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SHORT REPORT
Serological responses to *Cryptosporidium* antigens in inhabitants of Hungary using conventionally filtered surface water and riverbank filtered drinking water

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SUMMARY

In this study the putative protective seroprevalence (PPS) of IgG antibodies to the 27-kDa and 15/17-kDa *Cryptosporidium* antigens in sera of healthy participants who were and were not exposed to *Cryptosporidium* oocysts via surface water-derived drinking water was compared. The participants completed a questionnaire regarding risk factors that have been shown to be associated with infection. The PPS was significantly greater (49–61%) in settlements where the drinking water originated from surface water, than in the control city where riverbank filtration was used (21% and 23%). Logistic regression analysis on the risk factors showed an association between bathing/swimming in outdoor pools and antibody responses to the 15/17-kDa antigen complex. Hence the elevated responses were most likely due to the use of contaminated water. Results indicate that waterborne *Cryptosporidium* infections occur more frequently than reported but may derive from multiple sources.

Key words: *Cryptosporidium*, infectious disease epidemiology, water-borne infections.

The protozoan pathogen, *Cryptosporidium parvum*, is responsible for gastrointestinal diseases resulting in watery or mucoid diarrhoea. Cryptosporidiosis can be chronic and life-threatening in immunocompromised and immunosuppressed individuals, such as AIDS patients and people living with cancer. Oocysts, the transmittable form of the organism, are able to survive in the environment for a prolonged period of time. They have been isolated from untreated and treated drinking-water supplies, swimming pools, rivers and reservoirs throughout the world including Hungary [1], causing a threat to public health.

Despite the detection of *Cryptosporidium* oocysts in water, no outbreaks of the disease have been reported in Hungary. The lack of detected outbreaks may indicate that oocysts detected in water are either not viable or are of a species or genotype that is not pathogenic to humans. An alternative explanation may be that the regular consumption of water with low concentration of oocysts induces protective immunity which actually reduces the risk of illness [2, 3]. In the latter case visitors in a community with repeated low-level

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exposure to pathogenic strains experience increased risk of illness compared to residents [4].

Two groups of human IgG antibodies to the 27-kDa and 15/17-kDa antigens are typical in *Cryptosporidium* infection. The 15/17-kDa antigen complex produces a strong response in the first 6 months post-infection (p.i.) and is detectable for 10 months p.i., thus usually indicating recent infection. The response to the 27-kDa antigens is weaker but steadier, and detectable for >1 year p.i. [5].

The aim of our study was to identify the antibody responses of inhabitants and the putative protective seroprevalence (PPS) of *Cryptosporidium* infection in two Hungarian settlements, Füzér and Mátrafüred, using conventionally filtered surface water and to determine factors associated with increased risk of infection. Western blot analysis with two different *Cryptosporidium* antigen markers, i.e. 15/17-kDa and the 27-kDa, was used to investigate antibody responses of healthy inhabitants of each settlement and of a control city (Budapest) using riverbank filtered water. The results of the serological assay were compared to potential risk factors associated with the participants.

Füzér is an agricultural area, located in the north-eastern part of Hungary at the Hungarian–Slovakian border; the water supply of the settlement was from Brook Nagy. Mátrafüred is a tourist spot, located in the northern part of Hungary, between the Mátra Hills. The drinking water was extracted from the water reservoir, which is on the Brook Csatorna, receiving water from nine springs located 50–500 m distance from the brook. In a previous study we examined the level of *Cryptosporidium* oocysts in Hungarian water-treatment plants using the U.S. EPA 1623 protocol [1], where the oocysts were concentrated using chemical flocculation of 10 l of raw water and Filta-Max filtration of ~100 l of drinking water followed by immunomagnetic separation. The oocysts were examined using epifluorescent and differential interference contrast microscopy after fluorescein isothiocyanate and 4′,6-diamidino-2-phenylindole (DAPI) staining.

Water-treatment plant at Füzér and Mátrafüred were found to be inadequate with regard to the removal of *Cryptosporidium*. The amount of *Cryptosporidium* oocysts in drinking water had been above the action level of 1–3 oocysts/10 l for years. The analysis of water samples taken at both settlements on three different occasions between 2006 and 2008 showed that the oocyst level in the drinking water of Füzér and Mátrafüred varied between 0·5 and 8·2 oocysts/10 l, and the oocysts were also detected in raw and filter backwash water with concentration of 0·2–8·1 oocysts/10 l.

Budapest, the capital of Hungary, was selected as the control city. The water supply of the capital and its suburbs originates from riverbank filtration, and *Cryptosporidium* oocysts have never been detected in the drinking water supply. Several previous studies confirmed the effectiveness of riverbank filtration for the removal of oocysts [1, 3].

For the serological analysis, blood samples were randomly obtained from a total of 184 healthy inhabitants in Füzér and Mátrafüred and 98 healthy inhabitants in Budapest. Individuals participated voluntarily after a clear explanation of the research objectives. The sample collection was performed with the permission of the Council of Health Science and volunteers signed a written informed consent. If the subjects were children informed consent was obtained from their parents/guardian. The study was organized with the help of the local general practitioners. Samples were collected in Füzér and Mátrafüred in June 2007 and in Budapest in September 2007. The sera samples were collected in sterile containers and stored at –20 °C for further analysis.

Frost *et al*. [6] reported that people who had lived for short time in an area where tap water contained few oocysts, or who often consumed bottled water, had low seropositivity for *Cryptosporidium* markers. Consequently, only inhabitants consuming local tap water on a regular basis for at least 5 years were included in our study. Participants had never been diagnosed with a *Cryptosporidium* infection. The gender balance in all samples was 30·2% male and 69·8% female. Age varied between 9 and 88 years with mean of 47 years at Füzér, 51 years at Mátrafüred and 42 years at Budapest ($P < 0{\cdot}001$).

In order to measure the IgG response to 27-kDa and 15/17-kDa antigens, the blood samples were analysed by a Western blot assay as described previously [7]. Antigen proteins for the Western blot assay were extracted from *C. parvum* oocysts of Iowa strains (Bunch Grass Farm, USA). The same positive control (derived from individuals with laboratory-confirmed infection) was used on all blots. The intensity of serological responses to the antigens was digitally analysed by the Molecular Imager Gel Doc System (Bio-Rad Laboratories Inc., USA). The system calculates the pixel density of the manually selected bands of the Western blot assay. The intensity of bands belonging

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Table 1. *Mean antibody levels in Western blot units (WBU) and putative protective seroprevalence (PPS) to* Cryptosporidium *antigens with 95% confidence intervals (CI)*

		27-kDa antigen		15/17-kDa antigen complex	
Settlement	*N*	Antibody level (95% CI*)	PPS (CI)	Antibody level (95% CI)	PPS (95% CI)
Budapest	98	16 WBU (12–22)	21% (14–31)	18 WBU (11–27)	23% (16–33)
Füzér	100	51 WBU (42–62)	61% (50–70)	45 WBU (36–54)	55% (46–67)
Mátrafüred	84	31 WBU (24–38)	50% (39–61)	46 WBU (32–61)	49% (37–60)

* CIs were evaluated using bootstrap due to the highly skewed distribution of the response.

to unknown samples was standardized with the positive control. The antibody levels are presented as Western blot units (WBU), where the negative control is set at 0 WBU and the positive control set at 100 WBU. The mean antibody levels of the inhabitants for the settlements were calculated. Previous findings suggested that antibody levels $\geqslant$20% of a positive control was needed for the reduction of the risk of illness [5]. In the current study, samples with $\geqslant$30 WBU were considered positive and the PPS for the settlements were calculated accordingly.

The results of the Western blot assay suggested an elevated seropositivity for *Cryptosporidium* in people regularly consuming C*ryptosporidium*-contaminated water. The antibody levels observed for the 27-kDa and the 15/17-kDa antigens were 51 WBU and 45 WBU at Füzér and 31 WBU and 46 WBU at Mátrafüred, respectively (Table 1), which were twice those observed in the population of the control city (16 WBU for the 27-kDa and 18 WBU for the 15/17-kDa antigens). The PPS for Füzér and Mátrafüred (49–61%) also suggested significantly elevated immune responses in inhabitants compared to the PPS of the control city (21% and 23%; Table 1).

Our results confirm the previous findings where serological responses were compared in Hungarian females using surface water, groundwater, karst water and riverbank filtrated water [3]. That study revealed that antibody levels to the 27-kDa and 15/17-kDa antigens in women using bank-filtered water were almost one third of the level observed in women using conventionally filtered and disinfected surface water. Kozisek *et al.* [8] also found significantly higher responses to the 27-kDa and 15/17-kDa antigens in a Czech population consuming filtered and disinfected surface water than in those consuming riverbank filtered water. All these findings suggest that a high level of subclinical C*ryptosporidium* infections occurs in people consuming surface-derived water. However, the effect of risk factors other than the source of drinking water that may result in elevated IgG levels has not been studied in previous research.

In our study the blood donors participated in a personal interview and completed a comprehensive, precoded, validated, written questionnaire for collection of data on potential risk factors [9]. The questionnaire covered demographic data, family life, education, travel history. Individuals were interviewed directly or in the case of young children the parents/guardian were interviewed. The interviewers had been trained prior to the interviews.

Based on the answers given to the questionnaire, four groups of variables with a possible effect on the antibody levels of the 27-kDa antigen and the 15/17-kDa antigen complex were identified:

Group 1: Food and water. Individuals were asked whether they consumed boiled/purified tap water, bottled water or water originating from private well and if so, whether the well was near to a lake or river. Participants were asked about the frequency of fresh salad consumption, whether they washed raw fruits and vegetables before consuming and if so, whether the fruits or vegetables were peeled if their skin was eatable.

Group 2: Animals and untreated water. Individuals were asked whether they had been associated with farm/zoo animals or pets (mature and/or cubs) and whether they consumed untreated surface water (lake, river, etc.) in the previous 12 months.

Group 3: Swimming habits. Individuals were asked whether they had swum or bathed in an outdoor or covered pool, lake, river or brook in the previous 12 months.

Group 4: Household. Individuals were asked whether in the previous 12 months they had been associated with a baby/used diapers; whether they had taken care of a family member having diarrhoea; whether they had pipe repair work done in their house;

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Table 2. *Results of the logistic regression model using Akaike's Information Criterion (AIC) and the likelihood ratio test*

	Seropositivity (27-kDa antigen)		Seropositivity (15/17-kDa antigen)	
	AIC*	*P* value	AIC*	*P* value
Null model	326·2706		308·9207	
Null model + settlement	295·7114	<0·0001	298·3190	0·0007
Food and water	309·5663	0·1906	316·8416	0·4266
Animals and untreated water	302·0101	0·5931	306·5376	0·8785
Swimming habits	301·4569	0·6891	296·6983	0·0473
Household	299·5550	0·1169	305·9660	0·3227
	Antibody levels (27-kDa antigen)		Antibody levels (15/17-kDa antigen)	
	LogLik†	*P* value	LogLik†	*P* value
Null model	−323·394		−315·0609	
Null model + settlement	−303·003	<0·0001	−305·1296	0·0005
Food and water	−280·882	0·2249	−282·2253	0·1518
Animals and untreated water	−298·969	0·4270	−302·1170	0·6444
Swimming habits	−297·739	0·2299	−300·2308	0·2795
Household	−292·533	0·2825	−292·4513	0·1154

* Smaller values of AIC correspond to the better model.
† The higher the absolute value of the log-likelihood (LogLik) test, the better the model.

whether they had outdoor activities or travelled abroad. Participants were asked whether in the previous 4 months they or their family members had diarrhoea lasting >4 days.

In addition, demographic variables such as gender, age, marital status and education level, categorized into six classes, were taken into account. The association between risk factors and seropositivity was modelled using logistic regression. Analysis of variance was used to access statistical significance of groups of factors. Tukey tests were used for *post-hoc* pairwise comparisons of odd ratios (OR). The continuous response (i.e. the absolute value of the antibody levels) was modelled using the two-stage model which was necessary due to zero inflation, which could not have been accounted for by, for example, the tobit model. The likelihood ratio test was used to access statistical significance of groups of factors. All analyses were run using R software [10].

Only observations with non-missing records for all the variables were used for model selection and fit ($n = 232$). The results of the model comparison are shown in Table 2. No group of variables was found to have a statistically significant effect on the antibody levels and seropositivity with the exception of swimming habits. In particular, bathing or swimming in an outdoor pool was associated with 50% higher odds of a positive response ($\geqslant 30$ WBU) to the 15/17-kDa antigen complex ($P = 0{\cdot}0197$). As only the level of antibodies to the 15/17-kDa antigen complex showed correlation with bathing in an outdoor pool, the results indicate recent exposure, which was expected as the study was undertaken during/after the summer season.

After adjustment for demographic factors, the odds of positive response to the 27-kDa antigen were on average 7·56 times higher in Füzér than in Budapest ($P < 0{\cdot}0001$) and on average 3·84 times higher in Mátrafüred than in Budapest ($P = 0{\cdot}0005$). The twofold difference in the odds between Füzér and Mátrafüred was not statistically significant ($P = 0{\cdot}1370$). For the 15/17-kDa antigen complex, those living in Füzér had three times higher odds of a positive response than those in Budapest, whereas those living in Mátrafüred had 2·6 times higher odds than those in Budapest of having a positive response ($P = 0{\cdot}0091$ and $P = 0{\cdot}0174$, respectively). The difference between Füzér and Mátrafüred was, again, not statistically significant (OR 0·87, $P = 0{\cdot}9162$).

The results of the multiple analyses of these factors and serological responses confirmed that there

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is a close relationship between positive responses and sources of drinking water used. No statistically significant predictor variables were identified other than bathing or swimming in outdoor pool suggesting that risk factors other than contaminated drinking water made little contribution to the immune response. However, the lack of significant associations between risk factors and seropositivity may have been a result of the sample size. Nonetheless, our findings support the previous observation suggesting that where oocysts have been in evidence in drinking water for a lengthy period, the population demonstrate a strong serological response against antigens of the pathogen. Furthermore, our study highlights that in such studies the contribution of all known risk factors to immune response should be evaluated.

Since 2008 the water quality problems of Füzér and Mátrafüred have been resolved, both settlements now receive drinking water from another water source. Therefore, it would be interesting to determine the impact of the change of water sources of the settlements on the antibody levels and PPS.

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DECLARATION OF INTEREST

None.

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