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MICHAŁ POLUS[1], RENATA KOCWA-HALUCH[1]

OCCURRENCE OF *CRYPTOSPORIDIUM*, *GIARDIA* AND *TOXOPLASMA* IN SURFACE WATERS IN THE AREA OF CRACOW

Despite some knowledge on the risks from exposure to intestinal parasitic protozoa such as *Cryptosporidium*, *Giardia* and *Toxoplasma*, no occurrence studies of these protozoa were conducted in Cracow area. Therefore, the purpose of the work was to examine the presence of *Cryptosporidium*, *Giardia* and *Toxoplasma* in surface water samples including intakes and recreational waters. The study showed that the vast majority of water samples were positive for *Cryptosporidium* sp., and some of the samples were positive for *Giardia intestinalis*. None of the tested water samples showed the presence of *Toxoplasma*. Given the limitations of the research methods used and the limits of detection, these results indicated that the concentration of protozoan cysts/oocysts may range from <1 to dozens oocysts/cysts per liter of water. These concentrations are comparable to the values given in reports of major worldwide outbreaks of cryptosporidiosis or giardiasis, which indicating a need to further investigate Cracow's surface waters.

1. INTRODUCTION

The conclusions of several hundred reports describing outbreaks of cryptosporidiosis and giardiasis indicates the potential for surface waters worldwide to contain these intestinal parasitic protozoa in natural waters [1, 2]. It has been shown that much of the surface water is contaminated with detectable levels of *Giardia intestinalis* cysts or *Cryptosporidium* oocysts. This applies to numerous developed countries from around the globe (U.S.A., Canada, U.K.), in which the percentage of positive environmental samples is in the range from a few to nearly 90% [3–7]. It is relatively difficult to assess the prevalence of *Toxoplasma* oocysts in the environment and one can only present certain estimations based on the prevalence of toxoplasmosis in domestic cats, wild cats and other felidae [8]. Some reports have stated concentration of oocysts/cysts

[1]Cracow University of Technology, Faculty of Environmental Engineering, ul. Warszawska 24, 31-155 Cracow, Poland, corresponding author M. Polus, e-mail: mpolus@vistula.wis.pk.edu.pl

higher than 100 per liter. Because the infective dose for an adult human is relatively low (about 10 oocysts/cysts), it seems from the epidemiological risk of intestinal parasitic protozoa is highly significant [9]. The main concern is the elimination of parasitic protozoa from drinking water, which is not a simple task, due to the small size of the cysts/oocysts, in part overcome by filtration, the significant resistance of the invasive forms of protozoa to environmental factors, and standard disinfectants simultaneously used in the water treatment process [9].

Besides the threat of consumption of treated water containing cysts of intestinal parasitic protozoa, there are also other possible sources of infection, such as swimming pools or open bathing waters [10]. Even enclosed swimming facilities, using regular chlorination, do not entirely eliminate the risk of illness coming from swallowing this water. Only in the U.S.A. in the last quarter century, more than 10 000 cases of cryptosporidiosis was described due to recreation in water parks or swimming pools. Similar risk of infection is associated with the use of lakes and other water recreation locations [10, 11].

In Poland, no extensive studies were done for the assessment of protozoa of the genus *Cryptosporidium*, *Giardia* and *Toxoplasma* in natural waters. Epidemiological data, especially concerning the incidence of cryptosporidiosis, is not complete. In this study, we examined several surface waters from the Cracow area, ranging from drinking water source intakes to recreational sites. Results were the detection of intestinal protozoa DNA by nested-PCR. This approach does not give accurate quantitative information on the concentration of parasites in a sample of water, however nested PCR is very sensitive and combined with the limits of detection, may indicate actual concentration ranges. It also allows work on smaller volumes of water samples and greatly simplifies the preliminary concentration of the material. Because the subject is surface water, and not treated water, the presence of parasitic DNA can be recognized as the presence of potentially infective oocyts/cysts.

2. METHODS

Water samples of 4 dm^3 were collected monthly from designated locations in the period from June to September 2012 (Fig. 1). The samples were subjected to filtration through a standard mixed cellulose ester membrane filter (Millipore type RAWP) with the diameter of 90 mm and porosity of 1.2 μm. In the case of very large water turbidity, filtration was done on either a few membranes and then the collected pellets were combined or by initial sample prefiltration using fiberglass in order to remove suspended solids (Millipore type AP25). After filtration, the membrane filter was transferred to a Petri dish, flooded with 5 cm^3 of 0.01% Tween-20 (in PBS) and gently scraped. The collected suspension was overlaid on an aqueous sucrose solution (d = 1.15 g/cm^3) and centrifuged (1250*g*, 10 min, 4 °C). The entire top fraction togeth-

er with the interphase was transferred to a new test tube, diluted to 50 cm^3 with cold PBS and centrifuged (4500*g*, 15 min, 4 °C). The pellet was washed twice with 25 cm^3 of PBS and centrifuged (4500*g*, 15 min, 4 °C). The pellet was finally suspended in 1 cm^3 of PBS and transferred to a standard 1.5 cm^3 test tube [12, 13].

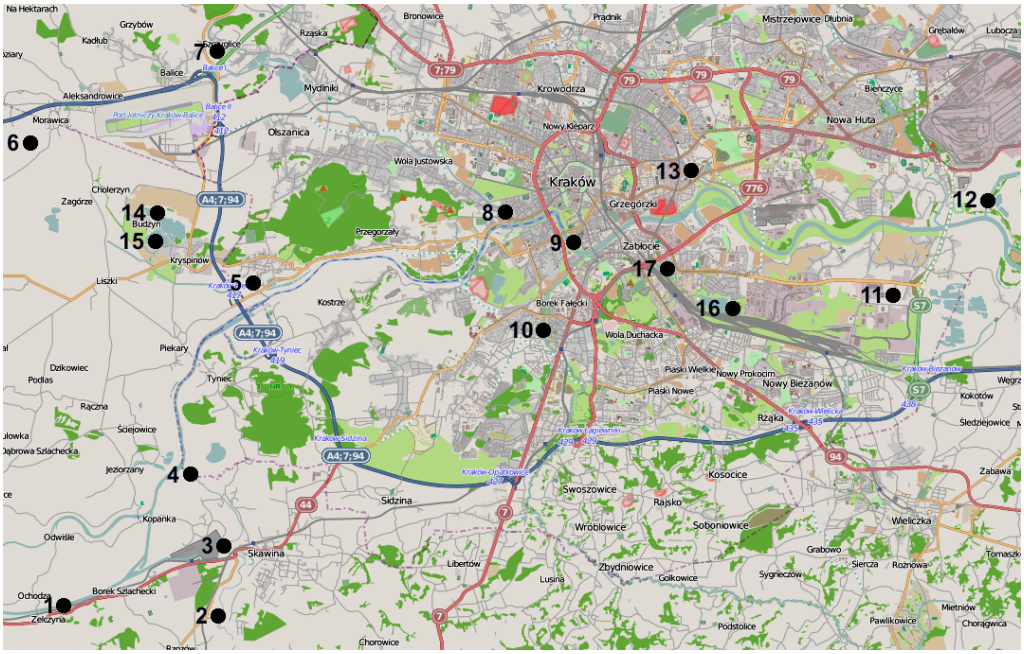

Fig. 1. Location of water sampling points. Numbering as in Table 2

Purification of *Cryptosporidium* oocysts and *Giardia* cysts was performed by immunomagnetic separation (IMS). IMS was adopted from the manufacturer's protocol by eliminating the 10 cm^3 IMS and beginning at the 1.5 cm^3 stage, and eliminating dissociation (Life Technologies, Dynabeads® GC-Combo Kit). In brief, pellets gathered in the earlier steps were suspended in 1 cm^3 of 1× SLA buffer (100 µl of 10× SLA and 100 µl of SLB in 1 cm^3 of water) in a 1.5 cm^3 flip tube and 10 µl of each Dynabeads®. The tubes were then placed on a rotary shaker and subjected to gentle stirring/rotation (15 rpm) for 2 h. Then, the tubes were placed in the DynaMag-2 and gentle swinging was performed for 2 minutes until the suspension of magnetic beads accumulated onto the walls of the tubes. Buffer was discarded and the remaining beads were rinsed twice in 1 cm^3 of 1× SLA buffer and subsequently in cold PBS. The material retained in the preceding step was centrifuged (14 000*g*, 10 min, 4 °C), and the supernatant was discarded. The pellet was suspended in 600 µl of lysis buffer (10 mM Tris, 0.5% SDS, pH 8.0).

The lysate was incubated at 37 °C for 30 min after which it was subjected to 5 freeze/thaw cycles (5 min in liquid nitrogen and 5 min at 60 °C per cycle) [14]. Samples were then incubated for 15 h (overnight) with proteinase K (200 μg/cm^3, 52 °C) while being vigorously shaken in a thermomixer. Then, tubes were incubated for 15 min at 95 °C to denaturate the proteinase K and single extraction with 500 μl of phenol:chloroform:isoamyl alcohol mixture (25:24:1 v/v, pH 8.0) was made. Lysate was centrifuged (7500*g*, 15 min, 4 °C) and the upper, water phase was transferred to a new 1.5 cm^3 test tube. DNA was precipitated by addition of 100 μl of 10 M ammonium acetate and 800 μl of isopropanol. The samples were then centrifuged (20 000*g*, 30 min, 4 °C). Pellets were washed once with 1 cm^3 of cold 70% ethanol, centrifuged (20 000*g*, 15 min, 4 °C), air-dried (1 h) and suspended in 50 μl of molecular biology grade water.

Extraction of *Toxoplasma* DNA was performed in the same way: water samples of 4 dm^3 were treated as described above. Only IMS step was omitted since there are no commercially available IMS kits for *Toxoplasma*.

The PCR was performed in a final volume of 25 μl in thermal cycler Mastercycler Personal (Eppendorf) using 2.5 μl of DNA under the following conditions: 0.7 unit of DNA polymerase DNA (Biotools, Spain), 2 mM of Mg^{2+}, 0.2 mM of dNTPs, 250 nM of each primer. In the case of the nested-PCR, 0.5μl of outer-PCR was used as a template. Genomic DNA of *Cryptosporidium parvum* (ATCC PRA-67D), *Giardia intestinalis* (ATCC 30888D) and *Toxoplasma gondii* (ATCC 50174D) were used as positive controls and quantitative standards in the PCR reactions. The concentration of standard solutions of genomic DNA was determined based on the measurements in the Qubit fluorometer (Invitrogen). The set of primers used in this study (Table 1) allowed accurate detection of 0.1 pg of genomic DNA of each of the three protozoa in a single-step PCR [15–17]. The primers are designed so that they can detect all *Cryptosporidium* species and species of *G. intestinalis* and *T. gondii*.

Table 1

The sequence of the primers used in the PCR reactions (outer and nested) [14–17]

Species	Outer-PCR	Nested-PCR
Cryptosporidium sp. (18S rRNA)	5′-agtgcttaaagcaggcaactg 5′-cgttaacggaattaaccaga	5′-tagagattggaggttgttcct 5′-ctccaccaactaagaacggcc
amplicon:	655-667 bp (species dependent)	429–455 (species dependent)
Giardia intestinalis (giardin gene)	5′-cataacgacgccatcgcggctctcaggaa 5′-ttagtgctttgtgaccatcga	5′-aagtgcgtcaacgagcagctc 5′-ttagtgctttgtgaccatcga
amplicon:	405 bp	171 bp
Toxoplasma gondii (B1 gene)	5′-tgttctgtcctatcgcaacg 5′-acggatgcagttccttttctg	5′-tcttcccagacgtggatttc 5′-ctcgacaatacgctgcttg
amplicon:	~2 kb	532 bp

All reactions, including nested-PCR, were performed using one and the same thermal profile: 94 °C/3 min, 40 cycles involving the phase of 94 °C/45 s: 55 °C/60 s: 72 °C/60 s, and final extension 72 °C/10 min. Electrophoresis was carried out on 1.5% standard agarose containing SYBR Green (Molecular Probes). In the cases of weak fluorescence, gels were additionally stained in SYBR Gold (Molecular Probes). Additional control assessments performed on seeded water samples showed PCR sensitivity of 10 *C. parvum* oocysts and 50 *G. intestinalis* cysts (Waterborne, Inc.).

3. RESULTS AND DISCUSSION

The applied method allowed the detection of parasitic DNA with sufficient oocysts/cyst recovery and adequate sensitivity [18]. Achieved sensitivity is similar to that reported by other authors [19, 20]. This did not prove the presence of live cysts/oocysts in water, but rather indicated the presence of its genetic material. If these were analyses of treated water samples, the criticism would be justified. Inactivation of microorganisms may not automatically cause degradation of DNA. However, it can be assumed that in natural waters (reservoirs, rivers and other watercourses) the presence of DNA may indicate that viable oocysts/cysts are present as presumably naked DNA would rapidly degrade [20, 21].

Table 2

Summary of water sampling points and the results of four consecutive months (June–September 2012)

	Sampling point	*Cryptosporidium* sp.				*Giardia intestinalis*			
		VI	VII	VIII	IX	VI	VII[1]	VIII	IX
1	Wisła River, Czernichów	+	–	+	–	–	n	–	–
2	Skawinka River, Skawina	+	+	–	+	–	n	–	–
3	Skawinka River, Skawina/intake	+	+	+	+	–	n	–	–
4	Skawinka River, Skawina/park	+	+	+	+	–	n	–	–
5	Sanka River, Mników/estuary	+	+	+	+	–	n	–	–
6	Sanka River, Kraków/intake	–	–	–	–	–	n	–	–
7	Rudawa River, Zabierzów	+	–	+	+	–	n	–	–
8	Rudawa River, Kraków/Salwator	+	–	+	+	–	n	–	–
9	Wisła River, Kraków/Wawel	+	–	+	+	–	n	–	–
10	Wilga River, Kraków/estuary	+	–	+	+	–	n	–	–
11	Drwina River, Kraków/estuary	–	+	+	+	–	n	–	–
12	Dłubnia River, Kraków/estuary	–	–	+	+	–	n	–	+
13	Białucha River, Kraków/estuary	–	+	+	+	–	n	–	–
14	Kryspinów I reservoir	+	–	+	+	+	n	+	–
15	Kryspinów II reservoir	+	+	+	+	+	n	+	–
16	Bagry reservoir, Kraków	–	+	+	+	–	n	+	+
17	Płaszów reservoir, Kraków	–	+	+	+	–	n	–	–

[1]n means no tests performed.

Table 2 contains a summary of the locations where water samples were collected (during the period June–September 2012). There are rivers flowing through Cracow area (item 1–13) and open water recreational sites (item 14–17). Some locations are placed on the same river, above and below the point of discharge of waste water from municipal treatment plants (items 2/3, and 5/6). *Cryptosporidium* was found in 75% of water samples in the observation period. Only one site (Sanka River at Mników intake) remained free of *Cryptosporidium* sp. The second water intake test point (Skawinka River) was positive for *Cryptosporidium* in three of the four examinations made. It can therefore be seen, that both the Wisła river itself, and all tributaries leading into it in the vicinity of Cracow are contaminated with *Cryptosporidium* oocysts. *Giardia intestinalis* was detected in only a few positions (item 14–17) which are reservoirs used for recreation.

Toxoplasma gondii DNA was not detected in any sample. It cannot, however, be argued that *T. gondii* does not occur at all. The essential difficulty in determining the presence of *T. gondii* in surface water is the lack of standardized detection techniques – in particular, the lack of a good immunomagnetic separation method and convincing PCR protocols. Figure 2 shows the differences in the quality of the results depending on the application of IMS. It is clear that more reliable results arise when using IMS. This means that the most critical step of the whole procedure is the isolation of DNA from the surface water. Thus, the purification of DNA requires further research. Therefore, the results obtained by our team in detection of *T. gondii* are to be treated as a prelude to further work.

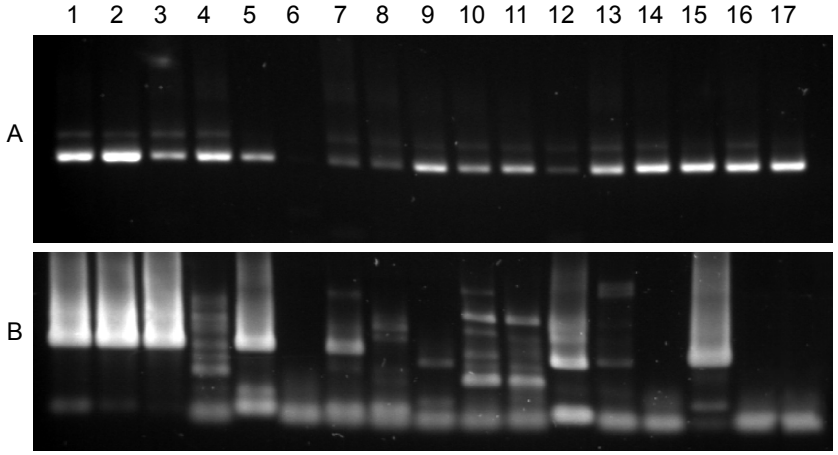

Fig. 2. Impact of IMS on the results of PCR. The figure shows an example of *C. parvum* detection result (September 2012): A – IMS step was made, B – no IMS. Sampling points labeled as in Table 2

A short comment should be made to the positive results obtained. Although the results of PCR have a purely qualitative meaning, the threshold sensitivity indicates that

the minimum number of oocysts of *Cryptosporidium* sp. or cysts of *G. intestinalis* in the volume of water (4 dm^3) is higher than 10 (*Cryptosporidium*) or 50 (*Giardia*). This means the minimum concentrations of 2.5/dm^3 and 12.5/dm^3, respectively.

In some cases of cryptosporidiosis outbreaks, the concentrations of oocysts in water samples were determined. The concentration levels vary mostly in the range of 0.13/dm^3 to 5/dm^3 (in few cases, more than 50 oocysts per liter) [22]. Assuming that the infective number of oocysts of *Cryptosporidium oocysts* is in the range of 10–100, the achievement of such a dose requires the consumption of a few to tens of liters of water, which seems generally unlikely and rather suggests that the actual concentration of oocysts reported by the authors discussing outbreaks was much higher [23, 24]. Otherwise, it would be concluded that the obtained results of this study demonstrate enormous epidemiological risk coming from natural waters in the city of Cracow. The annual reports of the Regional Sanitary Epidemiological Station in Cracow (WSSE Kraków) indicate an increase in the last five years in the number of cases of full-blown intestinal infections of unknown origin (beginning in 2009, a statutory order was introduced for physicians to report all catarrhal diarrhea of possibly infectious origin, the number of cases in subsequent years, is steadily increasing consecutively from 2009 until 2012: 1066, 1146 , 1448 and 2584 cases have been reported, respectively) [25]. At the same time the number of reported cases of giardiasis is growing, from 8 to 58, in the last three years, but there is no indication whether *G. intestinalis* infections were associated with contact with contaminated water [25]. It is highly likely that a significant proportion of cases of unknown etiology is caused by *Cryptosporidium* oocysts and *Giardia* cysts present in surface waters. Demonstrated in this study the prevalence of *Cryptosporidium*, relatively low prevalence of *Giardia* and the relatively small number of reported cases of cryptosporidiosis and giardiasis is probably due to the general good practice in Poland of boiling water before consumption.

4. CONCLUSIONS

High prevalence of *Cryptosporidium* sp. in surface waters in Cracow has been revealed. Also, *Giardia intestinalis* was detected in relatively numerous samples. Because the missing data on the prevalence of waterborne parasitic protozoa in the rest of the Lesser Poland province, it seems necessary to perform further efforts aimed at assessing the water safety.

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