-rabies vaccination in the red fox population in Bosnia and Herzegovina.
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Declaration of competing interest

None.

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