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Methods used for concentrating oocysts of *Cryptosporidium* spp., spores *Encephalitozoon* spp. and *Enterocytozoon* spp. and their occurrence in Slovak water samples

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The number of outbreaks of water-borne diseases caused by parasites seems to have increased in recent years. Nevertheless, the occurrence of these pathogens in water generally pays little attention. Waterborne transmission is a major route in the epidemiology of the parasite and therefore poses a serious public health problem. *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. parasites are recognised worldwide as a common cause of diarrhoea. In most cases, it is a dilapidated or poorly maintained standard sanitation and water supply. It is important to perform periodic tests on protozoa, which are often lacking in small laboratories. Since it is necessary to filter large volumes of water for reliable diagnostics and consequently, it is difficult to concentrate them in a large volume of filtrate, it is not easy to detect their presence in the water. Various filtration methods are used to filter these pathogens from water, but cryptosporidial oocysts and microsporidia spores still occur in most of the world's and Slovak recreational waters. Therefore, it would be appropriate to use the abilities of gill-breathing aquatic animals that filter cryptosporidial oocysts and microsporidia spores from the water by absorbing them with food. Zeolite can also purify water by capturing high concentrations of contaminants, including cryptosporidial oocysts and microsporidial spores.

KEYWORDS

***Cryptosporidium* spp, *Encephalitozoon* spp, *Enterocytozoon* spp, filtration, PCR**

1 Introduction

One of the leading causes of death in children under the age of 5 is acute diarrhoea, especially in developing countries (Mohammad et al., 2021). The reason may also be the widespread occurrence of zoonotic parasites *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. in the environment, including waters, which are transported by the faecal-oral route (Omolabi et al., 2021). Although infections caused by these three parasites are poorly reported, their prevalence is relatively high. Dong et al. (2020) examined the incidence of *Cryptosporidium* in the general population, patients, school-aged children and the healthy population, with the highest estimated prevalence of *Cryptosporidium* infection in Mexico (69.6%, 95% CI 66.3–72.8), Nigeria (34.0%, 95% CI

12.4–60.0), Bangladesh (42.5%, 95% CI 36.1–49.0), Republic of Korea (8.3%, 95% CI 4,4–13,2) Although the worldwide prevalence of *Encephalitozoon* spp. and *Enterocytozoon* spp. is around 10% (Kucerova-Pospisilova et al., 1998; Halanova et al., 2013; Pan et al., 2015; Zang et al., 2021), in China is up to 41% – 44% incidence of *Enterocytozoon bieneusi* (Cao et al., 2020). Whereas infection can also be transmitted to humans by water and the environment (Guy et al., 2021), not only by direct contact; cryptosporidiosis, encephalitozoonosis and enterocytozoonosis are among the most common water-borne diseases. Oocysts and spores are transported by rivers, reducing their viability and concentration. Clinical surveillance and monitoring of aquatic pathogens are essential tools to detect and prevent further spread and minimise the outbreak's extent. However, clinical trials are usually limited to sufficiently ill individuals to seek treatment, leading to underestimating the prevalence of the disease (Yulfi et al., 2021) and providing a delayed outbreak indicator in the community. Analysis of large volumes of water is also needed to screen for pathogens from source and treated drinking water, as amounts from one infectious virion to 500 kL of water are considered sufficient concentrations for viral pathogens to detect them (Zahedi et al., 2021). Microbial contaminants, including bacteria, viruses and unicellular parasites, are the most important components to be controlled in reclaimed water due to potential effects on human health due to short-term exposure and ingestion. Most effects occur shortly after exposure, although the chronic effects of infections are also known. The highest number of conditions reported after contact with drinking, recreational and environmental water containing *Cryptosporidium* was in the USA in 2014, where up to 24 people lost their lives due to this parasite (Teel et al., 2022). One death after infection with the parasite *Encephalitozoon* spp. has been reported by tap water (Collier et al., 2021). Deaths against *Enterocytozoon* spp. are not yet known. Nevertheless, an epidemic has occurred in connection with this parasite, in which 200 people have become infected due to poor treatment of drinking water and its contamination (Cotte et al., 1999). The remaining parasites were also identified in rivers, lakes, drinking and wastewater. Cervero-Aragó et al. (2021) detected from surface waters in Austria *Cryptosporidium* spp. in 60% of samples. According to Ruan et al. (2021) overall prevalence rate of *Encephalitozoon* spp. in water was 58.5%. Still, subgroup analysis showed that the prevalence rate in wastewater treatment plants was much higher than in other waters (up to 74.1%). Also, the overall detection rate of *Enterocytozoon bieneusi* in water was high, up to 64.5% (Ruan et al., 2021). Overview transmission of *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon bieneusi* through water is essential for disease risk assessment and for designing effective preventive measures.

In Slovakia, diseases caused by the parasites *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon bieneusi* are among the Diseases reported with positive laboratory results. Cryptosporidiosis was first reported in seven HIV/AIDS patients (Čatár and Sobota, 1987).

In 2012, one case was reported in Slovakia, while in 2013, up to 12 cases of cryptosporidiosis were reported (RÚVZ, 2012; ÚVZ, 2013). They were recorded in two siblings in 2013, of whom only a seven-year-old boy showed clinical signs of the disease. Using DNA typing, *C. hominis* species was identified in these children (Ondriska et al., 2013).

In 2014, one case of an infection caused by *Cryptosporidium* spp. ÚVZ (2015).

At present, other infections of children from Roma settlements or children's homes in eastern Slovakia are also known, which were caused mainly by *C. hominis* and *C. muris* (Hasajová et al., 2014).

Human cryptosporidiosis was also caused by *C. parvum* in an immunocompetent patient in Slovakia, with the probable transmission of infectious oocysts occurring by direct contact with infected calves from Zemplínska Teplica and subsequent poor hand hygiene (Mravcová et al., 2020).

In 2003, Čisláková et al., 2003 microsporidia infections in 14% of immunocompromised patients; in 2013, Halánová et al. identified *E. bieneusi* genotype A and *E. cuniculi* genotype I in 30.5% of children living in Roma settlements and 2019 Halánová et al. detected *E. intestinalis* in up to 33% of immunocompromised patients and 5.7% of immunocompetent people.

2 The world's most widely used techniques for concentration and isolation of *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. from water

Although methodologies for detecting pathogens are well described and mastered today, sampling and concentration are still, to some extent, a problem. Costs and logistics are more demanding when larger samples are needed. A promising concentration method appears to be the hollow fibre dead-end ultrafiltration method DEUF (Kahler and Hill, 2020), which serves for the concentration of pathogens in water samples with different physico-chemical properties. It is designed to be performed in the field with minimal equipment and setup. In the DEUF process, water flows into the ultrafilter through the hollow fibre membranes and out of the pores of the ultrafilter, while the microbes are trapped in the hollow fibres. Ultrafilters can filter 10–50 L of turbid surface water or hundreds of litres of ready-made drinking water. The volume of filtered water thus depends on the water quality characteristics and the predicted concentrations of the target microorganisms. Several methods of filtering parasites from water samples are used worldwide, but the most common are filtering techniques using filters, which are summarized in Table 1. After filtration, the ultrafilter is processed in the laboratory. Alternative water filtration techniques are also used, but they must meet the required technical parameters and performance characteristics.

To capture oocysts of *Cryptosporidium* spp. and spores *Encephalitozoon* spp. and *Enterocytozoon bieneusi* have proven other alternative water filtration techniques (Table 2), but must meet the required technical parameters and performance characteristics. Isolation of *Encephalitozoon* spp. and *Enterocytozoon* spp. from raw wastewater and treated wastewater is practised using a combined system (Yamashiro et al., 2017). Primary wastewater samples with a volume of at least 100 L are subjected to centrifugal and concentration techniques (Cantusio et al., 2006), including centrifugation and washing. Subsequently,

TABLE 1 Standard filtration techniques used in the world.

Filtered parasite	Used equipment and filter	Pore size (μm)	Material	Advantages	Disadvantages	References
Cryptosporidium spp.	Membrane filtration machine (EMD Millipore Corp, United States)	1.2	nitrocellulose membrane	Low cost simple method	Uncomfortable work with a cloudy sample, constant replacement of filters is necessary.	Kumar et al., 2016
	Filtration equipment (Millipore, Durapore-hydrofilná)	0.45	polyvinylidene fluoride (PVDF) membrane			Bonilla et al., 2015
	Filtration equipment, ISO method, 15,553	1.2	acetate-cellulose membrane	Relatively simply method, Reproducible results	Filter is not reusable, High cost method	Cervero- Aragó et al., 2021
	microfiltration membrane (Sterlitech, Kent, WA, United States)	2	-			Ma et al., 2019
	Membrane microfilters, Super Micro-Wynd filters	1	polypropylene membrane	Low cost method	Necessity to change filters often	Velická et al., 2007; Our study
Cryptosporidium spp., *Encephalitozoon* spp. *Enterocytozoon bieneusi*	Filter capsule (Envirocheck High Volume)	1	-	Larger filter area	High expenses	Teel et al., 2022; Stine et al., 2004
	Filtration equipment with a vacuum system (Millipore®, Merck, Darmstadt, Nemecko)	1.2	cellulose ester membrane			Branco 2012; Franco et al., 2001

TABLE 2 Alternative techniques used to concentrate *Cryptosporidium* oocysts, *Encephalitozoon* spp. and *Enterocytozoon bieneusi* from the environment.

Technique	Isolated parasite	Volume	Detection	References
Compressed foam filter system	*Cryptosporidium* spp.	10–20 L	ChemScan	Sartory et al., 1998; Rushton et al., 2000
Continuous flow centrifugation	*Cryptosporidium* spp.	100 L	IFA, PCR	Swales and Wright 2000; Fan et al., 2021
Size selective continuous filtration	*Cryptosporidium* spp., E. bieneusi	100 L	IFA	Oda et al., 2000
Combined system	*Encephalitozoon* spp., E. bieneusi	100 L	Nested PCR	Yamashiro et al., 2017
Model organisms	*Cryptosporidium* spp. *Encephalitozoon* spp.	—	Microscopy, Nested PCR, Real-time PCR	Sučik et al., 2020
Zeolite filtration	*Cryptosporidium* spp.	10 L	Nested PCR	Sučik et al., 2021; Moropeng et al., 2020

ChemScan-quantitative evaluation of minerals by scanning electron microscopy.
IFA, immunofluorescence assay.
PCR, polymerase chain reaction.

the wastewater samples are subjected to a membrane filtration technique (Franco et al., 2001).

3 Techniques used for the detection of cryptosporidia and microsporidia

3.1 Microscopic methods

For the detection of *Cryptosporidium* spp. the sample concentrate can also be subjected to microscopy using staining. The most used technique for staining oocysts is the Ziehl-Neelsen technique. The two-step Ziehl-Neelsen staining process stains all tissue cells pink with a basic fuchsin solution in the first step. In a second step, the tissue is incubated in an acidic alcohol solution, which decolorizes all cells except the acid-fast cells, which retain their color and appear red. The mechanisms by which this color is produced are not well understood, but it is thought that the interaction of basic fuchsin with components of the bacterial cell wall creates a new molecule that is responsible for the color (Van Deun et al., 2008). Fayer, Morgan, Upton (2000) also used the modified Ziehl-Neelsen technique (Casemore et al., 1985) to detect cryptosporidia. Oocysts of *Cryptosporidium* spp. appeared pinkish-red, almost spherical, and measured 4–6 μm. Saha et al. (2019) saw cryptosporidial oocysts as light red to dark red spherical bodies, containing granules or bubbles and measuring 8–10 μm. The

samples are always microscopically observed at least twice to avoid errors. Results are compared to the Centers for Disease Control and Prevention image gallery. Staining according to Kinyon, staining according to Miláček and Vítovec, dimethyl sulfoxide-carbol fuchsin staining and safranin-methylene blue are used to stain oocysts (Magi et al., 2006).

Light microscopic examination of stained clinical smears, especially stool samples, is an inexpensive method for the detection of microsporidia spores, although it does not allow their identification to the species level. The most commonly used staining technique is the Chromotrope 2R method or its modifications (Feng et Li, 2017).

Transmission electron microscopy (TEM) has long been the gold standard for the identification of microsporidia based on polar filament observation in organisms and is still important for observing and describing the ultrastructural features of developing and mature organisms, but it is too expensive, time-consuming, and unsuitable for routine diagnosis (Weber, Deplazes, Schwartz, 2000; Feng et Li, 2017). Weber's chromotropic staining was also used by Galván et al. for the examination of microsporidia. (2013). Using optical brighteners, spores are visualized under a fluorescence microscope due to the binding of the optical brighteners to the chitin in the spore wall. Depending on the reagent used as well as the wavelength, the microsporidian spore walls will fluoresce. When using Uvitex 2B, Rylux D (Ostacolor, Prague, Czech Republic) and a wavelength of 405-490 (light during observation, 510 nm), the spores appear as green-white or turquoise oval formations (Valenčáková et Sučik, 2020).

3.2 Immunological and serological methods

Luka et al., in 2019, in their studies, they presented a label-free interdigitated capacitive biosensor for the detection of oocysts of *Cryptosporidium* spp. in water samples. Specific anti-*Cryptosporidium* monoclonal antibodies (IgG3) were covalently immobilized on interdigitated gold electrodes as capture probes, and bovine serum albumin was used to prevent nonspecific adsorption. Antibody immobilization was confirmed by measuring the change in contact angle. Detection was achieved by measuring the relative change in capacitance/dielectric properties due to the formation of the *Cryptosporidium*-antibody complex. The biosensor was tested for different concentrations of *Cryptosporidium* spp.

The results show that the developed biosensor can accurately distinguish different captured cell numbers and densities on the biosensor surface. The number of *Cryprosporidia* oocysts captured on the electrode surface was confirmed using a fluorescein isothiocyanate (FITC) immunofluorescence assay. The response of the developed biosensor depends mainly on the concentration of *Cryptosporidium* spp. under optimized conditions. The biosensor demonstrated a linear detection range between 15 and 153 cells/mm2 with a detection limit of 40 cells/mm2. The developed label-free capacitive biosensor has great potential for the detection of cryptosporidia in environmental water samples. Furthermore, under optimized conditions, this label-free biosensor can be extended to detect other biomarkers for biomedical and environmental analyses.

Serological tests are more sensitive and specific compared to microscopy. A direct immunofluorescence test (IFA or DFA) was also used for the detection of cryptosporidia by Pignata et al. (2019), using fluorescently labeled *Cryptosporidium* monoclonal antibody (Cellabs, Sydney, Australia).

As an alternative to examining the presence of *Encephalitozoon* spp. and *Enterocytozoon bieneusi* in the host is the use of commercially produced monoclonal antibodies, diagnosed in people with AIDS (Thellier et al., 2005) through stool. However, at certain stages of infection and in cases of chronic infections, excretion of spores through feces is not the rule. Therefore, in the case of a small number of samples (up to 10 samples), it is necessary to supplement the diagnosis with the IFAT serological method or, in the case of a larger number of samples, ELISA for the detection of antibodies against clinically significant types of microsporidia.

The Elisa Reagent test kit (Jining Industry Co., Ltd., Shanghai, China) was used to detect *E. cuniculi* antibody by Wang et al., 2022. The presence of *E. intestinalis* and *E. bieneusi* was proved by immunofluorescence test using species-specific monoclonal antibodies Halánová et al., 2019 in immunocompromised patients in Slovakia.

3.3 Molecular methods

Over the past 20 years, PCR-based molecular methods for amplification of gene targets have been developed and increasingly applied to improve sensitivity and species specificity in the diagnosis of both cryptosporidiosis and microsporidiosis (Feng et Li, 2017; Dashti et al., 2022). Compared to traditional methods based on microscopy, these molecular methods can offer potential advantages such as increased sensitivity, higher specificity, faster time to result and easier interpretation.

Researchers in Pakistan (Abbas et al., 2022) amplified the 18S ribosomal RNA gene for the detection of cryptosporidia in water, soil and food using newly designed genus-specific primers through a Multiplex PCR reaction that uses several types of primers to amplify multiple fragments in a single DNA sample.

Standard PCR was used to detect cryptosporidia in a biofilm in the Philippines (Masangkay et al., 2022), which is a fast and simple method used to amplify DNA sequences *in vitro*, which is based on the principle of enzymatic replication of nucleic acids. To quantify the number of cryptosporidia in biofilms, Koh et al. (2013) used a quantitative polymerase chain reaction (qPCR) technique in which DNA molecules are labeled with a fluorescent dye that is used to monitor and quantify PCR products in real time. Real-time PCR was also used by Elwin et al. (2022) for genotyping *Cryptosporidium* spp. while monitoring the pool water on the water slide.

E. bieneusi (genotypes similar to C and D), *E. intestinalis* and *E. cuniculi* (genotypes I and III) were detected by classical PCR in the analyzed water samples using different pairs of diagnostic primers (Galván et al., 2013).

Cantusio et al. (2006) confirmed the presence of *E. bieneusi* (AM1, AM3, AM27) in raw wastewater using Nested PCR, which consists of two steps: after the initial 25-35 PCR cycles, another PCR is performed using new primers "nested" into the original primers, which reduces the risk of unwanted products. *E. intestinalis* and *E. bieneusi* species were also identified by microsporidial SSU-rDNA amplification in waste, surface and groundwater samples (Fan et al., 2021).

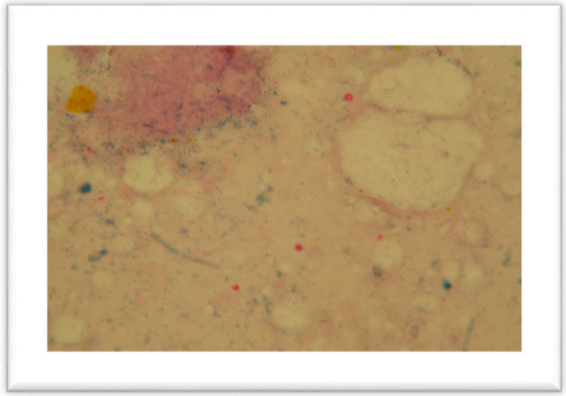

FIGURE 1
Cryptosporidium spp.—Kinyon staining.

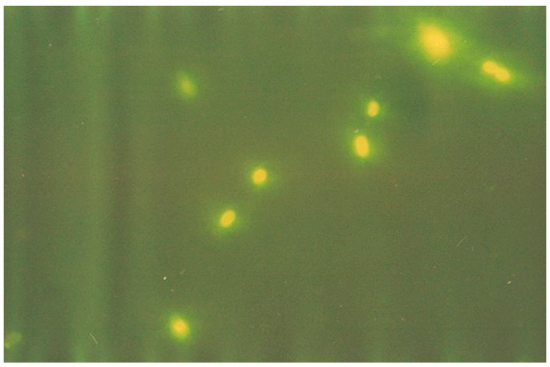

FIGURE 2
Encephalitozoon cuniculi stained by fluorescence.

4 Occurrence of *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. in the waters of Slovakia

Climate change and population growth are the causes of deteriorating river water quality. Predictions of climate change point to a higher frequency and intensity of extreme precipitation in many areas, while increased discharges of (treated) wastewater can affect faecal pollution, microbiological surface water quality and, ultimately, drinking water safety (Demeter et al., 2021).

Progress in the disposal and treatment of municipal wastewater is a basic prerequisite for sustainable development and environmental protection. At the same time, the existence of water management infrastructure also supports further social and economic growth in the regions (Cao et al., 2021). Slovakia has something to catch up with in terms of public sewers. In January 2018, 68% of the population reached the connection to the public sewerage system, but only less than 40% of the municipalities have a public sewerage system or only a partially built one. Building public sewers is a very important step in protecting groundwater quality. In particular, improper wastewater treatment or leaking sumps pose a significant risk to groundwater (MoEPSR, 2019).

Problems of protozoan parasites (Cryptosporidium) in the aquatic environment are comprehensive. Given that Slovakia is an industrial and agricultural country, we can expect that these microorganisms can contaminate water resources. A possible source of contamination can also be contaminated water with faeces used for irrigation. Therefore, the investigated areas (Figure 1) are interesting for studying these pathogens.

The presence of *Cryptosporidium* spp. in the waters of Slovakia is generally little studied, microsporidia *Encephalitozoon* spp. and *Enterocytozoon* spp. attention has not yet been paid to this area.

The pioneer in this issue was Velická, which in the period 2001–2003, using Micro-Wynd filters, isolated *C. parvum* from water reservoirs in Slovakia (Klenovec, Hriňová, Nová Bystrica, Málinec, Turček, Starina and Bukovec), while the sample volume was 10 L. After methyl violet staining, according to Miláček and Vítovec (1985) and microscopic determination, 41.4% of the samples were positive for *C. parvum*, and repeated determinations confirmed these findings. However, cryptosporidia have also been identified in several samples of already treated waters (Velická, 2007). Another researcher was Hatalová et al., who isolated *Cryptosporidium* spp. from the waters of eastern Slovakia using *Artemia metanauplii*, with a sample volume of 5 L. The observed parasite was found by Nested PCR in two of the five localities, namely, *C. parvum*, genotype IIaA16G1R1 in the Nad jazerom urban settlement lake in Košice and *C. hominis* genotype IeA11G3T3 in the Geča Lakes in the Košice Region (Hatalová et al., 2019). Kalinová et al. (2019), in their study, examined water samples from the Nitra region, used membrane microfilters made of a mixture of cellulose acetate and cellulose nitrate to capture cryptosporidia, and the sample volume was also 5 L. DNA sequencing resulted in four positives out of ten water bodies. The Jelenec and Vráble ponds were positive for *C. hominis*, genotype IeA11G3T3, Čierne Kľačany for *C. parvum*, genotype IIaA15G1R1 and *C. parvum*, genotype IIaA16G1R1 was found in the Slepčany reservoir.

To prove the presence of individual species of *Cryptosporidium* spp. microscopic methods were used, using the staining method of Miláček, Vitovec (1985), and molecular methods, namely, Nested PCR (Xiao et al., 1999).

5 *Cryptosporidium* spp. and *Encephalitozoon* spp. researched from water samples by our workplace using model organisms

At our workplace, we have analyzed the presence of *Cryptosporidium* spp. and *Encephalitozoon* spp., in aquatic animals: *Rivulogammarus fossarum, Lymnaea stagnalis, Unio tumidus, Dytiscus marginalis, Lymnaea stagnalis.* from 5 water reservoirs (Bukovec, Kechnec, Čaňa1, Geča, Čaňa2). Ziel Neelson staining with Kinyoun modification (Ma and Soave, 1983) was used to detect cryptosporidia (Figure 1). Microsporidia were stained by fluorescence (Vávra et al., 1993). Subsequently, we observed the gut of the animal samples or gills in animals with gills using a fluorescence FL-800 microscope (Figure 2). DNA analysis of *Cryptosporidium* spp. was performed by Nested PCR, using primers Xiao F1/R1 and Xiao F2/R2 (Xiao, 2010). Real-time PCR was used to diagnose *Encephalitozoon* spp. with primers 530F and 580R (Danišová et al., 2021). After receiving the sequences and

TABLE 3 Overview of *Cryptosporidium* spp. and *Encephalitozoon* spp. in Slovak water samples.

Place of collection	Type of water surface	Species	Genotype	Water filtration method	Researcher
Jelenec	pond	*C. hominis*	IeA11G3T3	Membrane microfilter	Kalinová et al., 2017
Čierne Kľačany	pond	*C. parvum*	IIaA15G1R1	Membrane microfilter	Kalinová et al., 2017
Vráble	pond	*C. hominis*	IeA11G3T3	Membrane microfilter	Kalinová et al., 2017
Slepčany	reservoir	*C. parvum*	IIaA16G1R1	Membrane microfilter	Kalinová et al., 2017
Košice- Nad Jazerom	lake	*C. parvum*	IIaA16G1R1	*Artemia franciscana*	Hatalová et al., 2019
Geča	lake	*C.hominis*	IeA11G3T3	*Artemia franciscana*	Hatalová et al., 2019
Geča	lake	*E. cuniculi*	—	*Lymnaea stagnalis*	Sučik et Valenčáková 2020
Nová Bystrica	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Klenovec	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Málinec	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Hriňová	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Turček	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Starina	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Bukovec	reservoir	*C. parvum*	—	Filter Super Micro- Wynd	Velická 2007
Čaňa1	lake	*C. parvum*	—	*Unio tumidus Lymnaea stagnalis*	Sučik et Valenčáková 2020
Čaňa1	lake	*E. cuniculi*	—	*Unio tumidus*	Sučik et Valenčáková 2020
Čaňa2	lake	*E. cuniculi*	—	*Dytiscus marginalis*	Sučik et Valenčáková 2020

comparing them with the homologous sequences from the GenBank using the BLAST program, the presence of *Cryptosporidium parvum* species in the Čaňa1 and *Encephalitozoon cuniculi* reservoirs was confirmed in the Čaňa1, Geča, and Čaňa2 reservoirs (Table 3).

6 *Cryptosporidium* spp. researched from water samples using zeolite

Zeolite, which together with quaternary ammonium chloride (QAC) has antimicrobial properties as a filter medium, can also be used for the early inactivation of parasites (Abbaszadegan et al., 2006). Due to the high pore density, it has an effective surface and can thus capture high concentrations of contaminants, which can also be applied to treat drinking water.

We compared the ability of oocyst catchability and properties when handling two types of zeolite, with smaller and larger particles. We came to the conclusion that easier handling was with coarser grained zeolite, it also achieved better results of oocyst filtration, even in a shorter time (Table 4).

TABLE 4 Comparison of success and filtration time of cryptosporidia by zeolites X and Y.

Zeolite type	Filtration time (min)	Oocyst catchability
X: 0,2–0,6 mm	**15**	4/4 samples
Y: 0–0,3 mm	**30**	3/4 samples

It doesn´t have any deeper meaning

7 Discussion

This systematic review aimed to compare the methods of concentration *Cryptosporidium* spp. oocysts and spores *Encephalitozoon* spp. and *Enterocytozoon* spp., to point out the possibility of using model organisms as filters and describing these pathogens' presence in waters in Slovakia. The occurrence of pathogens *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. is a relatively common phenomenon in drinking water sources and represent a significant problem due to the difficulty of detecting their presence. Both oocysts and spores are resistant to the many drinking water disinfectants used, so it is almost impossible to prevent their presence in the water. Due to the high prevalence of *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. in water, emphasis should be placed on its more frequent diagnosis. The mentioned genotypes, found in Slovak water samples, were also found in the other countries, specifically in lakes freely accessible to people for swimming, located in the cottage area, where the sewerage system has not been built yet. Specifically, the IeA11G3T3 genotype is anthroponotic in nature; it was the most common subtype contributing to morbidity in Israel (Grossman et al., 2019), and was found in HIV-positive patients in Thailand (Sannella et al., 2019), Zambia (Mulunda et al., 2020) or Mexico (Urrea-Quezada et al., 2018). Genotype IIaA16G1R1 has been identified in cancer patients not only in Slovakia (Hatalová et al., 2018) but also in

Slovenia, Estonia, Romania, Canada, Mexico and Tasmania (Soba et Logar, 2008; Koehler et al., 2014; Lassen et al., 2014; Valenzuela et al., 2014; Iqbal et al., 2015; Vieira et al., 2015). There are also studies confirming this genotype in rivers in Romania (Imre et al., 2017) or in donkeys and horses in Algeria (Laatamna et al., 2015).

As the zoonotic species *C. parvum*, *C. hominis*, and *E. cuniculi* have been identified in several bathing water samples, it can be assessed that little attention is paid to this issue given the risk these parasites pose to human and animal health.

For prevention, it would be appropriate to use gill-breathing aquatic animals to filtrate parasites, which absorb cryptosporidia and microsporidia spores of *Encephalitozoon* spp. and *Enterocytozoon* spp. as the results show, gill-breathing aquatic animals are suitable for detecting opportunistic pathogens in water. These species have been studied, among others, by researchers in the Czech Republic. The model organisms that effectively absorb oocysts have been used *Margaritifera* spp., *Rotifera* spp., *Anostraca* spp., *Bivalvia* spp. and *Gastropoda* spp. (Križanová, 2007; Rousková, 2008; Kociánová, 2009). This method for filtering opportunistic pathogens from water practised on aquatic organisms appears simple, ecological, economical and time-consuming.

The data suggest that zeolite has removed observed microorganisms from water so that it could be helpful for water and wastewater treatment of small volumes, as evidenced by multiple other world studies (Salazar et al., 2004; Abbaszadegan et al., 2006; Moropeng et al., 2020).

Due to its high pore density, it has an effective surface and thus can capture high concentrations of contaminants, which can also be applied to drinking water treatment. However, the disadvantage is the time-consuming method, so it cannot be used in practice to filter large volumes (100 L and more). Laboratory and field test data in the United States since the mid-1970s suggest that zeolite filter beds have 1.7 to 1.9 times the solids loading capacity/ft3 and excellent filtration performance compared to multimedia, even in tests. gravity filtration (Hansen, 1997; Fuger, 2003).

Based on more than 100 laboratory and field tests (2/3 using pressure vessels and 1/3 using gravity beds) since the mid-1990s, which represent commercial, residential and industrial water filtration projects, it has been concluded that in terms of solids loading capacity, high purity zeolite outperformed multimedia, sand/anthracite and sand because it more efficiently removed fine particles in the 0.5 μ to 10 μ range that escape from the conventional medium (Johnson and Desborough, 2010).

On the contrary, the filtration method consisting of filtration apparatus and filters remains less time-consuming, which is more suitable for filtration of larger volumes and is, therefore, the most used in practice. Several filtration devices and filters made of different materials have been tested, and the multifunctional use of this method is also an advantage.

However, what remains a problem is the neglect of reporting the occurrence of these parasites in the waters and the ignorance of the professional public about the reporting of these diseases. The United Kingdom could be an inspiration in this direction, where the Drinking Water Inspectorate (DWI) requires water companies to carry out risk assessments at all their water supply points and to determine the level of risk posed by *Cryptosporidium* spp. represents the quality of the final treated water. UK regulations also require companies to design and operate appropriate treatment and disinfection on an ongoing basis. Proven non-compliance with this regulation is a criminal offence in this country (Zahedi et al., 2015). Australia has also issued a warning against these zoonoses occurring in the Northern Territory (NT). People are warned before *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon bieneusi* by the Australian Veterinary Association, alerting to the possibility of indirect infection through contaminated water (AVA, 2021). So far, the only standardized method for detecting pathogens *Cryptosporodium* spp., *Encephalitozoon* sp. and *Enterocytozoon bieneusi* is United States Environmental Protection Agency (USEPA) Method 1623.1. An EnviroChek HV filter cartridge with a porosity of 1 μm is used to filter parasites from water samples, filtering 10–50 L of raw water. Immunomagnetic separation is used for the concentration of biological material and differential interference microscopy and fluorescence microscopy for detection, using fluorescein isothiocyanate and 4′,6-diamidino-2-phenylindole for cell staining (Fradette et Charette, 2022). The United States has also issued an EPA document on these parasites (USEPA, 1994) and its supplement (USEPA, 2001b), which contains sufficient information to conclude that *Cryptosporidium* spp., *Encephalitozoon* spp. and *Enterocytozoon bieneusi* can cause health problems. They occur in public water mains at levels that may pose a risk to human health.

8 Conclusion

Cryptosporidium spp., *Encephalitozoon* spp. and *Enterocytozoon* spp. are highly prevalent water parasites. These pathogens were detected in both wastewater and treated wastewater, and identical genotypes were detected from different treatment plants, which pose a high risk of infection to humans and animals. To reduce the number of infections caused by this parasite, a set of guidelines on wastewater use would be needed, as water is one of the most common sources of infection. Greater emphasis should also be placed on detecting and disinfection domestic water. Although filtering methods for this purpose are already known, the general public neglects their importance. This is not the case in Slovakia, despite the fact that *Cryptosporidium* spp. and *Encephalitozoon* spp. occurred in almost all of the water bodies studied, which are normally used for recreation. Model organisms eating these parasites as well as zeolite with its antimicrobial properties seem to be suitable options for preventing the occurrence of infection.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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