Presence of Giardia cysts and Cryptosporidium oocysts in drinking water supplies in northern Spain

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Abstract

Aims: To evaluate the prevalence of Cryptosporidium and Giardia in surface water supplies from the province of Álava, northern Spain, and to investigate possible associations among the presence of these pathogenic protozoa with microbiological, physicochemical and atmospheric parameters.

Methods and results: A total of 284 samples of drinking and recreational water supplies were analysed. Cryptosporidium oocysts were found in 63.5% of river samples, 33.3% of reservoirs samples, 15.4% and 22.6% of raw water samples from conventional and small water treatment facilities (respectively), 30.8% of treated water from small treatment facilities, and 26.8% of tap water from municipalities with chlorination treatment only. Giardia cysts were found in 92.3% of river samples, 55.5% of reservoirs samples, 26.9% and 45.2% of raw water samples from conventional and small water treatment facilities (respectively), 19.2% of treated water from small treatment facilities, and 26.8% of tap water from municipalities with chlorination treatment only. The presence of Cryptosporidium and Giardia had significant Pearson’s correlation coefficients (P < 0.01) with the turbidity levels of the samples, and a number of significant associations were also found with the count levels for total coliforms and Escherichia coli. The samples were positive for Cryptosporidium significantly (P < 0.05) more frequently during the autumn season than during the spring and winter seasons. No significant differences were found in the seasonal pattern of Giardia. A moderate association (r = 0.52) was found between rainfall and the presence of Cryptosporidium oocysts.

Conclusions: Cryptosporidium and Giardia are consistently found at elevated concentrations in surface waters for human consumption from the province of Álava, northern Spain.

Significance and Impact of the Study: Water treatments based on rapid filtration process and/or chlorination only are often unsatisfactory to provide safe drinking water, a situation that represents an important public health problem for the affected population because of the risk of waterborne outbreaks.

Keywords
Cryptosporidium, Giardia, drinking water, public health, recreational water, water quality.

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Introduction

Protozoan enteroparasites of the genera Cryptosporidium (phylum Apicomplexa) and Giardia (subphylum Sarcomastigophora) have emerged over the past decades as major waterborne pathogens. Both parasites are among the major causative agents of gastroenteritis in humans and can potentially shorten the life spans of immunocompromised individuals. These micro-organisms are transmitted via the faecal–oral route, with the consumption of contaminated drinking water and use of recreational waterways significant avenues for acquisition of infection in developed countries (Slifko et al. 2000). Cryptosporidium and Giardia are ubiquitous in the aquatic environment and their transmission stages (oocysts and cysts, respectively) may remain viable for several months under...
a range of environmental conditions (Smith et al. 1995). In addition, Cryptosporidium oocysts and Giardia cysts are resistant to conventional disinfectants at the concentrations and exposure times commonly used, and their infectious doses in humans have been estimated to be as low as 30 oocysts for Cryptosporidium (DuPont et al. 1995) and 10 cysts for Giardia (Adam 2001). Taking together, these data indicate that Cryptosporidium and Giardia represent a significant threat to public health.

More than 160 waterborne outbreaks of cryptosporidiosis and giardiasis have been reported worldwide, with most cases reported in the US and UK (Sliisko et al. 2000; Craun et al. 2002). This situation has become a major concern for water utilities and sanitary authorities that are responsible for providing safe drinking water supplies for human consumption. In the US, the US Environmental Protection Agency (USEPA) initiated an effort in 1996 to collect data related to the biology and epidemiology of these protozoa, and to evaluate their risk to human health. As a result, the USEPA has implemented national drinking water regulations and developed innovative technologies to improve the detection, monitoring and surveillance of these micro-organisms in drinking water (U.S. Environmental Protection Agency 2002). Similar initiatives have been implemented in other countries including England, Wales (Drinking Water Inspectorate 2003), Canada (Health Canada 2004) and New Zealand (Ministry of Health of New Zealand 2005). The new European Drinking Water Directive (Directive 98/83/CE) establishes the goal that all the state members should provide drinking water supplies with the absence of pathogenic organisms. However, for practical purposes, the highly variable sensitivities of the methods available for the detection of Cryptosporidium and Giardia and problems associated with the determination of the (oo)cysts viability/infectivity make the establishment of maximum acceptable concentrations very difficult (Messer and Wolpert 2003). Concentrations of ≥3–30 (oo)cysts per 100 l in treated water have been proposed as an ‘action level’, where the possibility of an outbreak may exist (Haas and Rose 1995; Wallis et al. 1996).

The province of Álava (northern Spain) extends over 3037 km², has winter/summer temperatures ranging from −6°C to 16°C and 7°C to 38°C, respectively, and its annual precipitation is in the range of 650–900 l m⁻². It has an estimated population of 294 360 people, of which 223 257 are living in the capital, Vitoria-Gasteiz. The province bears a significant cattle farming activity whose inhabited areas in the province bear a significant cattle farming activity whose population is responsible for providing drinking water supplies with minimal treatment, usually only chlorination. Because Cryptosporidium oocysts and Giardia cysts are resistant to this disinfectant, these communities (estimated total population of approx. 50 000 people) are potentially at risk of waterborne outbreaks of cryptosporidiosis and/or giardiasis. These facts, together with the lack of previous data in the province of Álava, have induced the Department of Health of the Basque Government to carry out the present study with the aims of (i) evaluating the prevalence of Cryptosporidium and Giardia in surface water supplies; (ii) estimating the efficiency of treatment plants in removing these parasites; and (iii) determining the relationship with microbiological, physicochemical and atmospheric parameters.

Materials and methods

Sample collection and filtration

A total of 284 samples of water supplies for human consumption and/or recreation were analysed. Samples were collected over a 30-month period between April 2000 and September 2002, and comprised natural surface waters from rivers (n = 52) and reservoirs (n = 36), raw (n = 26) and treated (n = 31) water from conventional water treatment facilities (CWTF, facilities that include coagulation, flocculation, sedimentation, filtration and disinfection processes), raw (n = 31) and treated (n = 26) water from small water treatment facilities (SWTF, facilities that include rapid filtration and/or disinfection processes only), and tap water (n = 82) from municipalities with chlorination treatment only. Sampling was mainly directed to those points where Cryptosporidium and Giardia were suspected. Sampling points were tested at an interval of approx. 2 months, although when increased numbers of pathogens in waters were observed surveillance was intensified to weekly intervals. Samples were collected in the field by using a portable sampling apparatus equipped with a peristaltic pump. A minimum of 100 l of water was filtered through a polypropylene MicroWynd D-PPPY filter (Cuno Europe, France) of 1-µm nominal pore size at a flow rate of 3–5 l min⁻¹. However, less volume of water was processed in some...
samples with high turbidity because of filter clogging. Filters were returned to the laboratory in clean polythene containers, typically within 2–5 h after filtration. Upon receipt, the samples were stored refrigerated at 4°C until processed. Water sub samples were also independently collected following the standard procedures for subsequent physicochemical and microbiological analysis.

Sample elution and concentration

Samples were processed within 24 h after filtration. Briefly, filters were kept in the housing with the water that remained after sampling and subjected to a crosscurrent air flow for 15 min to unpack the fibres and facilitate the removal of cysts/oocysts. Filters were then cut longitudinally and the fibres divided into an outer, middle and inner layer. Portions were washed separately three times for 5 min in 1 l of PBS buffer pH 7.2 containing 0.1% SDS, 0.1% Tween 80, and 0.01% Antifoam A (Sigma-Aldrich, Dorset, UK). In order to increase the cyst/oocyst recoveries, fibre portions were sonicated in a water bath for 5 min at 40 Hz between the second and the third wash. The material that was washed from the fibres was centrifuged at 1050 g for 10 min using 50-ml centrifuge tubes in a swinging-bucket rotor, and the supernatant carefully discarded by aspiration. The packed pellet volume was recorded, and the pellet resuspended in a suitable volume of deionized water and sonicated in a water bath for 10 min at 40 Hz to prevent cysts/oocysts aggregation.

Sample purification

Cysts/oocysts in the samples were isolated from other particulate material by immunomagnetic separation using the commercial kit Dynabeads GC-Combo (Dynal Biotech, Bromborough, UK), as described in the method 1623 (U.S. Environmental Protection Agency 1999). Briefly, the procedure involves adding magnetic beads labelled with Cryptosporidium- and Giardia-specific monoclonal antibodies to 10 ml of resuspended pellet and allowing the antibody-antigen reactions to bind the (oo)cysts to the beads. The sample is then magnetized, separating the (oo)cyst-magnetic bead complex from the sample debris, which is then discarded. The beads are then detached and the (oo)cysts are added to a well slide for sample screening, allowed to air-dry completely, and fixed with acetone.

Sample staining and examination

The identification and enumeration of cysts/oocysts was carried out by immunofluorescence assay using the commercial kit Crypto/Giardia IF Test (Cellabs, Brookvale, Australia), according to the manufacturer’s instructions. Well slides were washed three times with abundant PBS buffer pH 7.2, and after adding mounting medium, the coverslip was sealed with nail polish. The slides were systematically examined by using epifluorescence microscope (Zeiss Standard Lab 16; Carl Zeiss, Göttingen, Germany) at 400× magnification, searching for brilliant apple-green fluorescing round to oval objects. Magnification was increased to 1000× for confirmation of presumptive samples and differential interference contrast microscopy was used for identification of internal morphological features such as number of sporozoites or nuclei, and presence of axonemata or median bodies. Estimations of the total amount of cysts/oocysts were calculated considering the volume of water filtered and the fraction of the pellet analysed. Positive and negative staining controls were routinely included.

Physicochemical analysis

Turbidity and free chlorine were measured in the laboratory immediately after the arrival of the samples. Turbidity was measured for each thoroughly stirred sample with a Hach 2100N turbimeter (Hach, Loveland, CO, USA) and the results were expressed in nephelometric turbidity units (NTU). Free chlorine was measured in treated water samples only by using the DPD chlorine test kit (LaMotte, Chestertown, MD, USA) and expressed in milligram per litre.

Analyses of *Escherichia coli* and total coliforms

*Escherichia coli* counts were determined by filtering 100-ml sample through a 0.45-μm pore size cellulose filter (Millipore, Bedford, MA, USA). The filters were incubated at 36°C for 24 h on the Chromocult® coliform agar (Merck Biosciences, Nottingham, UK) and the dark blue- to violet-coloured colonies were considered to be *E. coli*. Total coliforms counts were determined by filtering 100-ml samples through 0.45-μm pore size cellulose filter (Millipore). The filters were incubated at 36°C for 24 h on the m-Endo total coliform agar (Millipore) and the deep red with distinct green metallic sheen colonies were counted as total coliforms.

Stock suspension preparation and enumeration

Human stools from patients infected with *Giardia lamblia* were obtained from the Donostia Hospital, San Sebastián (Spain). Stools from calves with cryptosporidiosis were kindly provided by Dr Enrique Pérez, Faculty of Veterinary, University of Extremadura (Spain). Faecal samples were filtered through a 0.5-mm sieve and concentrated by
centrifugation at 1050 \( g \) for 10 min. Cysts/oocysts were isolated by using percoll-sucrose gradient (specific gravity: 1.09–1.10) and centrifugation at 1050 \( g \) for 10 min. Purified cysts/oocysts were stored at 4°C in PBS buffer pH 7.2 supplemented with 10 000 U ml\(^{-1}\) penicillin and 10 mg ml\(^{-1}\) streptomycin (Sigma-Aldrich) to prevent bacterial growth. Spiking suspensions containing 10 000–15 000 cysts/oocysts were prepared in reagent water with 0.01% Tween 20. Stock and spiking suspensions were enumerated by using both haemocytometer chamber and well slide for immunofluorescence detection (U.S. Environmental Protection Agency 1995). A total of 10 different haemocytometer chambers were counted and averaged for each cyst/oocyst suspension to achieve optimal counting accuracy. Well slide counting was performed by analysing a 20-\(\mu l\) aliquot of each cyst/oocyst suspension, in triplicate. Stock suspensions of cysts/oocysts were used for no more than 12 weeks following the purification.

**Initial precision and recovery of the method**

In order to determine the initial recovery achieved using the method, 12 independent spiking experiments were carried out by filtering 50 l of distilled water as described above. Spiking suspensions with 2500 cysts of *Giardia* and 5000 oocysts of *Cryptosporidium* were sequentially delivered into the inlet tube of the sampling housing by injection using a syringe with a 21-gauge needle. This procedure avoids the loss off cysts/oocysts that may remain attached to the internal wall of the carboy. The percent recovery \((R)\) of the method was calculated by using the following equation:

\[
R = \frac{N}{T} \times 100
\]

where \(N\) is the number of cysts/oocysts detected and \(T\) the number of cysts/oocysts spiked. The average percent recovery and the SD of the recoveries for *Cryptosporidium* and *Giardia* were also calculated.

**Quality control of the method**

In order to determine the inter-assay variation of the method for the recovery of *Cryptosporidium* oocysts and *Giardia* cysts, a spiking experiment was carried out every 15 field samples processed, as previously described. The acceptance criterion for *Cryptosporidium* and *Giardia* recoveries during routine use of the method was defined as their initial mean recovery ±1.5 SD.

**Meteorological data**

Rainfall data recorded at representative weather stations were utilized to study the extent to which heavy rains influence the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in water for human consumption. The obtained precipitation data were used to calculate weekly sums and monthly arithmetic means.

**Statistical analysis**

Pearson’s correlation coefficient and nonparametric Spearman’s rho were calculated to evaluate how physicochemical, microbiological and atmospheric parameters are related with cysts/oocysts rates. Spearman’s rho test was chosen because it is less sensitive to extreme values than the standard Pearson’s correlation coefficient. Chi-squared test was used to estimate possible significant differences between the seasonal prevalence of *Cryptosporidium* and *Giardia* and rainfall. Values of \(P < 0.05\) were considered statistically significant. All the analyses were performed with the Statistical Package for Social Sciences 12.0 for Windows (SPSS Inc., Chicago, IL, USA) software.

**Results**

**Precision of the method**

The initial average percent recovery of the method \((n = 12)\) was 16.7% (SD: 7.7) for *Cryptosporidium* oocysts and 41.5% (SD: 10.6) for *Giardia* cysts. The acceptable recovery intervals (accuracy) ranged from 5.2% to 28.2% for *Cryptosporidium* oocysts and from 25.5% to 57.5% for *Giardia* cysts. Inter-assay variation during routine use of the method was investigated by assaying a total of 18 independent spiking experiments carried through out the course of the study (Fig. 1). Only 3/18 (16.7%) of the *Cryptosporidium* and *Giardia* seeded samples failed to fall inside the acceptable range for (oo)cysts recovery.

**Prevalence of Cryptosporidium and Giardia in water samples**

Over the course of the 30-month study period, a total of 284 water samples were analysed for the detection of *Cryptosporidium* oocysts and *Giardia* cysts. Both protozoa were frequently found in all water sources, except those from CWTF (Table 1). Natural surface water from rivers and reservoirs showed the highest rates of protozoa occurrence, with concentrations that reached 1767 *Cryptosporidium* oocysts and >25 000 *Giardia* cysts per 100 l. CWTF achieved at least 3-log (99.9%) (oo)cyst removal, and no protozoa were detected in the finished water. However, SWTF only achieved 0.53 log\(_{10}\) *Cryptosporidium* oocysts and 1.62 log\(_{10}\) *Giardia* cysts removals,
from 26% to 31% and from 19% to 27%, respectively. The detected concentrations were always <8 (oo)cysts per 100 l.

### Physicochemical parameters

Physicochemical and microbiological data of the water samples studied are shown in Table 2. Turbidity values varied between 0.3 and 181 NTU, with river and reservoir water samples recording the highest levels (mean: 15.3 and 107 NTU, respectively). CWTF achieved an average fivefold reduction of turbidity levels (mean: 0.4; SD: 0.2). In contrast, a much lower performance of 2.7-fold reduction rate was accomplished by SWTF (mean: 3.1; SD: 9.4). In order to evaluate the efficiency of the disinfection process, free chlorine levels were measured in finished and tap water. The tested samples showed values ranging from 0.1 to 1.08 mg ml\(^{-1}\) (mean: 0.31–0.40; SD: 0.22–0.42).

### Microbiological parameters

Occurrence of E. coli and total coliforms was determined as these micro-organisms are traditional faecal indicators. The presence of E. coli was detected in all the categories of samples, except those from CWTF-treated water (Table 2). As expected, the highest counts were obtained in natural surface and raw water samples (means ranging from 4.6 to >100 CFU 100 ml\(^{-1}\)). Although absence of E. coli was recorded in CWTF-finished water, low levels of this indicator were frequently observed in SWTF-treated water samples (mean: 170 CFU 100 ml\(^{-1}\); SD: 30.9). The presence of E. coli was also sporadically detected in tap water samples (mean: 0.04 CFU 100 ml\(^{-1}\); SD: 0.24). Total coliforms counts reveal the same pattern obtained for E. coli, although with slightly higher rates.

### Table 1

Descriptive statistics of the concentration of Cryptosporidium oocysts and Giardia cysts in the different water samples analysed.

<table>
<thead>
<tr>
<th>No. samples</th>
<th>Rivers</th>
<th>Reservoirs</th>
<th>Conventional water treatment facilities (CWTF) raw</th>
<th>CWTF treated†</th>
<th>Small water treatment facilities (SWTF) raw</th>
<th>SWTF treated†</th>
<th>Tap water§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium</td>
<td>52</td>
<td>52</td>
<td>36</td>
<td>36</td>
<td>26</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Giardia</td>
<td>92.3</td>
<td>33.3</td>
<td>55.5</td>
<td>15.4</td>
<td>26.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% positive samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (n 100 l(^{-1}))</td>
<td>0–1767</td>
<td>0–25 000</td>
<td>0–380</td>
<td>0–1320</td>
<td>0–276</td>
<td>0–50</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean (n 100 l(^{-1}))</td>
<td>113.2</td>
<td>6522.8</td>
<td>17.6</td>
<td>63.3</td>
<td>17.2</td>
<td>46</td>
<td>0.0</td>
</tr>
<tr>
<td>SD</td>
<td>279.5</td>
<td>9452.7</td>
<td>31.7</td>
<td>110.8</td>
<td>26.7</td>
<td>50</td>
<td>0.0</td>
</tr>
</tbody>
</table>

†Conventional water treatment facilities that include coagulation, flocculation, sedimentation, filtration and disinfection processes.
‡Small water treatment facilities that include rapid filtration and/or disinfection processes only.
§Tap water with chlorination treatment only.
Table 2 Descriptive statistics of other physicochemical and microbiological parameters of the water samples analysed

<table>
<thead>
<tr>
<th></th>
<th>Turbidity (NTU)</th>
<th>Free-chlorine (mg l⁻¹)</th>
<th>Escherichia coli (CFU 100 ml⁻¹)</th>
<th>Total coliforms (CFU 100 ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>SD</td>
<td>Range</td>
</tr>
<tr>
<td>Rivers</td>
<td>0.3–181</td>
<td>15.3</td>
<td>32.6</td>
<td>–</td>
</tr>
<tr>
<td>Reservoirs</td>
<td>0.3–100</td>
<td>10.7</td>
<td>25.4</td>
<td>–</td>
</tr>
<tr>
<td>Conventional water</td>
<td>0.7–7.8</td>
<td>2.0</td>
<td>1.8</td>
<td>–</td>
</tr>
<tr>
<td>Treatment facilities (CWTF) raw†</td>
<td>0.3–10</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1–1.08</td>
</tr>
<tr>
<td>Small water treatment</td>
<td>0.4–95</td>
<td>8.5</td>
<td>17.5</td>
<td>–</td>
</tr>
<tr>
<td>facilities (SWTF) raw‡</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tap water§</td>
<td>0.3–13.4</td>
<td>1.7</td>
<td>2.0</td>
<td>0.1–0.75</td>
</tr>
</tbody>
</table>

†Conventional water treatment facilities that include coagulation, flocculation, sedimentation, filtration and disinfection processes.
‡Small water treatment facilities that include rapid filtration and/or disinfection processes only.
§Tap water with chlorination treatment only.

Table 3 Correlation between the number of Cryptosporidium oocysts detected and the physicochemical and microbiological parameters of the water samples analysed

<table>
<thead>
<tr>
<th></th>
<th>Turbidity</th>
<th>Free-chlorine</th>
<th>E. coli</th>
<th>Total coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson</td>
<td>Spearman’s</td>
<td>Pearson</td>
<td>Spearman’s</td>
</tr>
<tr>
<td></td>
<td>correlation</td>
<td>rho</td>
<td>correlation</td>
<td>rho</td>
</tr>
<tr>
<td>r</td>
<td>P</td>
<td>r</td>
<td>P</td>
<td>r</td>
</tr>
<tr>
<td>Rivers</td>
<td>0.001</td>
<td>0.092–0.075</td>
<td>0.596–</td>
<td>–</td>
</tr>
<tr>
<td>Reservoirs</td>
<td>0.057**</td>
<td>0.900–0.000</td>
<td>0.251–</td>
<td>0.140–</td>
</tr>
<tr>
<td>Conventional water</td>
<td>0.721**</td>
<td>0.000–0.000</td>
<td>0.532–</td>
<td>0.005–</td>
</tr>
<tr>
<td>treatment facilities (CWTF) raw†</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Small water treatment</td>
<td>0.906**</td>
<td>0.000–0.000</td>
<td>0.171–</td>
<td>0.365–</td>
</tr>
<tr>
<td>facilities (SWTF) raw‡</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SWTF treated‡</td>
<td>0.932**</td>
<td>0.000–0.000</td>
<td>0.297–</td>
<td>0.140–0.381</td>
</tr>
<tr>
<td>Tap water§</td>
<td>0.316**</td>
<td>0.004–0.004</td>
<td>0.214–</td>
<td>0.053–0.103</td>
</tr>
</tbody>
</table>

†Conventional water treatment facilities that include coagulation, flocculation, sedimentation, filtration and disinfection processes.
‡Small water treatment facilities that include rapid filtration and/or disinfection processes only.
§Tap water with chlorination treatment only.
*Correlation significant at the 0.05 level; **Correlation significant at the 0.01 level.

Correlation between physicochemical parameters and protozoa

The statistical analysis of the association between physicochemical and microbiological parameters and the occurrence of Cryptosporidium oocysts and Giardia cysts is summarized in Tables 3 and 4. A strong linear correlation (P < 0.01) between the presence of Cryptosporidium oocysts and the turbidity of the water was found in all the sample categories, except those from rivers. Similar results were obtained for Giardia cysts in samples from reservoirs, SWTF-treated water, and tap water. No significant correlations (P > 0.05) were found between the incidence of these protozoa and the levels of free chlorine in drinking water samples.

Correlation between microbiological parameters and protozoa

A significant correlation between Cryptosporidium oocysts and E. coli was observed in raw (P < 0.05) and treated water samples (P < 0.01) from SWTF. This parasite was also found to be highly correlated with the presence of total coliforms (P < 0.05) in all the categories of samples, except those from rivers, SWTF-treated waters, and tap waters. Finally, a number of significant associations between the
Occurrence of *Giardia* cysts and the *E. coli* total coliform counts were detected in samples from rivers, reservoirs, CWTF raw water and SWTF-treated water.

**Seasonality of prevalence**

The distribution of results by season (Fig. 2) revealed that autumn had a significantly higher incidence of *Cryptosporidium* oocyst positive samples than spring ($\chi^2 = 2.92$, $P < 0.1$) and winter ($\chi^2 = 3.85$, $P < 0.05$). No significant differences were detected in the seasonal pattern of *Giardia* cysts.

<table>
<thead>
<tr>
<th>Turidity</th>
<th>Free-chlorine</th>
<th><em>E. coli</em></th>
<th>Total coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson</td>
<td>Spearman’s</td>
<td>Pearson</td>
</tr>
<tr>
<td></td>
<td>correlation</td>
<td>rho</td>
<td>correlation</td>
</tr>
<tr>
<td></td>
<td>$r$</td>
<td>$P$</td>
<td>$r$</td>
</tr>
<tr>
<td>Rivers</td>
<td>$-0.161$</td>
<td>$0.255$</td>
<td>$0.26$</td>
</tr>
<tr>
<td>Reservoirs</td>
<td>$0.452**$</td>
<td>$0.060$</td>
<td>$0.172$</td>
</tr>
<tr>
<td>Conventional water treatment facilities (CWTF) raw†</td>
<td>$0.316$</td>
<td>$0.116$</td>
<td>$0.150$</td>
</tr>
<tr>
<td>CWTF treated†</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Small water treatment facilities (SWTF) raw‡</td>
<td>$0.229$</td>
<td>$0.224$</td>
<td>$0.335$</td>
</tr>
<tr>
<td>SWTF treated‡</td>
<td>$0.848**$</td>
<td>$0.000$</td>
<td>$0.393$</td>
</tr>
<tr>
<td>Tap water§</td>
<td>$0.194$</td>
<td>$0.082$</td>
<td>$0.325**$</td>
</tr>
</tbody>
</table>

†Water treatment facilities that include coagulation, flocculation, sedimentation, filtration and disinfection processes.
‡Small water treatment facilities that include rapid filtration and/or disinfection processes only.
§Tap water with chlorination treatment only.

*Correlation significant at the 0·05 level; **Correlation significant at the 0·01 level.

**Influence of rainfall on the occurrence of Cryptosporidium and Giardia**

Mean monthly rainfall values were calculated and plotted against the percentage of monthly positive samples for *Cryptosporidium* and *Giardia* (Fig. 3). On the basis of Pearson’s correlation coefficient, moderate associations were observed between the rainfall and the presence of *Cryptosporidium* oocysts ($r = 0·52$). The occurrence of *Giardia* cysts was weakly correlated with the rainfall data ($r = 0·34$). Interestingly, peak prevalences for both protozoa were achieved in October (autumn period), in coincidence with the highest rate of rainfall. A second *Giardia* peak was observed in April (spring period), also associated with elevated rainfall levels.
Discussion

During the past 15 years, an increasing number of waterborne outbreaks caused by *Cryptosporidium* and *Giardia* have been documented worldwide (Lisle and Rose 1995; Slifko et al. 2000; Crun et al. 2002; Fricker et al. 2002), showing a trend in which protozoa and viruses are replacing bacterial pathogens as agents of primary concern in waterborne disease (Briancesco and Bonadonna 2005). Because of this situation, detection of *Cryptosporidium* oocysts and/or *Giardia* cysts in surface water, especially in reservoirs for drinking water supply, is of great public health importance. In order to provide safe drinking water, drinking water systems must optimize five key elements: source water protection, adequate treatment, secure distribution, appropriate monitoring and appropriate response to adverse monitoring results (Huck and Coffey 2004).

In Spain, the prevalence of *Cryptosporidium* and *Giardia* has been well documented in humans (Rodríguez-Hernández et al. 1996), livestock (Quilez et al. 1996; Castro-Hermida et al. 2002) and molluscs (Freire-Santos et al. 2000; Gómez-Couso et al. 2004, 2005). However, very few epidemiological surveys have been conducted on the occurrence of these protozoa in surface water for human consumption (Rodríguez-Hernández et al. 1994; Montemayor et al. 2005) and no significant waterborne outbreaks of cryptosporidiosis/giardiasis have been reported. In addition, although the European Community environmental legislation states that water intended for human consumption should not contain pathogenic organisms (Directive 98/83/CE), in Spain there are no specific regulations relating to *Cryptosporidium* and *Giardia* tolerable limits in drinking water.

In the present study, the initial recovery results (*Cryptosporidium*, 16.7%; *Giardia*, 41.5%) obtained during the optimization of the analytical system were satisfactory, meeting the acceptance criteria proposed by the method 1623 (U.S. Environmental Protection Agency 1999). Reliable values were also achieved in the inter-assay precision tests, assuring the reproducibility of the method. These facts demonstrate that the system performance is suitable for analytical purposes.

Data obtained in this long-term survey show that *Cryptosporidium* and *Giardia* (oo)cysts were consistently detected in surface, raw and finished water samples from the province of Álava (northern Spain), showing their ubiquitous distribution. In most cases *Giardia* prevalence was higher than that of *Cryptosporidium*, corroborating the tendency observed in other countries (Hörman et al. 2004; Rimhanen-Finne et al. 2004; Briancesco and Bonadonna 2005).

Water treatment facilities play a key role in the process to provide safe drinking water for human consumption. Despite their small size, *Cryptosporidium* oocysts and *Giardia* cysts can be effectively removed from water supplies by conventional particle separation processes. However, small number of these protozoa can be found in finished water even in the absence of treatment problems (States et al. 1997). In our study, CWTF achieved three orders of magnitude removal for (oo)cysts, and no protozoa were found in the treated water. These data are consistent with those obtained in other investigations at pilot- and full-scale conventional water treatment plants (reviewed by Betancourt and Rose 2004). In addition, significant correlations between water turbidity level and presence of *Cryptosporidium* oocysts have been documented in previous reports (Falabi et al. 2002; Hsu and Yeh 2003; Hsu 2003). The observation that consistent removal rates of (oo)cysts are achieved when the treatment facilities produce water of consistently low turbidity (≤0.3 NTU) has suggested that turbidity is a useful in-plant measure of the degree of (oo)cyst removal (Nieminsky et al. 1995; Hsu and Yeh 2003). In the present study, CWTF achieved fivefold turbidity removals, with an average plant effluent turbidity of 0.4 NTU. We also found a strong association between turbidity and presence of *Cryptosporidium* oocysts and *Giardia* cysts in most of the categories of water analysed. These findings strongly support the use of turbidity removal as a reliable indicator of the effectiveness of removal of (oo)cysts in water treatment plants. Finally, no *E. coli* counts were detected in finished water from CWTF and total coliforms were only detected in one sample at very low concentration (mean: 0.12 CFU 100 ml⁻¹). Overall, these results reveal that the CWTF system components involved in the removal of water particles and disinfection were intact and operating correctly, assuring the production of safe drinking water.

With regard to SWTF, removals of 0.53 log10 for *Cryptosporidium* oocysts and of 1.62 log10 for *Giardia* cysts were obtained. However, both protozoa were found in 30-8% and 19-2% of the finished water samples, respectively (means: 7-8 oocysts/100 l, and 1-3 cysts/100 l). SWTF also achieved 2.7-fold turbidity removal, producing an average effluent turbidity of 3.1 NTU. As expected, these data show that SWTF performance is inferior in comparison with CWTF, demonstrating that water treatments based on rapid filtration process and/or disinfection only are clearly insufficient for removing protozoa and reducing turbidity levels to acceptable limits. In addition, *E. coli* and total coliforms counts were also found in finished water from SWTF, indicating inadequacy of the disinfection procedures in some of the SWTF.

*Escherichia coli* and total coliforms are used as indicator organisms worldwide for faecal contamination and
microbial water hygiene (Edberg et al. 2000). In this investigation, counts of these micro-organisms ranging between 4·6 and >100 CFU 100 ml⁻¹ were found in an elevated proportion of the surface water samples analysed. It is important to take into consideration that the Zadorra Reservoir system bears a considerable cattle farming activity, with up to 7000 cows, 8500 pigs, 25000 sheep and 700 horses. This picture suggests that livestock faecal products are probably the main source of environmental and water contamination. Some reports have investigated the possible association between the prevalence of Cryptosporidium and Giardia and the presence of other micro-organisms, with unclear results (Payment and Franco 1993; Hörmann et al. 2004; Rimhanen-Finne et al. 2004; Briancesco and Bonadonna 2005). We found a number of significant correlations between the presence of Cryptosporidium and Giardia and samples being positive for E. coli and/or total coliforms. These findings indicate that micro-organism counts may be used as predictors for the presence of these protozoan parasites.

The analyses of the tap water samples from municipalities with chlorination treatment only show relevant data. The presence of E. coli and total coliforms was sporadically recorded at very low levels (average E. coli, 0.04 CFU 100 ml⁻¹; average total coliforms, 0.89 CFU 100 ml⁻¹), demonstrating an acceptable performance of the disinfection treatment. However, Cryptosporidium and Giardia were detected in the 26·8% of the samples, at average concentrations of 2·2–2·3 (oo)cysts/100 l. These data indicate that some of the tap water samples analysed contained (oo)cysts at concentrations in the range considered as ‘action level’ by Haas and Rose (1995) and Wallis et al. (1996). Although the viability and the genotype of the (oo)cysts have not been assessed in this study, this situation represents a potential risk for waterborne infection for an estimated 50 000 population in the province of Álava.

This 30-month survey has also shown that Cryptosporidium and Giardia (oo)cysts are present in surface water throughout the year, with the highest frequency of occurrence during the autumn. A second peak for Cryptosporidium was recorded during the summer, whereas Giardia prevalence was more homogeneously distributed during the rest of the year. The seasonality of these parasite protozoa has been investigated in few studies, with diverse results. High frequencies of samples positive for Cryptosporidium and Giardia in environmental water have been linked to activities associated with agricultural practices and cattle farming such as calving, lambing and muck spreading (Kemp et al. 1995; Ong et al. 1996; Casemore et al. 1997). Additionally, runoff from precipitation has been proposed as a mechanism for entry of these organisms into surface waters (Bodley-Tickell et al. 2002) and previous studies have reported moderate correlations between rainfall and the presence of Cryptosporidium and Giardia (Atherholt et al. 1998; Thurman et al. 1998; Bodley-Tickell et al. 2002). However, unclear seasonality or association with rainfall has been found in some other investigations (Carrington and Miller 1993; Robertson and Gjerde 2001). In the present study, we found a reasonable correlation (r = 0.52) between the rainfall and the presence of Cryptosporidium, with oocysts peak concentration in October coinciding with the annual highest rate of rainfall. A weak association was found for Giardia (r = 0.34), but again cysts peak concentrations were reached in October with a second lesser peak in April. These data suggest that monitoring of Cryptosporidium and Giardia must be intensified during autumn rainfall months, the period of highest prevalence of these protozoa in surface water.

To address this situation, the Department of Health of the Basque Government, in co-ordination with the local authorities, has initiated a number of actions directed to assure the quality of the most vulnerable drinking water supplies in the province of Álava. These include protection of watersheds susceptible to be contaminated by human or domestic animal faecal waste, construction of new compact water treatment water facilities and improvement of the disinfection procedures by implementation of UV light irradiation-based systems where the construction of compact water treatment facilities was not feasible. In addition, the sensitivity of the detection assay has been enhanced by adoption of the Envirochek capsule filters (Pall Gelman Sciences, Ann Arbor, MI, USA) for routine use, as recommended in the Method 1623 (U.S. Environmental Protection Agency 1999). In our hands, these capsules achieved a preliminary initial average percent recovery (n = 4) of 33·4% (SD: 2·2) for Cryptosporidium oocysts, and 48·2% (SD: 3·9) for Giardia cysts (Carmena et al., unpubl. data).

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