Presence of Cryptosporidium spp. and Giardia duodenalis through drinking water

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ABSTRACT
To evaluate the presence of Cryptosporidium spp. and Giardia duodenalis in the influent and final effluent of sixteen drinking water treatment plants located in a hydrographic basin in Galicia (NW Spain) – in which the principal river is recognised as a Site of Community Importance (SCI) – estimate the efficiency of treatment plants in removing these protozoans and determine the species and genotypes of the parasites by means of a molecular assay. All plant samples of influent and final effluent (50–100 l) were examined in the spring, summer, autumn and winter of 2007. A total of 128 samples were analysed by method 1623, developed by US Environmental Protection Agency for isolation and detection of both parasites. To identify the genotypes present the following genes were amplified and sequenced: 18S SSU rRNA (Cryptosporidium spp.) and b-giardina (G. duodenalis). The mean concentrations of parasites in the influent were 0.0–10.5 Cryptosporidium spp. oocysts per litre and 1.0–12.8 of G. duodenalis cysts per litre. In the final treated effluent, the mean concentration of parasites ranged from 0.0–3.0 oocysts per litre and 0.5–4.0 cysts per litre. The distribution of results by season revealed that in all plants, the highest numbers of (oo)cysts were recorded in spring and summer. Cryptosporidium parvum, C. andersoni, C. hominis and assemblages A-I, A-II, E of G. duodenalis were detected. Cryptosporidium spp. and G. duodenalis were consistently found at high concentrations in drinking water destined for human and animal consumption in the hydrographic basin under study, in Galicia (NW Spain). It is important that drinking water treatment authorities rethink the relevance of contamination levels of both parasites in drinking water and develop adequate countermeasures.

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1. Introduction

Cryptosporidium spp. and Giardia duodenalis are common food and waterborne protozoa that affect humans and a wide range of domestic and wild animals (Fayer, 2004). These parasites are among the major causal agents of diarrhoeal disease in humans and animals worldwide, and can even potentially shorten the life span of immunocompromised hosts. Transmission is sustained by both a zoonotic and an anthroponotic cycle (Fayer et al., 2000; Slifko et al., 2000; Thompson, 2000). The infected hosts, whether humans or animals, shed very large numbers of transmissive stages – oocysts or cysts – in their faeces, thereby increasing environmental contamination. Moreover, the (oo)cysts are very resistant to harsh environmental conditions and disinfectants, at the concentrations commonly used in water treatment plants to reduce...
bacterial contamination (Karanis et al., 2002; WHO, 2002; Center of Disease Control and prevention, 2006), although it has been shown that G. duodenalis cysts are less resistant than Cryptosporidium spp. oocysts to higher concentrations of chlorine and ozone (Sterling, 1990). Drinking water resources become contaminated when faeces containing the parasites are deposited or flushed into water (Smith et al., 2007; Reynolds et al., 2008). Drinking water, even from well-managed treatment plants, can cause illness when it contains sufficient numbers of viable and infective (oo) cysts to G. duodenalis cysts have been shown to survive in water for up to 2 months at temperatures as low as 8 °C (Meyer and Jarroll, 1980), and Cryptosporidium spp. oocysts can survive for up to 1 year at 4 °C in artificial seawater (Tamburrini and Pozio, 1999). The number of parasites required to induce infection is relatively small. In fact, the infectious dose (ID50) has been estimated to be as low as 83–123 oocysts for Cryptosporidium spp. (DuPont et al., 1995; Chappell et al., 2006) and 19–50 cysts for G. duodenalis cysts (Adam, 2001).

Most drinking water must be pumped from its source or directed into pipes or holding tanks typically located in the headwaters of river systems. Other sources of drinking water include reservoirs, rivers, canals and/or low land reservoirs. However, (oo)cysts can enter surface waters from discharges from wastewater treatment plants, biosolids, urban runoff, or agricultural runoff, thereby causing infections (Cacciò et al., 2001). In many countries, there are no requirements for testing surface waters for the presence of these parasites, although it has been shown through outbreaks that the pathogens can be shed into recreational and drinking water (Karanis et al., 2006; Giangaspero et al., 2007; Sunderland et al., 2007). Moreover, infected animals can also act as reservoirs of pathogens, and even though they show no obvious signs of disease or clinical symptoms (Castro-Hermida et al., 2005, 2007a,b), they may ultimately spread the pathogens to human food (Hancock et al., 2001) or directly to humans (Enriquez et al., 2001). In many countries, there are no requirements for testing surface waters for the presence of these parasites, although it has been shown through outbreaks that the pathogens can be shed into recreational and drinking water (Karanis et al., 2006; Giangaspero et al., 2007; Sunderland et al., 2007).

The present study involved the analysis of samples of influent and effluent from sixteen drinking water treatment plants distributed in the Tambre river hydrographic basin (1530 km² in surface; 42°46′ to 43°10′ N, 8°00′ to 9°03′ W). This basin is one of the most important in this study region (Galicia, NW Spain) and the Tambre river was declared a Site of Community Importance (SCI), by the European Commission Habitats Directive (92/43/EEC) in 1992. Nevertheless, there is significant farming activity in this hydrographical basin, predominantly cattle farming (livestock census: 69,803), the runoff from which may be a potential source of contamination in surface water. Moreover, it is a supply of water destined for human consumption, providing drinking water to approximately 200,000 people. These drinking water sources are generally open to varied recreational uses, including swimming, especially during the spring and summer. The water is treated in conventional large and medium-size water plants and treatment includes coagulation, flocculation, and clarification through sedimentation, filtration and disinfection processes. However, some small communities (approximately 6195 people) use surface water supplies with minimal treatment, usually only chlorination. The specific aims of the present study were to evaluate the prevalence and concentrations of these parasites in sixteen drinking water treatment plants, to estimate the efficiency of treatment plants in removing these protozoans and to determine the species and genotype of these parasites by means of a molecular assay.

2. Materials and methods

2.1. Sample collection

Two samples (50 to 100 l), one of untreated water (influent) and other of treated water (final effluent) were collected in 16 drinking water treatment plants (Fig. 1) during the spring, summer, autumn and winter of 2007 (32 samples per season). The holding times of each step in the process were taken into consideration when collecting the samples, so that comparable water samples were obtained at these points in the treatment process.

Samples were taken immediately to the laboratory and processed with the Filta-Max Automatic System (IDEXX Laboratories, Inc., Westbrook, ME, USA). Filtration cartridges were set up according to the manufacturer’s instructions, and all samples were filtered at recommended flow rates. The filtration elution and final concentration of the sample were performed with the same equipment, and by following the manufacturer’s instructions. Briefly, this involved placing a membrane

Fig. 1 – Geographic location of the drinking water treatment plants (●) in the Tambre River hydrographic basin (Galicia; NW Spain). All of them were sampled in this study. Santiago de Compostela (●), with 93,458 inhabitants, is the main city in this area.
filter (3-µm pore size; polysulphone) in the base of the sample concentrator, inserting the filter module into the apparatus, adding 600 ml of phosphate-buffered saline (PBS) containing 0.01% Tween 20 (PBST) to the reservoir, and unscrewing the filter housing to allow expansion of the foam pads. The foam pads were washed by pumping the plunger and then transferring the PBST elution volume into the magnetic particle concentrator and filtering the entire volume to approximately 20 to 25 ml, under vacuum. The process was repeated with a second volume (600 ml) of PBST and the resulting concentrate was pooled with the first concentrate and then filtered under vacuum to produce a final volume of around 20 ml. This was then transferred into a 50-ml centrifuge tube. The filter membrane was transferred to a small sealable plastic bag, and 8–10 ml of PBST was added, the membrane was kneaded manually, and the spent wash volume was pooled with the primary eluate. The membrane washing procedure was repeated twice. The sample was resuspended to 50 ml with PBST and centrifuged at 1500×g for 5 min. The supernatant was aspirated and the resuspended pellet was transferred to a Leighton tube and subjected to Immunomagnetic Separation technology (IMS).

2.2. Sample analysis for parasite occurrence

The IMS procedure was performed as described in US EPA method 1623 (USEPA, 2001). Briefly, each 10-ml sample concentrate was added to a Leighton tube containing 1 ml of 10× SL buffer A and 1 ml of 10× SL buffer B (Dynabeads® GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway). One hundred microlitres of Cryptosporidium and Giardia IMS beads were added to each tube, and samples were incubated for 1 h at room temperature with constant rotation. The Leighton tubes were then placed in a magnetic particle concentrator and gently rocked for 2 min through an angle of 90°. The supernatant was decanted; the membrane was transferred to a small sealable plastic bag, following the manufacturer’s instructions, but the beads were washed several times before the beads and parasites were dissociated and the isolated parasites were resuspended in Tris–EDTA buffer. DNA was isolated with a QIAamp® DNA Mini kit (QIAGEN GmbH, Germany), according to the manufacturer’s instructions.

2.3. Staining procedure for recovered (oo)cysts

The samples were dried, fixed in acetone, and 50 µl of fluorescein isothiocyanate (FITC)-conjugated anti-Cryptosporidium sp. and anti-Giardia sp. MAb (Aqua-Glo; Waterborne Inc., New Orleans, La) were placed in each well. The slides were placed in a humidified chamber, incubated (37 °C for 45 min), and excess FITC-MAb was aspirated. Any remaining FITC-MAb was removed by adding 50 µl of PBS to each well, leaving the slides for 5 min, and aspirating the excess PBS. A 50 µl aliquot of 4’,6’-diamidino-2-phenylindole (DAPI) solution (0.4 µg/ml in PBS) was introduced into each well. The slides were left at room temperature for 15 min, and excess DAPI solution was removed by washing the slides twice in PBS and once in distilled water. The slides were placed in the dark until dry, then a 10 µl aliquot of mounting medium (2% diazabicyclooctane [DABCO] in 60% glycerol 40% PBS) was placed in each well, and slides were sealed for subsequent examination under epifluorescence optics (Nikon Eclipse 90i microscope. Nikon Corporation, Tokyo, Japan), as described in method 1623 (USEPA, 2001). Cryptosporidium sp. oocysts were identified on the basis of their size, shape, and the presence of a suture on the oocyst wall, at a magnification of 400×. Giardia sp. cysts were identified by their size, shape and the pattern and intensity of immunofluorescence assay staining (i.e., bright green fluorescence of the cyst wall). Parasites in each slide well were enumerated, and the mean numbers were used to extrapolate to concentrations of parasites per litre of sample. Positive and negative controls, as recommended in the Method 1623, were used. The (oo)cysts used as positive controls were those provided with the MAb’s (Aqua-Glo; Waterborne Inc., New Orleans, La). Oocyst concentrations were calculated taking into account the mean recovery percentages of Cryptosporidium spp. oocysts (50%±13%) and G. duodenalis cysts (41%±5%) determinate by McCuin and Clancy (2003) using Filta-Max system and IMS. Important variations of the turbidity and temperature of water samples that could modify the efficiency of the filters were not observed. In each season, inter-assay test was carried out, according to method 1623, in order to confirm the good performance of the detection system.

2.4. DNA extraction

Samples containing relatively high concentrations of nucleated (oo)cysts were selected for DNA extraction from the frozen samples as described in Section 2.2. The parasites were isolated by IMS (Dynabeads® GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway), following the manufacturer’s instructions, but the beads were washed several times before the beads and parasites were dissociated and the isolated parasites were resuspended in Tris–EDTA buffer. DNA was isolated with a QIAamp® DNA Mini kit (QIAGEN GmbH, Germany), according to the manufacturer’s instructions.

2.5. Gene amplification and sequencing

Cryptosporidium species and genotypes were determined by a technique based on a two-step nested PCR protocol and sequencing of PCR products. An 830 bp fragment of the 18S SSU rRNA gene was amplified with primers 5′-TTCTAGAGCT-AATACATGCG-3′ and 5′-CCCTAATCTTGGAAAACAG GA-3′ for primary PCR and 5′-GGAGGGTGTGATTATATTTATTAAG-3′ and 5′-AAGGATTAGGAAACTGCT A-3′ for secondary PCR (Xiao et al., 1999). The primary PCR mixture contained 1× PCR buffer, 6 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 100 nM (each) primer, 2 units of Taq polymerase (New England BioLabs), and 10 µl of purified DNA in a final volume of 50 µl. Each of 45 cycles consisted of 94 °C for 40 s, 55 °C for 40 s,
Table 1 – Arithmetic mean, maximum (max) and minimum (min) numbers of Cryptosporidium spp. oocysts and G. duodenalis cysts in the influent and effluent samples from drinking water treatment plants over a period of 12 months

<table>
<thead>
<tr>
<th>Plant</th>
<th>Treatment</th>
<th>Oocysts per litre</th>
<th>Cysts per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Influent</td>
<td>Effluent</td>
</tr>
<tr>
<td>1</td>
<td>T1</td>
<td>7.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>T2</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>9</td>
<td>T3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>10.5</td>
<td>0.0</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>0.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

T1 Water facilities that only include sedimentation and chlorination.
T2 Small water treatment plants that include rapid filtration and/or disinfection processes.
T3 Conventional water treatment facilities that include coagulation, flocculation, sedimentation, filtration and disinfection.

and 72 °C for 1 min 30 s after an initial hot start at 94 °C for 4 min and ending with 72 °C for 7 min. A secondary PCR product was then amplified from 5 μl of the primary PCR. The secondary PCR mixture was identical except that the MgCl₂ concentration was 3 mM. Each of 45 cycles consisted of 40 s, 55 °C for 30 s, and 72 °C for 1 min after an initial hot start at 94 °C for 4 min and ending with 72 °C for 7 min.

For molecular typing of G. duodenalis a semi-nested PCR and partial sequencing of the β-giardin locus were performed (Cacciò et al., 2002). In the primary reaction, a 753 bp fragment was amplified with the forward primer G759 5′-AACGGCGAGACCT-CACCCCGAGTGCC-3′ and the reverse primer G759 5′-AGGCG-CGCCCTGGATCTTCGAGACGAC-3′. In the sequential semi-nested PCR reaction, a 384 bp fragment was amplified with the forward inner primer G736 5′-CATAAACGACGAGCTGC-3′ and the reverse primer G759, as previously described by Cacciò et al. (2002). The PCR mix contained 1× PCR buffer, 3 mM MgCl₂, 200 μM (each) deoxynucleoside triphosphate, 200 nM (each) primer, 2.5 units of Taq polymerase (New England BioLabs), and 5 μl of purified DNA as template for primary steps and 2.5 μl of primary PCR product for secondary steps, in a final volume of 50 μl. PCR was performed as follows: an initial denaturation step of 94 °C for 5 min, 40 cycles of 30 s at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension cycle of 7 min at 72 °C.

In all cases, for each reaction, a negative (water) and a positive control (bovine (oo)cysts) were added. The PCR products were submitted to 1.2% agarose gel electrophoresis, stained with ethidium bromide and the gel image recorded under UV light. Gel images were captured with a gel documentation system (Gel Doc XR system, BioRad).

Following successful PCR, the products were purified (GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare Europe GmbH, Germany) and sequenced on both strands (MWG Biotech, Germany). Chromatograms and sequences were examined with Chromas (http://www.technelysium.com.au/chromas.html) and BioEdit (http://www.mbio.ncsu.edu/BioEditpage2.html). Sequence searches were conducted with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. Statistics

The normality of the data was tested with the Kolmogorov-Smirnov test. For normally distributed results, differences in the concentrations of Cryptosporidium spp. and G. duodenalis in the final effluents from the influents in the drinking water treatments plants and throughout the year were compared by pairwise multiple comparison procedures (Student–Newman-Keuls method) and one-way ANOVA. Results that did not comply with the assumptions of normality were tested by Kruskal–Wallis test (nonparametric ANOVA) and Dunn’s Multiple Comparison test. Analyses were carried out with GraphPad InStat® for Windows, version 3.05 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at P<0.05.

3. Results

3.1. Prevalence of Cryptosporidium spp. and G. duodenalis in water samples

A total of 128 samples (64 from untreated water and 64 from finished water) were collected for a period of one year. Cryptosporidium spp. oocysts were detected in both influent and effluent of 14/16 (87.5%) and 12/16 (75.0%) plants respectively throughout the year, while G. duodenalis cysts were observed
in both influent and effluent from all plants, 16/16 (100%), in the different seasons. In positive water samples, no empty (i.e., without internal characteristics, ghosts) or DAPI negative Cryptosporidium spp. oocysts or G. duodenalis cysts were found.

The mean concentrations of parasites in influent samples were 0.0–10.5 Cryptosporidium spp. oocysts per litre and 1.0–12.8 G. duodenalis cysts per litre. In final treated effluent, the mean concentration of parasites ranged from 0.0–3.0 oocysts per litre and 0.5–4.0 cysts per litre. There were no significant differences in the mean concentrations of the (oo)cysts per litre of either influent or effluent sample throughout the year in the different treatment plants. Furthermore, the concentration of (oo)cysts per litre during all 4 seasons of the year in the final effluent did not differ significantly among the 16 drinking water treatment plants, and therefore the treatments carried out in the different plants were ineffective for both protozoans (Table 1). Surprisingly, in 5 plants (31.2%) the concentration of (oo)cysts per litre was higher in the treated than in the untreated water, although the differences were not statistically significant (Table 1).

The lowest reductions in the protozoans were observed in plants 4, 5, 8 and 9. These plants only include small water treatment facilities with rapid filtration and/or disinfection processes. This system of processing drinking water resulted in the lowest removal efficiency in comparison with the other plants, although significant differences were not observed.

The distribution of results by season revealed that in all drinking water treatment plants, the highest numbers of (oo)cysts were found in spring and summer (Fig. 2A and B). There was a significantly higher (P < 0.05) incidence of Cryptosporidium spp. oocysts in influent and effluent samples collected in summer than those collected in winter, and there were no significant differences among the other seasons (Fig. 2A). In the case of G. duodenalis the concentrations of cysts per litre were significantly higher, in both the influent and effluent samples, in summer (P < 0.05) than in autumn and winter (Fig. 2B).

Comparison of the 2 seasons in the year when most parasitic forms were detected (spring and summer), revealed that in the zone of capture or in the effluent, potentially infective stages of both parasites were observed in 15 (93.7%) plants. In the spring, treatments were totally ineffective for Cryptosporidium spp. and G. duodenalis in 7 (43.7%) and 6 (37.5%) plants, respectively. In these facilities, the concentrations of both parasitic stages were greater in the drinking water (2–8 oocysts per litre; 2–12 cyst per litre) than in the influent water (0–2 oocysts per litre; 0–2 cyst per litre), although the differences were not statistically different.

Fig. 2 – Removal efficacy of Cryptosporidium spp. oocysts (A) and G. duodenalis cysts (B) by drinking water treatment plants, according to season. Means in the same season with a different letter are significantly different. Means in different season with an asterisk are significantly different between each other.

Fig. 3 – Typical agarose gel with the diagnostic profile for the 18S SSU rRNA gene from Cryptosporidium spp. (A) and the β-giardin from gene G. duodenalis (B).
PCR amplification of 18S SSU rRNA gene sequences was successful for samples containing Cryptosporidium spp. oocysts. For G. duodenalis isolates, PCR amplification of the 753-bp fragment of the β-giardin gene was performed (Fig. 3A and B). Samples that contained relatively high concentrations of nucleated (oo)cysts were selected for processing. The samples were collected from the influent and final effluent of the 16 plants during spring and summer. At least two PCR products from each plant were sequenced. Unfortunately, sequencing of the DNA of (oo)cysts from only 6 plants was achieved.

Cryptosporidium parvum was detected in the influent from plants 2 and 15, and C. andersoni and C. hominis in the influent from treatment plants 11 and 12 respectively (Table 2). G. duodenalis cysts of assemblage A-II were detected in plant number 12. In other samples two assemblages (A-I+E) and (A-II+E) were detected, in plants 2, 11 and 1, and in plants 6 and 15 respectively (Table 2).

The results obtained by the sequencing and/or PCR-restriction fragment length polymorphism of the (oo)cyst samples from the final effluent were similar to those identified in the influent. Nevertheless, in plants 1 and 15, cysts of assemblage A-II were identified in the final effluent in the plants in which two assemblages (A-II+E) were detected in the influent (Table 2).

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>Influent</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium spp.</td>
<td>G. duodenalis</td>
<td>Cryptosporidium spp.</td>
</tr>
<tr>
<td>1</td>
<td>C. parvum</td>
<td>A-I+E</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>A-II+E</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>A-II+E</td>
</tr>
<tr>
<td>8</td>
<td>C. parvum</td>
<td>A-II+E</td>
</tr>
<tr>
<td>14</td>
<td>C. andersoni</td>
<td>A-I+E</td>
</tr>
<tr>
<td>15</td>
<td>C. hominis</td>
<td>A-II</td>
</tr>
</tbody>
</table>

ND: DNA extraction was not achieved.

### 3.2. Molecular identification of parasites

are of particular concern because of their capacity to cause the simultaneous infection of large numbers of people. Wastewater outfall and rainfall-initiated runoff from urban, suburban and rural land can carry encysted stages of these pathogenic protozoa into untreated waters used for agricultural and recreational purposes and, in some locations, as a source of drinking water. Moreover, (oo)cysts can withstand normal water disinfection processes, and have been found in significant quantities in the final effluents of wastewater treatment plants (Robertson et al., 2006).

This study examined the prevalence and concentrations of Cryptosporidium spp. and G. duodenalis in sixteen drinking water treatment plants located in the same hydrographical basin in which pumping areas for the plants are located close to recreational areas, river beaches and in some cases grazing areas, mainly for cattle. Moreover, the efficiency of treatment plants in removing the protozoans was estimated and the species and genotypes were determined by means of a molecular assay in order to identify host sources of Cryptosporidium spp. and G. duodenalis that contribute to contamination events and the risks that they pose to human health. The analysis of samples from sixteen plants revealed (oo)cysts in both influent and effluent samples from 14/16 (87.5%) and 12/16 (75.0%) plants respectively throughout the year, while G. duodenalis cysts were observed in both influent and effluent samples from all plants, 16/16 (100%), in the different seasons. In contrast, the occurrence that we report in untreated water and treated final effluents samples is higher than reported in different countries or zones (LeChevallier et al., 1991; Briancesco and Bonadonna, 2005; Karanis et al., 2006; Carmen et al., 2007).

This contradiction can probably be explained by the higher level of environmental contamination or the lower protection of surface waters to the load of contaminant (oo)cysts in the area of study. In this study, all drinking treatment plants were located in the same hydrographical basin, and monitoring was carried out during all four seasons of the year, in both the influent and the effluent. The results corresponding to examination of the influent samples demonstrate that Cryptosporidium spp. and G. duodenalis infections are widespread throughout this region of Spain, with giardiosis being more widespread and occurring at greater intensity of infection than cryptosporidiosis. This is supported by epidemiological data in other countries (Fayer, 2004; Di Giovanni et al., 2006).

In this study no direct relation between the loading of population equivalent and the parasite concentrations was

### 4. Discussion

The great majority of apparently water-related health problems are the result of microbial (bacteriological, viral, protozoan) contamination, and the enteropathogens Cryptosporidium spp. and G. duodenalis are especially important in this respect (Fayer et al., 2000). These faecal protozoans are of principal concern in setting health-based targets for safety. Parasitological water quality often varies rapidly and over wide areas. Short-term peaks in concentrations of (oo)cysts may considerably increase the risk of cryptosporidiosis and giardiosis and may trigger waterborne outbreaks of these diseases. Furthermore, by the time contamination is detected, many people may have been exposed. For these reasons, end-product testing, even when frequent, cannot be relied on to ensure the safety of drinking water. Failure to ensure drinking water safety may expose the community to the risk of outbreaks of intestinal and other infectious diseases. Outbreaks associated with drinking water...
observed. This may be due to the location of some plants, as the hydrographical basin includes areas where livestock farming predominates, especially cattle farming, and where there are numerous pastures and cultivated areas that are fertilized throughout the year, as well as areas dedicated to both large and small game hunting; there may therefore be uncontrolled contamination via animals, especially wild animals, which are important contributors of parasites in the contamination of drinking water (Ruecker et al., 2007). Wild animals were frequently observed during this study, residing in the natural vegetation buffer zones or directly within agricultural and urban settings.

The presence of high mean concentrations of Cryptosporidium spp. oocysts (0.5–3 oocysts per litre) and G. duodenalis cysts (0.5–4 cysts per litre) in the final effluent is of public health concern. The ID50 has been estimated to be as low as 83–123 oocysts for Cryptosporidium spp. (DuPont et al., 1995; Chappell et al., 2006) and 19–50 cysts for G. duodenalis cysts (Adam, 2001) and action levels of 10–30 oocysts per 100 litres and 3–5 cysts per 100 litres has been proposed by Haas and Rose (1995) and Wallis et al. (1996), respectively. During recent years, several waterborne outbreaks by Cryptosporidium spp. and G. duodenalis have been documented worldwide (Fayer, 2004; Yoder et al., 2004; Giangaspero et al., 2007; Sunderland et al., 2007). Nevertheless, in Spain very few epidemiological surveys have been carried out to establish the occurrence of these protozoa in drinking water, and no significant waterborne outbreaks of cryptosporidiosis and giardiasis have been reported (Rodríguez-Hernández et al., 1994, 1996; Montemayor et al., 2005; Gómez-Couso et al., 2005, 2006; Carmena et al., 2007).

Although Cryptosporidium spp. and G. duodenalis were found in large numbers of samples throughout the year, the highest numbers of (oo)cysts were found in spring and summer. The seasonality of these parasites has been investigated in a few studies, with diverse results. Although a similar seasonal pattern has been reported by some authors (Isaac-Renton et al., 1996; Cacciò et al., 2003; Montemayor et al., 2005; Carmena et al., 2007), it has not been confirmed by others (Hashimoto et al., 2001); thus, it is not clear whether or not seasonality is a general feature of Cryptosporidium spp. and G. duodenalis contamination. In the present study, the increase in parasitic forms, especially in the influent water in treatment plants in spring and summer may be caused by activities associated with agricultural practices (fertilization of cropped land with slurry) and farming activities, such as calving, lambing and grazing, especially of adult cows and heifers. The detection of C. hominis in the influent suggests that infected humans have a role in the contamination of these waters. Moreover, at these times of year, many people use the river beaches and recreational areas, especially in the holiday periods, to take part in sporting activities, including river fishing. These data suggest that monitoring of Cryptosporidium spp. and G. duodenalis must be intensified during spring and summer, the period of highest prevalence of these protozoa in drinking water treatment plants.

None of the treatment processes carried out in the 16 plants during the different times of year was totally effective, and significant reductions in the concentration of (oo)cysts per litre were not observed in the effluent samples. The average removal efficiencies at these water treatments plants ranged between 0.12-log and 1.04-log for Cryptosporidium spp. and between 0.04-log and 0.96-log for G. duodenalis. Nevertheless, Betancourt and Rose (2004) in conventional water treatment plants observed that protozoa removals were higher (1.5-log – 2.5-log). Major waterborne cryptosporidiosis and giardiosis outbreaks associated with contaminated drinking water have been linked to evidence of suboptimal treatment. Cryptosporidium spp. oocysts are more resistant than G. duodenalis cysts to removal and inactivation by conventional water treatment (coagulation, flocculation, sedimentation, filtration and disinfection processes). The majority of the data on performance of treatment processes in removing (oo)cysts from drinking water have been obtained from pilot tests, and few studies have been performed in full-scale conventional water treatment plants. Pilot studies have demonstrated that removal of the enteropathogens throughout all stages of the conventional treatment is largely influenced by the effectiveness of coagulation pretreatment, which along with clarification constitutes the first treatment barrier against protozoan breakthrough (Betancourt and Rose, 2004). In the present study, the highest percentages of elimination of parasitic forms were observed in plants that applied conventional treatment and that included coagulation pretreatment, although performance was still very poor and significant differences were not observed. The lower efficiency of the plants may be due to the fact that the plants are not permanently staffed, automated procedures are not available and therefore the plants function irregularly. 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 tolerable limits of Cryptosporidium spp. and G. duodenalis in drinking water. It is therefore urgent that the health authorities rethink the relevance of the contamination levels of both parasites in drinking water and that they develop adequate countermeasures with the aim of protecting human and animal populations.

As regards the determination of species and genotypes of these parasites by means of a molecular assay, unfortunately, the DNA of (oo)cysts from only 6 plants was finally able to be sequenced. This may have been due to inhibitors present in the water, the small volumes used or to loss of parasites during concentration and purification before carrying out the PCR. Although PCR is a highly sensitive and useful technique, so far, we have only been able to apply it successfully to samples with a high parasite load, which makes purification of the oocysts/cysts easier. The PCR technique is therefore of rather limited use with water samples with low parasite loadings. Cryptosporidium hominis (formerly C. parvum genotype I or human, involved in the anthropogenic route of transmission) and Assemblage A G. duodenalis (genotype A-II), responsible for anthropogenic transmission, were detected in the influent at plant 15, suggesting that the majority of (oo)cysts in the untreated water originated from human infections. The determination of species and genotypes of these parasites by means of a molecular assay helps in the identification of host sources of Cryptosporidium spp. and G. duodenalis that contribute to contamination. However, caution is required when extrapolating molecular data from water samples to determine host sources of contamination. In studies that determine the host specificity of Cryptosporidium spp. and G. duodenalis there is a direct association between the host being studied and the faecal material used for typing parasites (Ryan et al., 2003; Zhou et al.,...
C. andersoni (which infects the abomasums of juvenile and mature cattle) was detected in drinking water treatment plant 14. Moreover, only C. parvum was observed in the effluent in plants 1 and 8, whereas C. andersoni (which infects the abomasums of juvenile and mature cattle) was detected in drinking water treatment plant 14. Moreover, only C. parvum was observed in the effluent in plants 1 and 8, whereas C. andersoni (which infects the abomasums of juvenile and mature cattle) was detected in drinking water treatment plant 14.

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The basic and essential requirements to ensure the safety of drinking water are a “framework” for safe water, comprising health-based targets established by a competent health authority; adequate and properly managed systems (adequate infrastructure, proper monitoring and effective planning and management), and a system of independent surveillance. It is important that drinking water treatment authorities rethink the relevance of Cryptosporidium spp. and G. duodenalis contamination levels in drinking water and develop adequate countermeasures.


