Aquat Ecol (2007) 41:47–54
DOI 10.1007/s10452-006-9043-5

ORIGINAL PAPER

The use of rotifers in detecting protozoan parasite infections in recreational lakes

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Received: 29 July 2005 / Accepted: 5 April 2006 / Published online: 31 August 2006

Abstract Although well-known methods for the detection of intestinal parasitic protozoans in water samples exist, they are insufficiently sensitive, expensive, of little practical value in the routine monitoring of waterborne pathogens and time- and labour-consuming. In the investigation reported here we have assessed *Cryptosporidium* oocyst detection using both the so-called Method 1623[recommended by the U.S. Environmental Protection Agency (USEPA)] and a direct method involving the determination of oocysts of *Cryptosporidium* in rotifers as detection tools of surface water contamination by dispersive stages of intestinal protozoans. Rotifers were sampled from three lakes located near the city of Poznan (Poland). To detect the oocysts of *Cryptosporidium*, we applied the fluorescent in situ hybridisation technique, an immunofluorescent assay and an enzyme immunoassay. Oocysts of *Cryptosporidium* were detected both in water collected from the lakes and in rotifers. The FISH technique applied to rotifers enabled the detection of biological contamination of surface water through an assessment of the dispersive stages of the parasite and was found to be more sensitive, less time-consuming and cheaper than the method recommended by the USEPA.

Keywords *Cryptosporidium* · Fluorescence in situ hybridisation · Rotifera – isolation and purification · Water – parasitology · Water pollution

Abbreviations

CSA	*Cryptosporidium*-specific antigen
EIA	Enzyme immunoassay
EPA	U.S. Environmental Protection Agency
FISH	Fluorescent in situ hybridisation
IFA	Immunofluorescent assay
ZN	Ziehl-Neelsen method

Introduction

Infections caused by parasitic intestinal protozoans such as *Giardia*, *Cryptosporidium* and

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Cyclospora have recently developed into an important health risk to both humans and animals. Dispersive stages of parasites, which are excreted with the faeces of infected hosts, are among the major sources of biological contamination of the environment, irrespective of the season. This is particularly true in aquatic ecosystems.

Oocyst, cysts and spores of intestinal protozoans are resistant to both environmental stressors and commercial disinfecting agents, and their frequent occurrence in water involves a risk of waterborne outbreaks. Moreover, the assumption that the existing methods of water treatment are sufficient for the elimination of intestinal pathogens should be revised since waterborne outbreaks of cryptosporidiosis, cyclosporosis and giardiosis have appeared despite the maintenance of accepted procedures of water treatment and the absence of *Escherichia coli*. To date, over 150 waterborne outbreaks of giardiosis, about 100 outbreaks of cryptosporidiosis and some outbreaks of cyclosporosis have been described, all of which could be traced back to water supply systems or to recreational swimming areas (lakes, swimming pools, water-parks etc.) (epidemiological reports: http://www.promedmail.org). Although there are well-known methods for detecting intestinal protozoans in water samples, these are mainly time- and labour-consuming, often very expensive and insufficiently sensitive. Currently, the most effective technology recommended by the U.S. Environmental Protection Agency (USEPA) relies on the so-called Method 1623 (Telliard 1999). However, for a greater degree of reliability in oocyst detection in contaminated water, water samples need to be examined using different techniques other than those used in Method 1623 (e.g. microscopic, enzyme immunoassay, indirect immunofluorescent assay, PCR-based analyses), which would improve the monitoring of recreational waters for waterborne pathogens (Quintero-Betancourt et al. 2002). Unlike Method 1623, the fluorescent in situ hybridisation (FISH) technique enables simultaneous identification of the species and assessment of its viability. In addition, the FISH technique is less complicated, cheaper and quicker than Method 1623. However, the real challenge is to develop a method that will both detect and identify intestinal parasitic protozoans and determine the viability of the dispersive stages of parasitic protozoans.

An alternative method for detecting these parasites uses various bivalve species for the identification of biological contamination of water. Although it is known that bivalves are excellent bioindicators of freshwater and sea contamination with (oo)cysts of *Cryptosporidium* and *Giardia* (Chalmers et al. 1997; Fayer et al. 1999; Freire-Santos et al. 2001; Gomez-Bautista et al. 2000; Graczyk et al. 1999; 2001; Lowery et al. 2001), the use of bivalves is often restricted in Poland – as in many other countries – as they are protected species.

Rotifers may be a useful tool in assessing surface water pollution based on some key characteristics of the phylum: (1) rotifers are among the most common biota of water ecosystems; (2) they consist of >2000 species globally, of which approximately 1350 have been reported in Europe (Dumont 1983) and 515 in Poland (Bielańska-Grajner and Radwan 1997); (3) the geographical distribution of the phylum is cosmopolitan; they live in marine and fresh waters; (4) rotifers feed mainly on algae, protozoans, bacteria, organic matter and periphyton (Gons 1979; Jürgens et al. 1994; Theil-Nielsen and Søndergaard 1999); (5) rotifers may be used for the monitoring of water quality (Mäemets 1983; Sládeček 1983; Saksena 1987); (6) rotifers ingest *C. parvum* and *Giardia* (oo)cysts (Fayer et al. 2000; Trout et al. 2002) and through predation they can remove *C. parvum* oocysts from aquatic ecosystems (Stott et al. 2003).

In a preliminary in situ investigation in which FISH was used, Majewska et al. (2003a, b) demonstrated that rotifers can detect water contamination by dispersive protozoan stages. Moreover, this technique enables viable (potentially infective) and nonviable (oo)cysts of *Giardia* and *Cryptosporidium* to be discriminated. The FISH technique applied to rotifers allows the investigator to simultaneously observe the pathogen's morphology, identify it to the species level, and determine its viability.

The aim of the present study was to compare two methods of pathogen detection in lakewater

samples: (1) an indirect method, in which Method 1623 was used to obtain suspended matter sedimented from water, which was later analysed using the Ziehl-Neelsen method (ZN), immunofluorescent assay (IFA), enzyme immunoassay (EIA) and FISH to determine the presence of oocystsor *Cryptosporidium*-specific antigen (CSA); (2) a direct method in which rotifers and EIA were used to determine the presence of CSA and FISH to determine the presence of oocysts (Fig. 1).

Materials and methods

Lakes

Rotifers and water samples were collected from three lakes located in the north-western part of Poznan (Poland): Kiekrz (surface area: 285 ha; maximum depth: 37.6 m; mean depth: 10.1 m), Strzeszyńskie (surface area: 34.9 ha; maximum depth: 17.8 m; mean depth: 8.2 m) and Rusałka (surface area: 36.7 ha; maximum depth: 9 m; mean depth: 1.9 m) (Fig. 2). These lakes are popular summer resorts for local inhabitants.

Sampling

Water samples from the lakes were collected at a depth of about 1.5 m, about 20–30 m from the lakeshore. All samples were collected in 2004 from fixed sites during the spring, summer and autumn. Two sets of material, each 5 l, were collected from each lake with a 5-l sampler. One set was examined using Method 1623 and the FISH technique, and the second was concentrated by filtering through a 15-μm plankton net to obtain rotifers. Both water samples were examined using Method 1623 and the FISH technique. The first sample was taken directly from the lake,

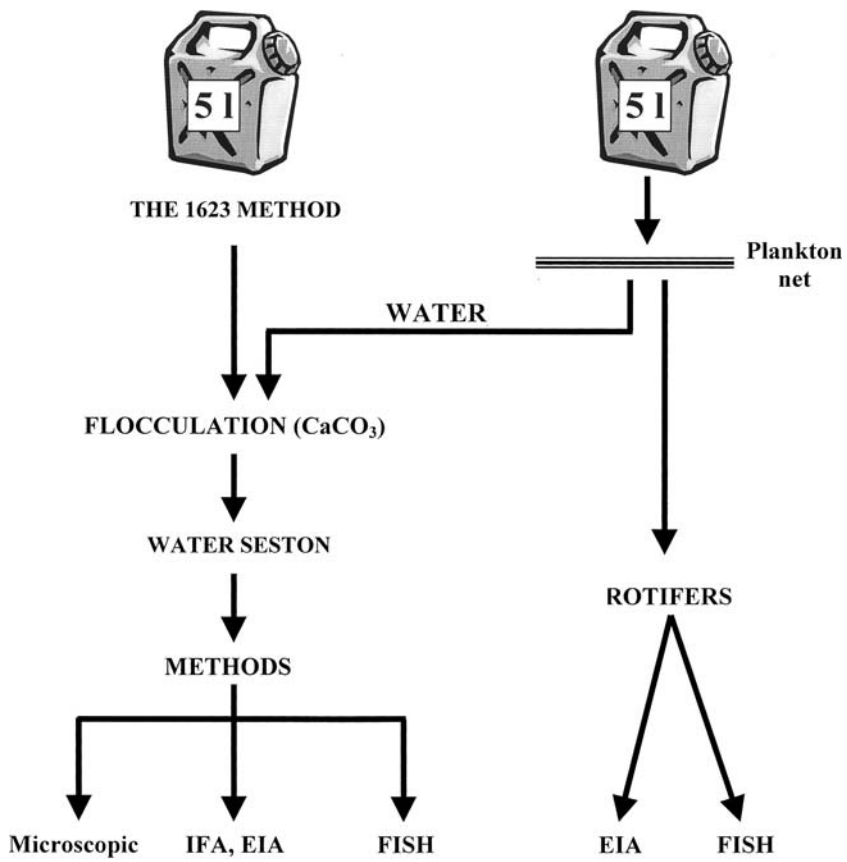

Fig. 1 Schematic diagram of applied methods

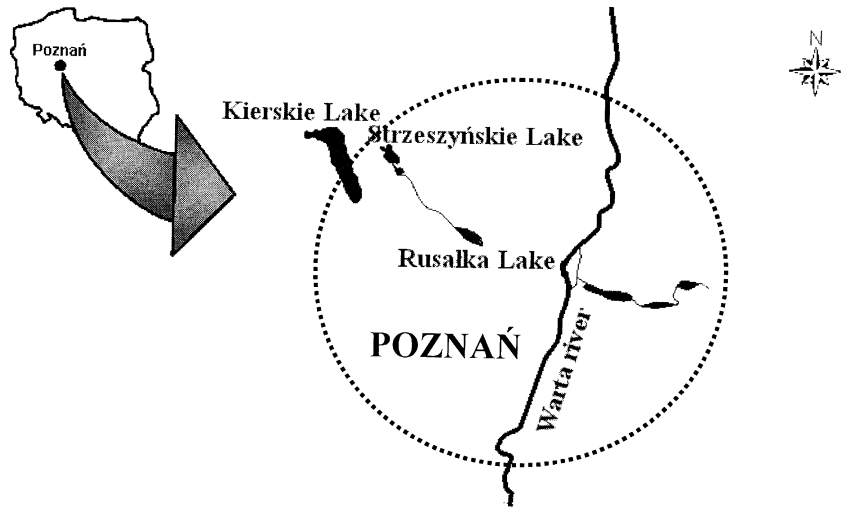

Fig. 2 Location of the Kiekrz, Strzeszyńskie and Rusałka lakes

whereas the second was first filtered through a 15-μm plankton net to obtain rotifers (Fig. 1). At the same time, replicate (three) samples were taken at each site for analysing the composition of the rotifer community in all three lakes. The collected material, each sample consisting of a total volume of 20 l, was sieved through a 15-μm plankton net and then fixed immediately with 4% formalin.

Rotifer samples

Rotifers from each concentrated sample were microscopically separated from the other zooplankton fractions (crustaceans) and homogenised (centrifuged at 8000 rpm for 5 min). To detect the *Cryptosporidium* oocysts as well as the coproantigen in the rotifers, we used the FISH technique, the IFA (Cellabs, Australia) and the EIA (ProSpecT *Cryptosporidium* Microplate Assay; Remel, Lenexa, Kan., USA). The FISH technique was modified according to Graczyk et al. (1997, 2003) and Vesey et al. (1998). The IFA and EIA protocols were based on the manufacturer's recommendations.

Water samples

The examination of water samples was based on the EPA recommendations as described in the 1623 Method. The seston was obtained using flocculation with $CaCO_3$ (Vesey et al. 1993). To detect the dispersive stages of parasitic protozoan the following methods were used (Fig. 1):

(a) microscopic – part of the suspended matter sedimented was stained according to the modified ZN method (Anonim 1991), and the slides were then examined microscopically at a magnification of 1000×;
(b) immunological – commercial tests (IFA and EIA) were used according to the manufacturers' protocols for the detection of *Cryptosporidium* oocysts and CSA in suspended matter sedimented;
(c) modified FISH technique.

FISH technique

The FISH technique is based on the hybridisation of fluorescently-labelled oligonucleotides with a specific fragment of parasitic 16S rRNA. All probes are designed for a species-specific sequence and are labelled with unique fluorochrome; consequently, the colour-based differentiation is species-specific. Because rRNA has a short life and is present in numerous copies only in viable cells, this technique allows visualization for viable and non-viable cells of parasites (Graczyk et al. 2003; Vesey et al. 1998).

For the FISH tests we used homogenised rotifers and suspended matter sediment. To each sample, 100 μl of acetone was added for permeabilisation, followed by 5 μl of Cry-1 probe. Each sample (20 μl) was then placed on a slide and, after an incubation period, examined by epifluorescence (UV-radiation at a wavelength of 490 nm).

Results

Kiekrz Lake

Cryptosporidium oocysts were detected in rotifers using the FISH technique only in the spring. The average size of *Cryptosporidium* oocysts was 5×5 μm (Table 1). The colour-based FISH reaction showed that the oocysts were viable. In the water sample obtained by plankton net filtration, the *Cryptosporidium* antigen was detected using the EIA test, and oocysts of *Cryptosporidium* were observed by the FISH technique. The parasite was not detected in the water sample taken directly from the lake.

In the summer (Table 1), no dispersive stages of *Cryptosporidium* were detected, neither in the water samples straight from the lake nor in those which were filtered through the plankton net. However, viable oocysts of *Cryptosporidium* were detected using the FISH technique in the rotifer samples. The size of the oocysts was 5×5.5 μm. In the autumn (Table 1), as in the summer, the parasite was not detected in the water samples; however, once again, *Cryptosporidium* oocysts were detected in the rotifers. The oocysts were non-viable, and their size was 5×5.5 μm.

The analysis of the rotifer community in Lake Kiekrz revealed the trend of a systematic decrease in numbers from the spring to the autumn. The highest abundance was recorded in the spring, when rotifers reached 2370 ind. l^{-1}. Later in the summer and autumn the densities were much lower, at 117 ind. l^{-1} and only 4 ind. l^{-1}, respectively. Six rotifer species dominated Kiekrz Lake, among which *Keratella cochlearis* f. *tecta* (Lauterborn), *K. quadrata* (Müller) and *Trichocerca pusilla* Lauterborn (Karabin 1985) indicated the eutrophic conditions of the lake. The dominating species varied on a seasonal basis: spring, *Polyarthra dolichoptera* Idelson, *Synchaeta lakowitziana* Lucks; summer, *K. cochlearis* f. *tecta*, *T. pusilla*; and autumn, *K. cochlearis* (Gosse), *K. quadrata*).

Strzeszyńskie Lake

In the spring season, oocysts of *Cryptosporidium* were detected in the rotifer sample, but they were not found in the filtered water samples (Table 1). On the other hand, *Cryptosporidium* oocysts and CSA were detected in water sampled directly from the lake. In the summer and autumn seasons, no dispersive stages of *Cryptosporidium* were recovered in either water taken up directly from the lake or in water filtered by the plankton net. Using the FISH technique, we detected *Cryptosporidium* oocysts only in rotifers sampled in the summer (Table 1). The oocysts were viable, and their size was 4.5×5 μm. The highest abundance was observed in the spring (519 ind. l^{-1}), followed by a sharp decrease in the summer (23 ind. l^{-1}) and autumn (6 ind. l^{-1}). *Keratella cochlearis* dominated throughout all three

Table 1 Results of water and rotifer examination for *Cryptosporidium* oocysts

Lakes/season	1632 Method[a]								Rotifers[a]	
	Water				Water from net				EIA	FISH[b]
	ZN	EIA	IFA	FISH[b]	ZN	EIA	IFA	FISH[b]		
Kiekrz										
Spring	–	–	–	–	–	+	–	+ (2)	–	+ (4)
Summer	–	–	–	–	–	–	–	–	–	+ (5)
Autumn	–	–	–	–	–	–	–	–	–	+ (6)
Strzeszyńskie										
Spring	–	+	+	+ (1)	–	–	–	–	–	+ (7)
Summer	–	–	–	–	–	–	–	–	–	+ (8)
Autumn	–	–	–	–	–	–	–	–	–	–
Rusałka										
Spring	–	–	–	–	–	+	–	+ (3)	–	+ (9)
Summer	–	–	–	–	–	+	–	–	–	+ (10)
Autumn	–	–	–	–	–	–	–	–	–	–

[a] –, Negative; +, positive

[b] Viable oocysts were: 2, 4, 5, 8, 10; non-viable oocysts were: 1, 3, 6, 7, 9; size of oocysts: (2), 5×4 μm; (4), 5×5 μm; (5), 5×5.5 μm; (6), 5×5.5 μm; (1), 5×6 μm; (7), 5×5 μm; (8), 4.5×5 μm; (3), 4.9×4 or 5×6 μm (9), 5×5 μm (10), 5×5.5 μm

seasons. In addition, *Trichocerca pusilla* dominated in the summer, and *Polyarthra dolichoptera* in the autumn. *T. pusilla* was the only eutrophic species found.

Rusałka Lake

In the spring, non-viable *Cryptosporidium* oocysts were detected in both the water filtered through the plankton net and in rotifers (Table 1). CSA was detected in the filtered water sample, whereas oocysts of the parasite were found in the rotifers. *Cryptosporidium* was not detected in water obtained directly from the lake. In the summer (Table 1), the dispersive stages of the parasite were not detected in water retrieved directly from the lake, but CSA was detected in water filtered by the plankton net. Using the FISH technique we detected *Cryptosporidium* oocysts in rotifers sampled in the summer (Table 1). There was a systematic trend towards increased densities from the spring to the autumn: from 10 ind. l^{-1} in the spring, to 118 ind. l^{-1} in the summer and 6701 ind. l^{-1} in the autumn. Three rotifer species dominated, among which only *Keratella cochlearis* f. *tecta* was a trophy indicator. There was a shift in the dominant species, with *Polyarthra dolichoptera* and *P. vulgaris* Carlin dominating in the spring, *K. cochlearis* f. *tecta* and *P. vulgari* dominating in the summer and *P. dolichoptera* dominating in the autumn.

Discussion

We observed that the FISH technique is more sensitive than the other methods used with respect to detecting *Cryptosporidium* oocysts and CSA. Most *Cryptosporidium*-positive results were obtained in the spring samples using different methods (EIA, IFA, FISH). *Cryptosporidium* oocysts detected by FISH in water samples and in rotifers from Kiekrz Lake were viable. However, *Cryptosporidium* oocysts detected by FISH in samples from Lakes Strzeszyńskie and Rusałka were nonviable. Moreover, using the FISH technique oocysts of *Cryptosporidium* were also recovered in seston obtained both from filtered water (Kiekrz and Rusałka lakes) and from the water sample taken directly from Lake Strzeszyńskie. It is important to note that the *Cryptosporidium*-positive results obtained by FISH were also confirmed by EIA and IFA.

The results obtained from a previous field research project (Trzebidzkie Lake) showed that rotifers may be an easily accessible tool with which to detect biological contamination of surface waters with dispersive stages of Protozoa (Majewska et al. 2003a). In addition, the results of the FISH demonstrated the presence of *Cryptosporidium* and *Giardia* (oo)cysts in *Brachionus diversicornis* (Daday) (Majewska et al. 2003b), which is an indicator species for the eutrophic state of water. In the present investigation the rotifers were homogenised for the FISH assay, thus making species determination of Rotifera which were positive for *Cryptosporidium* oocysts impossible.

In all rotifer samples, *Cryptosporidium* oocysts were detected only by FISH, with the exception of samples from the autumn season (Strzeszyńskie and Rusałka lakes). All oocysts detected in rotifers in the summer were viable in all lakes, in comparison to the non-viability of the rotifer samples in other seasons. Additionally, none of rotifer samples positive for *Cryptosporidium* oocysts by FISH were confirmed by the commercial EIA test, which detects CSA at approximately 20 ng ml^{-1}. This may have been due to the level of CSA in rotifer samples being lower than the sensitivity of EIA test and/or the rotifer organisms may have been able to destroy CSA.

The recovery of the most *Cryptosporidium*-positive samples from the spring season seems to be consistent with hydrological seasonality and the input of allochthonous matter into the lakes, which may contain faecal material or manure. Gołdyn (2000) has shown that it is during the spring circulation that the greatest amounts of nutrients are transported from the catchment area into the lake waters. The worst case of waterborne human cryptosporidiosis in the world occurred during the spring of 1993 in Milwaukee (USA) (Mackenzie et al. 1994, 1995). During this massive outbreak, 400,000 people were infected with *Cryptosporidium*, 4,000 of whom had to be hospitalised, with many of latter, in particular those with HIV infections or immunodeficient,

ultimately dying. Eisenberg et al. (2005) has suggested that this outbreak in the USA was caused by a transmission cycle due to infectious persons shedding pathogens into the sewage, environmental transport of these pathogens via Lake Michigan to the drinking-water plant and the subsequent infection of susceptible persons via exposure to the drinking water. This indicates a necessity for concentrating on the examination of the risk of waterborne outbreaks caused by the intestinal protozoan parasites during the spring season, when the potential of allochthonous enrichment is the highest.

It should be noted that in the present investigation all of the rotifers sampled during the spring were positive for oocysts of *Cryptosporidium* as assessed by the FISH method. We assume that the dominant species of rotifers at that time, which remove the oocysts of parasites from the aquatic ecosystem by ingesting them, may have taken part in oocyst transmission between particular links of the freshwater food chain, as was shown for Trzebidzkie Lake by Majewska et al. (2003a). In the case of the Kiekrz and Strzeszyńskie lakes, where rotifer densities in the spring were the highest compared with other seasons, the dominating species were *Polyarthra dolichoptera* and *Synchaeta lakowitziana* in the first lake and *Keratella cochlearis* in the second one. In Rusałka Lake, where the rotifer densities were extremely low in the spring, it seems probable that the dominating species, *Polyarthra dolichoptera* and *P. vulgaris*, which reached nearly 90% of the total rotifer community, were those that transmitted the *Cryptosporidium* oocysts.

In conclusion, the FISH technique used in combination with rotifers enables the detection of biological contamination of surface water and is an easier and more sensitive methodology than Method 1623. Moreover, it is more practical to apply in the routine monitoring of waterborne pathogens than Method 1623. According to our knowledge, the present study is the first field research carried out on the utilization of rotifers as detectors of water contamination by *Cryptosporidium* oocysts.

Acknowledgements This work was supported by an Interdisciplinary Grant between the Poznan University of Medical Sciences and Adam Mickiewicz University (Poznan, Poland, Grant No. 502-06-1-0006467) and a NATO Collaborative Linkage Grant (Brussels, Belgium, Grant No. CLG 979765).

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