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Detection of *Blastocystis* spp., *Cryptosporidium* spp. and *Encephalitozoon* spp. among wild animals from Eastern Slovakia

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RESEARCH ARTICLE

ABSTRACT

The aim of this study was to draw attention to the risk of transmission of *Encephalitozoon, Cryptosporidium* and *Blastocystis* infection due to high animal migration and to point out that even wild animals can be a source of many zoonotic diseases. *Encephalitozoon cuniculi, Cryptosporidium* spp. and *Blastocystis* spp. are frequent microscopic organisms that parasitise humans, domestic and wild animals. Two hundred and fifty-five faecal specimens were collected from wild boars, badgers, wolves, bears, foxes and deer from 15 locations in Slovakia. Sequencing of positive PCR products and subsequent sequence comparison with GenBank sequences identified *Blastocystis* spp. in five wild boars. The ST 5 ($n = 4$) and ST 10 ($n = 1$) subtypes were determined by genotyping. We identified *Encephalitozoon cuniculi* in five wild boars, and genotype II ($n = 5$) was determined on the basis of ITS repeat sequences. *Cryptosporidium scrofarum* was sequenced in wolves ($n = 4$) and wild boars ($n = 1$), while *Cryptosporidium suis* only in wild boars ($n = 2$). None of the wild boars had a mixed infection.

KEYWORDS

Encephalitozoon spp., *Cryptosporidium* spp., *Blastocystis* spp., wild animals, Slovakia, PCR

INTRODUCTION

Wild animals can be potential reservoirs of many zoonotic diseases (Fredriksson-Ahomaa et al., 2020). Contamination of the area with their faeces increases the risk of spreading infectious diseases. The most probable faecal-oral transmission of *Encephalitozoon* spp., *Cryptosporidium* spp. and *Blastocystis* spp. occurs by the human-to-human/animal-to-animal/animal-to-human and, perhaps, human-to-animal routes. Importantly, however, the infectious stages of these parasitic species have repeatedly been found in the environment. Water appears to be the most common source of infections caused by the three monitored pathogens. Whether it is drinking or recreational water, there is probably pollution by wild (but also domestic) animals, especially with their faeces and urine. These can enter the above-mentioned waters by the free movement of animals near water sources and by torrential rains, which move the faeces from higher places to the reservoirs. It is, therefore, necessary to investigate the presence of these parasites in wild animals to prevent possible epidemics or the spread of diseases from the point of view of epidemiology and epizootiology. It is also necessary to control the surrounding waters and monitor the occurrence of these parasites there (Russini et al., 2020). Detection and identification of any pathogen are difficult in wild animals, and absolute prevalence values are inaccurate. Because of their extensive genetic diversity, the first pathogen we focused on in wildlife animals are the controversial unicellular

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protists *Blastocystis* spp. Differences in the nucleotide sequences of the SSU rRNA gene of *Blastocystis* spp. isolates demonstrate substantial genetic heterogeneity (Stensvold and Clark, 2020). According to the consensus on the terminology of *Blastocystis* spp. subtypes proposed by Stensvold et al. (2007), *Blastocystis* spp. isolates are referred to as *Blastocystis* spp. 'subtypes' (ST). These designations are based on differences between SSU rRNA gene sequences and have been confirmed to be water sourced (Stensvold and Clark, 2020).

Other controversial pathogens we studied were microsporidia. They are a diverse group of eukaryotic, spore-forming, obligate intracellular pathogens that infect vertebrates and invertebrates. Due to their broad host range and low host specificity, they have gained scientific attention as human and animal pathogens. Three species are clinically relevant within the *Encephalitozoon* genus: *Encephalitozoon cuniculi, E. hellem* and *E. intestinalis*. These species of microsporidia most often infect immunosuppressed people (AIDS/HIV patients, bone marrow or organ transplant patients) as well as immunocompetent people, most often travellers or the elderly (Kicia et al., 2016).

Several groups have reported the occurrence of *Encephalitozoon* spp. in wildlife. *Encephalitozoon cuniculi* has been identified in primate hosts (Sak et al., 2011), carnivores (Åkerstedt and Kapel, 2003) and rodents in Europe and Japan (Tsukada et al., 2013; Danišova et al., 2015).

Today the *Cryptosporidium* genus consists of 44 described and recognised species. Many other genotypes/isolates from vertebrates and the environment have little biological and morphological data to determine their species (Feng et al., 2018). Wildlife and livestock have the potential to act as a biological reservoir for harmful protozoan parasites such as *Cryptosporidium* spp. (Xiao and Feng, 2008). Recent molecular studies have described 11 species of *Cryptosporidium* and almost 30 genotypes in various species of wildlife species (Feng, 2010). In this study, *Cryptosporidium* was found in fallow deer, antelopes (*C. parvum*), red deer (*C. ubiquitum*), South African antelopes, ibex and bison (*C. andersoni*). An interesting group are rodents (mice, rats, squirrels, voles, etc.), which can act as food sources for predators or as disease vectors. Their ubiquitous occurrence in the wild, rural environment and sharing of their habitats with livestock or contamination of drinking water have led to many studies on the prevalence of *Cryptosporidium* spp. in different geographical areas (Bednarska et al., 2007; Kváč et al., 2008; Fayer et al., 2010; Danišová et al., 2017; Horčičková et al., 2019).

MATERIALS AND METHODS

Sampling area and samples

Faecal samples (n = 255) from wildlife animals including 84 wild boars, 63 bears, 62 foxes, 26 wolves, 14 roe deer, 4 deer and 2 badgers, were collected from 15 randomly selected forest areas in Eastern Slovakia. As shown in Fig. 1, the localities included Červenica, Jablonov nad Turňou, Kavečany, Makovica, Perín, Poľana, Poltár, Rimavská Sobota, Rozhanovce, Seňa, Slovenská Ľubča, Smolník, Strážske, Tatra National Park (TANAP) and Zádiel. Animal excrements were collected from the earth around the feeding places in the forests in autumn and winter for two consecutive years (September–November, 2018–2019). The samples, placed in sterile plastic containers, were transported to

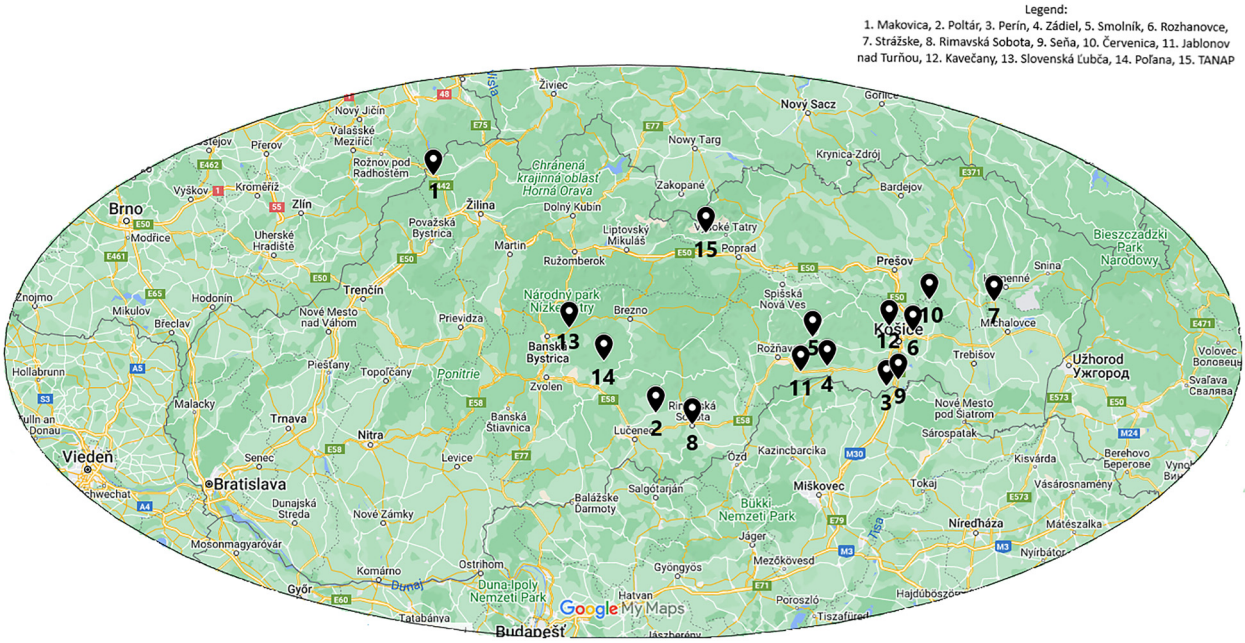

Fig. 1. Sampling localities in Eastern Slovakia

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the laboratory in a cool box and stored at 4 °C until processing. Within 7 days, all samples were processed.

DNA isolation

According to the manufacturer's instructions, DNA extraction was performed using a DNA-Sorb-B Nucleic Acid Extraction kit (Amplisens, Russia) from 100 mg faeces. Oocysts/spores from the samples were disrupted by a homogeniser, Precellys 24 (Bertin Technologies, Germany), at 6,500 rpm for 90 s with the addition of 1.0-mm zircon beads, 0.5-mm glass beads and 300 µL lysis solution. After isolation, the DNA samples were stored at –20 °C until used in PCR for molecular analysis.

PCR and sequence analysis

***Encephalitozoon* spp.** The real-time SYBR Green PCR amplification was used with specific primer pair ecfITSf and ecfITSr, described by Malceková et al. (2013). Primers amplify the ITS region, part of the small subunit (SSU) rRNA and part of the large subunit (LSU) rRNA genes of *E. cuniculi* (2920–2938 bp), *E. intestinalis* (1187–1205 bp) and *E. hellem* (2644–2627 bp) at an annealing temperature of 60 °C.

Electrophoresis and subsequent visualisation by UV light of wavelength 312 nm were used to analyse PCR products. Sequencing was performed in both directions, and a Chromas Pro Programme was used to align the sequences. Subsequently, the sequences were compared with known sequences in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The repetitive sequence 5′-GTTT-3′ in the ITS region was used as a genetic marker for genotyping (Talabani et al., 2010).

***Cryptosporidium* spp.** Nested PCR was conducted using a protocol modified in our laboratory to amplify the SSU region of DNA of *Cryptosporidium* species. In the first and second reactions, Xiao F1/R1 and Xiao F2/R2 primers were used from the protocol by Xiao et al. (1999) with a final amplicon of 826–864 bp (depending on the isolates). We made the third reaction to a higher specificity and a more accurate location of the polymorphic region of the 18S rRNA gene. In the third reaction, VKSS F1 and VKSS R2 were used (Leetz et al., 2007). The combination of genus-specific primers VKSS F1/VKSS R2 amplifies the gene section of 345–355 bp in length, which is 100 bp larger than that in the initial reaction by Leetz et al. (2007) and, therefore, it captures a larger section of the polymorphic region of the 18S rRNA gene, specific for the identification of *Cryptosporidium* spp. Secondary PCR products were analysed by electrophoresis in 1.5% agarose gel and visualised by UV light of wavelength 312 nm (Xiao et al., 2001).

All positive samples were sent for sequencing to confirm *Cryptosporidium* spp. after the nested PCR. The sequences were compared to the NCBI database sequences with BLAST.

***Blastocystis* spp.** The protocol for performing nested PCR to amplify the SSU DNA region of *Blastocystis* species was modified in our laboratory. Forward primer Blast F and reverse primer Blast R were used in the reaction from the protocol by Bohm-Gloning et al. (1997). The final amplicon was 466–479 bp (depending on the isolates). The PCR products were directly sequenced in both directions. The sequences were aligned and completed using the Bioedit programme (http://www.labtools.us/clustalw2/). The SSU rRNA sequences obtained were compared with those available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Sequencing and subsequent DNA analyses identified all monitored opportunistic pathogens. The hosts were predominantly wild boars, in which *Encephalitozoon* spp., *Cryptosporidium* spp. and *Blastocystis* spp. were identified, and *Cryptosporidium* spp. was also identified in wolves. The species *Encephalitozoon cuniculi*, for which genotype II was determined based on repetitive sequences in the ITS region, was identified in 5 wild boars from two areas, Makovica and Rozhanovce. In addition to one sample of wild boar from Rozhanovce, four samples of wolves were positive for *Cryptosporidium scrofarum* in the regions of Slovenská Ľupča, Poľana and TANAP. *Cryptosporidium suis* was detected in two samples of wild boar faeces in the Zádiel and Seňa areas. *Blastocystis* spp. subtypes ST 5 ($n = 4$) and ST 10 ($n = 1$) were identified in 5 wild boars in the area of Zádiel, Smolník and Strážske (Table 1, Fig. 1).

DISCUSSION

It is clear that protozoal infections, in which the infectious stages of transmission are excreted in the faeces, have the potential to contaminate the environment. These stages may be ingested by other possible hosts, whether wildlife, domestic animals or humans, which may result in infection and possibly disease. The most frequently observed eukaryotic gastrointestinal symbiont in humans and animals is *Blastocystis* spp., but *Encephalitozoon cuniculi* and *Cryptosporidium* spp. are also prevalent. The low specificity of *Blastocystis* spp., *E. cuniculi* and *Cryptosporidium* spp. for the host and the zoonotic potential suggests that the animals could serve as possible reservoirs for transmission. The prevalence and distribution of *Blastocystis* spp. in animal populations in Europe, acting as the focus of zoonotic diseases, are little studied. Overall, blastocystosis is reported only sporadically in wild animals, mostly in primates and zoo animals. Researchers from Italy also have studied *Blastocystis* spp. in wildlife. They found 3 STs in wild boars, of which ST15 was the most common (80.8% prevalence) (Russini et al., 2020). Another study to identify blastocystosis in wild boars was done in Iran and it revealed a prevalence of 25%–44% (Yaghoobi et al., 2016).

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Table 1. Encephalitozoon cuniculi, Cryptosporidium spp. and *Blastocystis* spp. in wild animals living in areas of Slovakia

Area	Animal	*n*	*Blastocystis* spp.	Subtype	*E. cuniculi*	Genotype	*Cryptosporidium* spp.
1. Makovica	wild boar	13	–	–	1	*E. cuniculi II*	–
GPS: 48.85114, 21.49256	bear	11					
2. Poltár	wild boar	5	–	–	–	–	–
GPS: 48.48833138,	deer	1	–	–	–	–	–
19.71999712							
3. Perín	badger	2	–	–	–	–	–
GPS: 48.5333 21.1875	fox	11	–	–	–	–	
4. Zádiel	wild boar	12	3	ST 5 (2), ST 10 (1)	–	–	*C. suis* (1)
GPS: 48.61615, 20.83275	fox	14	–	–			
5. Smolník	wild boar	26	1	ST 5	–	–	–
GPS: 48.7335, 20.6649	bear	23	–	–	–	–	
6. Rozhanovce	wild boar	10	–	–	4	*E. cuniculi II*	*C. scrofarum* (1)
GPS: 48.754304, 21.343105							
7. Strážske	wild boar	8	1	ST 5	–	–	–
GPS: 48.8735 21.83668	fox	12	–	–	–	–	–
8. Rimavská Sobota	deer	3	–	–	–	–	–
GPS: 48.38284 20.02239	roe deer	14	–	–	–	–	–
9. Seňa	wild boar	10	–	–	–	–	*C. suis* (1)
GPS: 48.7798, 21.20075,							
10. Červenica	bear	14	–	–	–	–	–
GPS: 48.87891, 21.44748,							
11. Jablonov nad Turňou	Fox	25	–	–	–	–	–
GPS: 48.59328, 20.67285,							
12. Kavečany							
GPS: 48.77547, 21.20589							
13. Slovenská Ľupča	wolf	9	–	–	–	–	*C. scrofarum* (1)
GPS: 48.766758, 19.272933							
14. Poľana	wolf	10	–	–	–	–	*C. scrofarum* (2)
GPS: 48.633 19.467							
15. TANAP	wolf	7	–	–	–	–	*C. scrofarum* (1)
GPS: N49°9′8″ E20°4′52″	bear	15	–	–	–	–	–
Σ animals		**255**	**5**		**5**		**7**

The occurrence of *Blastocystis* spp. was higher in non-human primates (NHPs) (31.8%). Nucleotide sequence analysis of the SSU rRNA gene revealed seven different *Blastocystis* spp. subtypes, such as ST1, ST2, ST3, ST10, ST11, ST13 and ST14 in wild animals. ST3 was the dominant subtype (41.9%, 13/31) detected in NHPs (Li et al., 2019). The first study in Slovakia took place in 2014 in bears, where ST3 was identified (Valenčáková et al., 2014). That study found ST5 (4/74) in wild boars, the subtype most common in pigs; ST10 was the second subtype identified. This subtype predominates mainly in ruminants but has also been found in other animals (Maloney et al., 2020). An important aspect is that *Blastocystis* subtype ST5 has been identified in humans (Stensvold and Clark, 2016). The ST10 subtype is considered a possible zoonotic infection, making wild boar a source of blastocystosis for humans and posing a potential risk to humans as well as pets. The research done by Němejc et al. (2014) confirmed 16 *E. cuniculi* mono-infections, 33 *Enterocytozoon bieneusi* mono-infections and 5 mixed *E. cuniculi*/*E. bieneusi* infections. The highest prevalence of microsporidial infection was found in wild boars in Austria and the Czech Republic. Sequence analysis of the ITS region revealed two genotypes of *E. cuniculi*, with genotype I detected only in the Czech Republic. Genotype II of *E. cuniculi* has been detected in Austria, Poland and Slovakia. In our study, we recorded *E. cuniculi* in two study areas with a prevalence of 6.49%. This is approximately the same as the prevalence of 7.7% found in a previous work by Luptáková et al. (2010). *Encephalitozoon cuniculi* was identified in wild animals from Poland, the Czech Republic and Slovakia, with the most common species of *E. cuniculi* genotype II (92.5%), followed by *E. cuniculi* genotypes I (1.5%) and III (6.0%) (Santaniello et al., 2021). There is little evidence of a prevalence of cryptosporidiosis among wildlife, especially wild wolves. Kvac et al. (2021) examined samples of wild animals from the Czech Republic, Poland and Slovakia for the presence of *Cryptosporidium* spp. by microscopy and PCR/sequence analysis. *Cryptosporidium* spp. was found in five samples from 179 red foxes, three samples from 100 grey wolves and one sample from 63 brown bears, which is similar to our reports. The territories of wolves often overlap with commercial forests and agricultural lands close to humans, bringing them into contact with domestic animals and humans. The fact that wolves mark their territory with urine and faeces increases the risk of human or animal infection.

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In conclusion, this work provides a snapshot of the prevalence of *E. cuniculi*, *Cryptosporidium* spp. and *Blastocystis* spp. in wild animals in Slovakia and draw attention to the danger of infection transmission due to the intensive migration of animals across the territories. Detection of a genotype of *E. cuniculi*, the species *C. scrofarum* and *C. suis* and the zoonotic subtypes of *Blastocystis* spp. underscores that wildlife can be a source of a large variety of zoonotic infections.

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