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Autor: Svoboda, Paul / Ruchti, Sandra / Bissegger, Christoph

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Occurrence of *Cryptosporidium spp.* Oocysts in Surface, Raw and Drinking Water Samples

Paul Svoboda, Cantonal Laboratory of Basel-Landschaft, Liestal

Sandra Ruchti, Christoph Bissegger and Marcel Tanner, Swiss Tropical Institute, Basel

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Introduction

The protozoan parasite *Cryptosporidium parvum* is an important pathogen of humans, and is also an enteric pathogen of a range of other mammals, particularly young livestock (1). The first cases of human infection with *Cryptosporidium spp.* were reported in 1976 (2, 3). Connected with the AIDS pandemic, *Cryptosporidium parvum* owing its potential for opportunistic infections, has become recognised as a pathogen of great public health importance.

Cryptosporidial infections are either asymptomatic or result in acute transient diarrhoea, which in some cases may be severe enough to require hospitalisation. However, in immunocompromised patients the infection can become a chronic condition with persistent clinical symptoms, and is potentially life-threatening. An effective chemotherapeutic treatment has not yet been developed (4).

The infectious stage of the parasite is the oocyst which is excreted in great numbers with the faeces of infected hosts. The infective dose is not accurately known, but is believed to be very low. Infection occurs by the faecal-oral route, either directly from person to person or animal to person (5), or indirectly through contaminated vehicles like food (6) and water (7).

Numerous waterborne outbreaks of cryptosporidiosis have been documented since 1983, mainly in the USA and in the UK (8). A massive outbreak in 1993 in Milwaukee, USA, where an estimated 403 000 persons suffered from watery diarrhoea, was caused by *Cryptosporidium spp.* oocysts that had invaded the city's water treat-

ment plant (9). This outbreak was considered to be responsible for the death of at least 69 individuals (10).

Therefore there is a need to assess the presence of *Cryptosporidium* in drinking water. In contrast to most enteric bacteria which are relatively easy to eliminate from contaminated waters, parasites like *Cryptosporidium* and some pathogenic human viruses are highly resistant to the effect of numerous disinfectant procedures (11). Therefore, the indicators commonly used as measure of disinfection are an inadequate measure of activity against *Cryptosporidium* oocysts. Hence, the occurrence of *Cryptosporidium* in raw and drinking water is of major concern to water treatment plants and the drinking water supply, and a sensitive and reliable testing method is essential. Effective concentration and detection techniques for *Cryptosporidium spp.* in water are difficult to develop, since oocysts occur in low numbers in drinking-water. The techniques available at present are inefficient, costly, tedious and time-consuming (12–14).

The present study was carried out using an improved IMS (immunomagnetic separation) method (15) to assess the contamination level of surface and drinking-water and the risk of a waterborne infection with *Cryptosporidium* in the canton Basel-Landschaft, from which data on cryptosporidiosis in children and the risk factors have already been established (5). It has to be emphasized that the water supply system in the canton Basel-Landschaft mainly consist of many small water facilities, which take their supply from ground or well water, i.e. nearly each community has its own water. Furthermore, agriculture with cattle is fairly common in this region and the geology is predominantly a lime-stone formation, an underground which has a high porosity and enables *Cryptosporidium spp.* oocysts to invade ground waters.

Materials and methods

Sample collection

River water

Samples were taken at four sampling locations (B1–B4) from the Lützel River in the Lützel Valley. The river is contaminated by sewage especially during time of heavy rains and impacted by agriculture. B1 is upstream and B2 downstream a water entry of a sewage treatment plant. B3 is located upstream and B4 downstream an entry of a small stream having a high run off from grazing land.

Raw water (untreated well water)

Samples were taken from two sources in a community in the Lützel Valley. The two sources were located downstream from the four sampling locations B1–B4.

Drinking water (treated well water)

Samples were collected from the water treatment plant that is treating the raw water by mechanical flocculation, sedimentation, rapid sand filtration and chlorine addition (0.05 mg/l).

During the 5-month study, weekly to biweekly samples of river, raw and drinking water were collected. Since river water quality is characterized by extreme variability, sampling was carried out when weather conditions were alternately fair or rainy, respectively. Flow and turbidity in each of the rivers were low as is characteristic of these rivers when weather conditions were fair. Rainfall sufficient to produce some runoff was always coupled by increased water turbidity.

Sampling was done in 20 liters polycarbonate containers that were transported to the laboratory within two hours. Care was taken during the sampling process to avoid sampling material floating on the surface and from bottom sediments. Water samples were analysed for the presence of *Cryptosporidium spp.* oocysts by applying the method of *Bissegger* et al. (15), which is described in brief below.

Water concentration by flocculation

Water samples were concentrated by flocculation with aluminium sulphate. For that purpose, 40 ml of 10 % aluminium sulphate were added to the water sample (20 l) while stirring well. The pH was adjusted to 5.8–5.9 with 1 M HCl. The flocculate was allowed to sediment at room temperature for 4 h, and the supernatant was subsequently aspirated by vacuum pump. The sediment was divided into 250 ml centrifuge bottles, and the vessel was rinsed with 50 ml 0.1 % Tween 80 and 100 ml demineralized water. The rinsing solutions were added to the centrifuge bottles. After centrifugation at 1500 x g for 10 min, the supernatant was discarded, and 40 ml citrate buffer (42 g citron acid monohydrate and 88.2 g tri-sodiumcitratedihydrate ad 500 ml demineralized water; pH = 4.7 (34)) were added. The bottles were well shaken on the vortex, the contents transferred to a conical centrifuge tube and left at room temperature overnight to dissolve the gelatinous precipitate of aluminium hydroxide. The next morning, 20 ml 0.1 % Tween 80 was added. The oocyst suspension was again well shaken and centrifuged for 10 min at 1500 x g. The supernatant was aspirated by vacuum pump and the pellet containing the oocysts retained.

Immunomagnetic separation (IMS)

The pellet (see above) was washed with 0.1 % Tween 80 and subsequently with PBS (1500 x g for 10 min). Dynabeads anti *Cryptosporidium* (Dynal AS, Norway; article no. 730.01) were used according to the manufacturer's instructions. The separated oocysts were transferred onto one well of a slide, dried, fixed and stained with a fluorescent monoclonal antibody (Crypt-a-Glo, Waterborne, Inc. Clinical and Environmental Parasitology Products, New Orleans, USA). The antibody solution was gently aspirated and washed out with oocyst free water. 10 µl of mounting medium (30 ml glycerol and 20 ml PBS and 1 g DABCO [diazabicyclooctane]) was pla-

ced onto the slide, covered with cover-slip and sealed with clear nail varnish. The oocysts were identified under an epifluorescence microscope at 400 x magnification on the basis of size (4–6 µm), shape (round to oval), and staining (apple green with a stronger marked outer ring).

Microbial contamination in river, raw and drinking waters

All river, raw and drinking water samples were also examined microbiologically. *Escherichia coli* and enteroccoci were determined measuring concentrations of colony-forming particles by using the standard membrane filtration method (16).

For the river water samples, 1 ml and 10 ml were analysed. For the raw and drinking water samples, volumes of 100 ml were analysed.

Statistical analysis

Each sample was defined by location, data of collection and water type. This information and results of each sample for the *Cryptosporidium* and bacteria were entered on a database and statistics program (EpiInfo 6.0. CDC). Statistical analysis was performed with STATA (analytical software, version 6.0, Statacorp, Texas, USA). Statistical significance was considered for $p < 0.05$.

Results

Occurrence of Cryptosporidium spp. oocysts in surface water

A total of 37 river water samples was examined between 9 June and 15 September 1998. *Cryptosporidium* oocysts were found in all 37 river water samples with oocyst numbers ranging from 1 to 22 oocysts per 20 l. The number of oocysts observed at the four locations were similar (fig. 1). Sample site B1 and B2 were selected to assess the impact of a sewage treatment plant. No significant difference in oocysts densities was found between B1 and B2 (Wilcoxon signed-rank test, $z = 0.31$). Results are summarised in table 1.

Occurrence of Cryptosporidium spp. oocysts in raw and drinking water

17 out of 26 (65 %) tested raw water samples and 3 out of 16 (18.7 %) drinking water samples were found to contain *Cryptosporidium* oocysts (table 2). The num-

Table 1
River water (Lützel) samples (20 l) from four location analyzed for *Cryptosporidium* oocysts

location	*mean*	*SD*	*median*	*range*
1 ($n = 9$)	8.2	4.4	9	3–14
2 ($n = 9$)	9.8	7.4	12	1–22
3 ($n = 9$)	8.3	4.8	8	3–18
4 ($n = 10$)	8.3	5.2	6	1–17

Table 2

Cryptosporidium spp. oocyst counts over a period of five month in two wells (untreated raw water) and drinking water

Date of sampling and analysis	*Number of oocysts in 20 l of water*		
	well 1	*well 2*	*drinking water*
9. 6. 1998	23	3	1
16. 6. 1998 R	14	0	0
30. 6. 1998	10	1	0
14. 7. 1998 R	3	0	0
21. 7. 1998	2	0	0
27. 7. 1998 R	0	0	0
11. 8. 1998	2	0	2
25. 8. 1998 R	8	1	0
1. 9. 1998	1	0	0
15. 9. 1998 R	19	4	0
22. 9. 1998	9	2	0
29. 9. 1998 R	0	0	0
1. 10. 1998 R	–	–	0
2. 10. 1998 R	–	–	5
12. 10. 1998	0	4	0
26. 10. 1998 R	–	–	0

R = Rainfall at the time of sampling

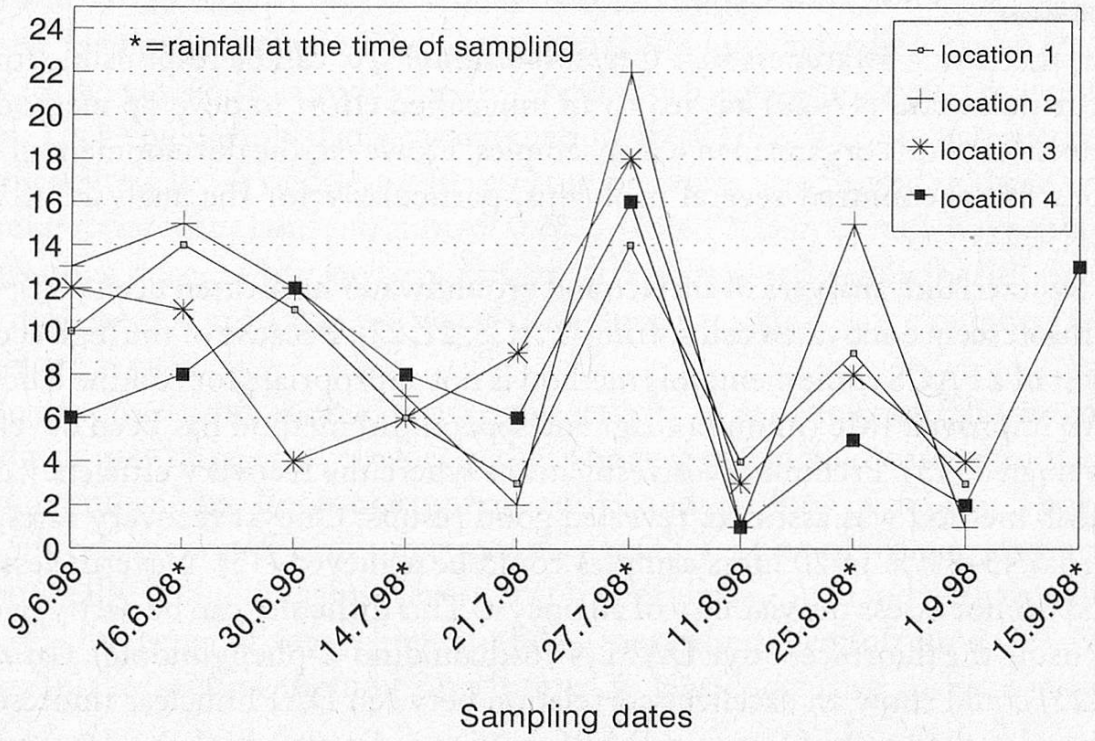

Figure 1 **Number of _Cryptosporidium_ oocysts in river water (Lützel) samples at four locations and ten different days over a period of 3 month (6.98–9.98)**

ber of oocyst observed in the well 1, averaging 8.4 oocysts/20 l (SD = 7.9, range = 0–23), was approximately 7 times higher than number in well 2, averaging 1.2 oocysts/20 l (SD = 1.6, range = 0–4).

Microbial contaminations in river, raw and drinking waters

Microbiological parameters were evaluated in all samples to investigate eventual association with parasite contamination. *Escherichia coli* and enterococci could not be found in two of the oocyst containing drinking water samples. One oocyst containing drinking water sample was found to be contaminated with 1 CFU *Escherichia coli* per 100 ml. The results are summarised in table 3.

Table 3
Microbial level of the three drinking water samples that also contain *Cryptosporidium spp.* oocyst

Date of sampling and analysis	*Number of oocysts/ 20 l*	*E. coli cfu/ 100 ml*	*Enterococci cfu/ 100 ml*
9. 6. 1998	1	0	0
11. 8. 1998	2	0	0
2. 10. 1998 R	5	1	0

R = Rainfall at the time of sampling

Discussion

The increasing awareness that *Cryptosporidium spp.* can be responsible for waterborne outbreaks (17–20) has led to an intensified effort to develop methods for the detection of this organism in water samples. However, the development of these methods has encountred several problems, particularly for the analysis of water samples.

In Switzerland, analyses of surface and groundwater have often been carried out using fluorescence activated cell sorting (FACS; 21, 22). Because of the high acquisition cost of a FACS equipment, this method is not appropriate for routine laboratories. An improved IMS (immunomagnetic separation) method has been developed and evaluated (15). Preliminary investigations where the recovery efficiency of the used IMS method was assessed, revealed good results. Oocyst recovery rates, ranging from 43–88 % in 20 liters samples could be achieved (15). Nevertheless, this method cannot assess the viability of an oocyst. This difficulty can be partly overcome by using the fluorescent dye DAPI (4', 6-diamidino-2-phenylindole). *Grimason* et al. (23) could show an excellent correlation between DAPI nuclear fluorescence and sporulated oocysts. However, DAPI staining indicates a biological state that is not necessarily synonymous with infectivity. Further studies are currently undertaken to explore this question.

In this study *Cryptosporidium ssp.* oocysts were found in water samples from the Lützel river as well as in raw and drinking water. The number of detected oocysts in river water ranged between 1 to 22 oocysts per 20 liters. The consistent appearance of oocysts in samples taken over a period of five months clearly demonstrates a continuous presence of this organism in water. This agrees with previous studies where these protozoan parasites were detected in high percentages of raw surface water samples (14, 24, 22). The highest number of oocysts was found after heavy rain, but no significant association was detected between rainfall and number of oocysts. Due to the diversity of the land use, the potential sources of contamination by *Cryptosporidium* are numerous and difficult to identify. A sewage treatment plant located upstream from the two wells could constitute an important part of these potential sources. In preliminary investigations *Cryptosporidium* oocysts were found in all samples of sewage effluent with concentrations ranging from 6 to 61 oocysts/20 l. The relevance of the sewage treatment plant as source of contamination will be further evaluated in the seasons to come.

The fact that oocysts could be consistently detected and that drinking water data (Cantonal Laboratory of Basel-Landschaft) from this facility where *E.coli* had been detected indicated that this source is under the influence of surface or river water infiltration which subjected them to contamination with enteric protozoas, raises the question of the puplic health importance. Routine water disinfection procedure system did not seem to guarantee efficient removal, and parasitic cysts and oocysts must be expected to be present in treated water from time to time. *Rose* et al. (25) could demostrate that a dose of 30 oocysts could initiate infection in 20 % of those exposed. Using the data from the *Cryptosporidium* human feeding study, *Haas* and *Rose* (26) developed an exponential dose-response *Cryptosporidium* risk assessment model. They could show that a contamination of 0.002–0.015 oocysts/l is contributing to endemic levels of disease. In our study oocysts were found in three samples of drinking water at a concentration of 0.05, 0.10 and 0.25 oocysts/l. Given this level of infectious doses, a few oocysts can create a substantial public health problem. Since most people in an area use water from the same supply, large numbers of consumers are at risk.

On the other hand, the occurrence and severity of epidemic waterborne cryptosporidiosis may be inversely linked to the incidence of endemic waterborne *Cryptosporidium* infections. Enteric protozoa tend to induce poor immunity. Thus, high levels of endemic waterborne infections may result in much of the population becoming partially immune against severe cryptosporidiosis. Such individuals will have a variable, but generally more limited, ability to transmit their infection (18). Nevertheless, further studies should be performed to give conclusive answers in this respect.

The use of «indicator organisms» to assess the microbiological quality of water is well established and has been practiced for almost a century. The measurement is based on the absence of the indicator organisms, principally *Escherichia coli* and *en-*

teroccoci. To evaluate the relationship between occurrence of parasites and potential indicators, microbiological analyses were performed. In agreement with other reports (21, 22) our field data indicate that *Escherichia coli* and *enterococci* seem to fail as indicators of parasites. *Enterococci* could not be found in any of the oocyst containing drinking water samples. *Escherichia coli* was found only in one of three oocysts containing drinking water samples.

Contamination of water supplies is important for the spread of cryptosporidial infection and has been linked to run-off from fields, pastures and areas of livestock, which is indicative of zoonotic transmission. But environmental *C. parvum* isolates were found to differ not only in viability, but also in their potential infectivity for man and animal, indicating intraspecies variation. *McDonald* et al. (27) examined isolates which came from a wide geographical range. They found two major parasite types, one associated with man, the other with animals. Interestingly some of the human isolates were not infective to immunosuppressed mice or they induced very mild infections whereas all animal isolates produced heavy infections. Moreover, parasites from AIDS patients with severe cryptosporidial infections produced relatively mild infections in calves whereas parasites from other AIDS patients with mild infections produced severe infections in calves. *Morgan* et al. (28) analysed different *C. parvum* isolates using specific primers. They revealed distinct genetic and biological differences between isolates of human and bovin origin. The variation in infectivity of isolates of *C. parvum* is probably a reflection of the differences in host specificity of the parasite and lends further support to the concept of the existence of distinct human and animal population of the parasite, but with a certain level of cross-infectivity. Thus, the ability to differentiate directly between human and animal isolates has important implications for studies on the transmission and zoonotic potential of *Cryptosporidium parvum.* In addition, the ability to differentiate will be of particular importance in outbreak situations. Therefore, all oocysts which were isolated from water samples in this study were preserved for further genotype analyses that are currently underway.

The study also indicates the need to obtain more data for a comprehensive risk assessment. Such data will become of great help for water utility managers and cantonal laboratories and can inform to decisions regarding watershed protection and changes in water treatment.

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Summary

In the course of a study, 37 surface, 26 raw and 16 treated drinking water samples have been analysed on the occurrence of *Cryptosporidium spp.* oocysts using flocculation with aluminium sulphate, an immunomagnetic separation and a fluorescent staining.

During the months of June and October 1998 *Cryptosporidium spp.* oocysts were found in all examined surface water samples. The number of detected oocysts in river water ranged from 1 to 22 oocysts per 20 liters. 17 out of 26 (65 %) analysed raw water samples and 3 out of 16 (18.7 %) drinking waters samples were *Cryptosporidium*-oocysts contaminated (1, 2 and 5 oocysts).

Zusammenfassung

Im Rahmen einer Studie wurden mittels Aluminiumsulfat-Flockung, immunmagnetischer Separation und Fluoreszenzfärbung 37 Oberflächengewässer-, 26 Rohwasser- und 16 aufbereitete Trinkwasserproben auf das Vorkommen von *Cryptosporidium*-Oocysten untersucht. In allen untersuchten Flusswasserproben konnten zwischen 1 und 22 *Cryptosporidium*-Oozysten nachgewiesen werden. 17 der 26 (65 %) Rohwasserproben und 3 der 16 (18,7 %) untersuchten Trinkwasserproben wiesen 1, 2 bzw. 5 *Cryptosporidium*-Oozysten auf.

Résumé

Dans le cadre d'une enquête la présence de *Cryptosporidium spp.* oocystes a été recherchée dans 37 échantillons d'eau de rivière, dans 26 d'eau non traitée et dans 16 échantillons d'eau potable en utilisant une floculation avec sulphate d'aluminium, une séparation immunomagnétique et une coloration fluorescente. Entre 1 et 22 *Cryptosporidium spp.* oocystes ont été trouvés dans toutes les échantillons d'eau de rivière. 17 des 26 (65 %) échantillons d'eau non traitée et 3 des 16 (18,7 %) échantillons d'eau potable contenaient des *Cryptosporidium spp.* oocystes (1, 2 et 5 oocystes).

Key words

Cryptosporidium spp., Drinking water, Protozoan parasite, Epidemiology, Immunomagnetic separation

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Korrespondenzadresse: Dr. Paul Svoboda, Kantonales Laboratorium Basel-Landschaft, Postfach, CH-4414 Füllinsdorf, E-Mail: paul.svoboda@vsd.bl.ch