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Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in surface water: A health risk for humans and animals

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ABSTRACT

The objective of the present study was to determine the degree of contamination by *Cryptosporidium* spp. and *Giardia duodenalis* in a river basin in a livestock farming area in Galicia (NW, Spain). Water samples (50 l) were collected at 22 points in the main basin (including 5 recreational areas), and at the source and mouth of the 3 most important rivers and at the mouth of a smaller, secondary river. Faecal samples were collected from dairy cattle selected at random from 18 herds farmed in the area. A total of 139 neonatal calves, 480 heifers and 697 cows were sampled. The prevalence, intensity of infection and the risk associated with the spread of infection by both enteropathogens were determined. Water and faecal samples were collected in spring, summer, autumn and winter of 2007. The species and genotypes of these parasites present in the water samples were identified. In both water and faecal samples, more parasitic stages were collected in spring and summer than in autumn and winter. In spring, *Cryptosporidium* spp. oocysts were detected in 33 (9.4%) cows from 13 (72.2%) herds, and *G. duodenalis* cysts were detected in 56 (16.0%) cows from 15 farms (83.3%); the intensity of infection ranged from 5 to 7895 *G. duodenalis* cysts per gram of faeces. Infective stages of *Cryptosporidium* spp. and *G. duodenalis* were also detected in respectively 26 (89.6%) and 27 (93.1%) water samples, in spring. The mean concentrations of parasites ranged from 2 to 1200 *Cryptosporidium* spp. oocysts per litre and from 2 to 400 *G. duodenalis* cysts per litre. *Cryptosporidium parvum*, *C. andersoni*, *C. hominis* and assemblages A-I, A-II, E of *G. duodenalis* were detected. The presence of both protozoans must be monitored in cattle, in sources of water used for recreational purposes and in artificial waterways used by farmers (water channels, animal drinking water and drainage systems).

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 (Smith et al., 2007; Reynolds et al., 2008). Transmission is sustained by both a zoonotic and an anthroponotic cycle (Fayer et al., 2000a;

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Slifko et al., 2000; Thompson, 2000). Many recent studies have investigated the prevalence of these protozoans in domestic ruminants (Castro-Hermida et al., 2005, 2007a,b; Fayer et al., 2006). Infections by *Cryptosporidium* spp. and *G. duodenalis* have been associated with economic losses, through the occurrence of diarrhoea in productive animals, and also death of animals, especially neonatal domestic ruminants (Fayer et al., 2000a; Adam, 2001). 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 (Karanis et al., 2002; WHO, 2002; Centre of Disease Control and Prevention, 2006). *Giardia 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 low. In fact, the infectious dose has been estimated to be as low as 10 *Cryptosporidium* spp. oocysts (Fayer et al., 2000a) or 10 *G. duodenalis* cysts (Adam, 2001).

Infected adult animals can 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). There are no requirements for testing surface waters for the presence of these parasites, although analyses of outbreaks have shown that the pathogens can be shed into recreational waters (Karanis et al., 2006; Giangaspero et al., 2007; Sunderland et al., 2007).

The present study was carried out in the Tambre river basin (1530 km^2 in surface; 42°46′–43°10′ N, 8°00′–9°03′ W). This river basin is one of the most important in this study region (Galicia, NW Spain) and the river Tambre was declared a Site of Community Importance (SCI), by the European Commission Habitats Directive (92/43/EEC) in 2001. Nevertheless, there is a significant level of farming activity in the area, predominantly cattle farming (livestock census: 69,803), the runoff from which may be a potential source of contamination in surface water. Moreover, the river is a source of water destined for human consumption, providing drinking water to approximately 200,000 people. The drinking water sources are generally open to varied recreational uses, including swimming, especially during the spring and summer. The overall aim of the present study was to establish the degree of contamination by *Cryptosporidium* spp. and *Giardia duodenalis* in this river basin. For this, the prevalence and intensity of infection by *Cryptosporidium* spp. and *G. duodenalis* in dairy cattle were determined and the risk associated with the spread of infection by both enteropathogens was estimated. Moreover, the prevalence and concentrations of these parasites were determined at 22 points in the main basin (including 5 recreational areas), and at the source and mouth of the 3 largest rivers and at the mouth of a secondary river. Finally, the species and genotype of *Cryptosporidium* spp. and *G. duodenalis* present in river water samples were identified by means of a molecular assay in order to clarify the identification of host sources of these parasites that contribute to environmental contamination.

2. Materials and methods

2.1. *Detection and quantification of* Cryptosporidium *spp. and* G. duodenalis *(oo)cysts from faecal samples*

Faecal samples were collected, in spring, summer, autumn and winter of 2007, from 139 neonatal calves, 480 heifers and 697 cows from 18 dairy herds in the Tambre river basin. Farms were selected at random and the only possible restriction was whether the farmers consented to the study. The samples were obtained by direct rectal sampling, with sterile plastic gloves or sterile rectal swabs, and the sampling date, origin, age and identification number were recorded for each animal. The samples were transported to the laboratory in a cool box and stored for a maximum of 24 h before analysis.

Identification of *Cryptosporidium* spp. oocysts in faecal samples from neonatal calves (<1 month) was carried out by the direct examination method of Heine (1982). The intensity of infection was evaluated semiquantitatively according to the average number of oocysts in 50 randomly selected microscopic fields at 1000× magnification. The categories established were 0: absence of oocysts; 1: ≤1 oocyst per field; 2: 2–5 oocysts per field; 3: 6–10 oocysts per field and 4: >10 oocysts per field. In heifers and cows, *Cryptosporidium* spp. oocysts were detected by a direct immunofluorescence technique with monoclonal antibodies (IFAT) (Aqua-Glo G/C Direct, FL, Comprehensive Kit. Waterborne, Inc., New Orleans, LA). The same technique was also used for detection of *G. duodenalis* cysts in all animals. Briefly, 2 g of faeces was diluted in phosphate buffered saline (PBS), pH 7.2, and filtered though a sieve (mesh size: 45 μm) into conical centrifuge tubes. Diethyl ether was then added at a proportion of 2:1; the tubes were shaken vigorously and centrifuged at 1000 × *g* for 5 min at 4 °C and the top 3 layers decanted off. The sediment was removed and washed in PBS by centrifuging at 1000 × *g* for 5 min at 4 °C. Aliquots of the sediment (100 μl) were fixed in acetone on slides at 4 °C for 10 min and processed with a commercial kit, which contains fluorescein isothiocyanate-conjugated monoclonal antibodies. All the sample volume was observed by fluorescence microscopy at 400× magnification. The number of oocysts per gram of faeces and the number of cysts per gram of faeces was calculated as follows: number of oocysts or cysts identified/(volume of sample examined (ml) × weight of faeces (g)). The sensitivity of this technique was 20 (oo)cysts per gram of faeces.

2.2. *Detection and quantification of* Cryptosporidium *spp. and* G. duodenalis *(oo)cysts from river water samples*

Water samples (50 l) were collected at 22 points in the main basin of the river Tambre (including 5 recreational areas) and at the source and mouth of the 3 largest rivers and at the mouth of a secondary river. The 29 sampling points were sampled in the spring, summer, autumn and winter of 2007.

The 116 samples were taken immediately to the laboratory and processed with the Filta-Max Automatic System (IDEXX Laboratories, Inc., Westbrook, ME). Filtration cartridges were set up according to the manufacturer's instructions, and all samples were filtered at recommended flow rates. The filter

elution and final concentration of the sample were determined with the same equipment, and by following the manufacturer's instructions. Briefly, this involved placing a membrane 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 20–25 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 to 10 ml, and the resuspended pellet was transferred to a Leighton tube and subjected to Immunomagnetic Separation technology (IMS).

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 tubes were removed from the magnetic particle concentrator, and 1 ml of 1× SL buffer A was added to each. The tubes were gently rocked to resuspend the bead-(oo)cyst complexes; the suspension was transferred to 1.5 ml polypropylene tubes, with a Pasteur pipette, and these tubes were placed in a second magnetic particle concentrator and rocked for 1 min. The supernatants were aspirated, the magnet was removed, and 50 μl of 0.1 N HCl was added to each sample, which was vortexed for a minimum of 10 s. The samples were allowed to stand for 10 min in an upright position, then vortexed for a further 10 s, and magnets inserted. The tubes were left undisturbed for 2 min. The beads collected at the back of the tube and the acidified suspension was transferred to the wells of a 2-welled slide, each containing 5 μl of 1.0 N NaOH.

The samples were dried, fixed in acetone, and 50 μl of fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* sp. and anti-*Giardia* sp. MAbs (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 placed in 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 the slides were sealed for subsequent examination under epifluorescence optics, as described in method 1623 (USEPA, 2001). *Cryptosporidium* spp. 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* spp. 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 were used, as recommended in Method 1623. The mean percentage recoveries, calculated by the method described by McCuin and Clancy (2003), of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts from source water samples by use of the Filta-Max system and IMS, were 50 ± 13 and 41 ± 9%, respectively.

2.3. DNA extraction, gene amplification and sequencing for river water samples

For selected samples that contained relatively high concentrations of nucleated (oo)cysts, the remaining river water (which had not been examined) was concentrated by centrifugation. The parasites were isolated by IMS (Dynabeads® GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway), following the manufacturer's instructions, although 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.

Cryptosporidium species and genotypes were determined by a technique based on PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the small-subunit (SSU) ribosomal RNA gene (18S SSU rRNA). A 2-step nested PCR protocol was used to amplify an 830 bp fragment of the 18S SSU rRNA gene, with primers 5′-TTCTAGAGCTAATACATGC G-3′ and 5′-CCCTAATCCTTCGAAACAG GA-3′ for primary PCR and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAG GAGTAAGGAACAACCTCC A-3′ for secondary PCR (Xiao et al., 1999). The primary PCR mixture contained 1× PCR buffer, 6 mM $MgCl_2$, 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, 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 $MgCl_2$ concentration was 3 mM. Each of the 45 cycles consisted of 94 °C for 40 s, 55 °C for 40 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 G7 5′-AAGCCCGAC-GACCTCACCCGCAGTGC-3′, 3′ and the reverse primer G759

5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′. In the sequential semi-nested PCR reaction, a 384 bp fragment was amplified with the forward inner primer G376 5′-CATAACGACGC CATCGCGGCTCTCAGGAA-3′and the reverse primer G759, as previously described by Cacciò et al. (2002). The PCR mix contained 1× PCR buffer, 3 mM $MgCl_2$, 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. The 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 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/BioEdit/page2.html). Sequence searches were conducted with BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Statistics

The prevalence and intensity of infection determined in the faecal samples were compared by a test of comparison of proportions and the Kruskal–Wallis test (non-parametric ANOVA). It was not possible to establish the intensity of infection by *Cryptosporidium* spp. in animals <1 month as oocysts per gram of faeces, because of the large numbers of oocysts excreted, and therefore these data were not included in the statistical analysis, and only the data from adult animals were compared.

For the river water samples, 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* at the different points 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 (non-parametric ANOVA) and Dunn's Multiple Comparison test.

In all cases, analyses were carried out with GraphPad InStat® for Windows, version 3.05 (GraphPad Software, San Diego, CA), and differences were considered significant at $P < 0.05$.

3. Results

3.1. Prevalence and intensity of infection of Cryptosporidium spp. and G. duodenalis in dairy cattle

Analysis of a single sample of each of the 1316 dairy cattle (neonatal calves, heifers and cows) selected at random during the study period revealed that 85 and 123 animals were shedding *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts, respectively, i.e., the prevalence of cryptosporidiosis and giardiosis in the Tambre river basin was 6.5 and 9.3%, respectively. Throughout the year, *Cryptosporidium* spp. oocysts were detected in 40 neonatal calves (28.8%), 20 heifers (4.2%) and in 25 cows (3.6%) from 18 dairy farms (100%). The mean intensity of oocyst shedding was 2 in the neonatal calves (2–5 oocysts per microscopic field at 1000× magnification), the intensity of infection in heifers and cows ranged between 2–100 and 5–84 oocysts per gram of faeces, respectively. *Giardia duodenalis* cysts were identified in 29 neonatal calves (20.9%), 45 heifers (9.4%) and in 49 cows (7.0%) from 18 dairy farms (100%). The number of cysts shed by neonatal calves ranged between 5 and 7895 cysts per gram of faeces, whereas the intensity of infection in heifers and cows ranged from 5 to 95 and from 5 to 82 cysts per gram of faeces. The mean prevalence of *Cryptosporidium* spp. and *G. duodenalis* on farms within the river basin was significantly higher ($P < 0.05$) in spring than in winter (Fig. 1).

Considering the animals sampled at each time of year and independently of age (neonatal calves, heifers and cows), there was a significant association between the season when sampling was carried out and the prevalence of infection. Thus, the prevalence of *Cryptosporidium* spp. and *G. duodenalis* was significantly higher in spring than in winter ($P < 0.05$) (Fig. 2). In spring only, *G. duodenalis* was significantly more prevalent than *Cryptosporidium* spp.($P < 0.05$) (Fig. 2). However, considering the age of the animals, it was found that (only) the prevalence of cryptosporidiosis was significantly higher ($P < 0.05$) in neonatal calves than in adult animals (heifers and cows) in all seasons, and there were no seasonal patterns in neonantal cryptosporidiosis or giardiosis (Tables 1A–D).

Comparison of the 2 seasons in which the greatest numbers of parasitic forms were detected, revealed that in spring and summer *Cryptosporidium* spp. oocysts were detected in 33 (9.4%) and 21 (6.0%) animals in 13 (72.2%) and 11 (61.1%) herds, respectively. The percentages of infection

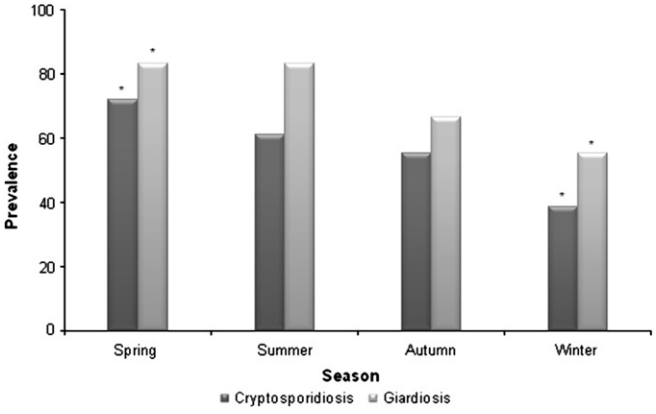

Fig. 1 – Seasonal prevalence of cryptosporidiosis and giardiosis on the farms within the river basin. Mean prevalences in different season indicated with an asterisk are significantly different from each other.

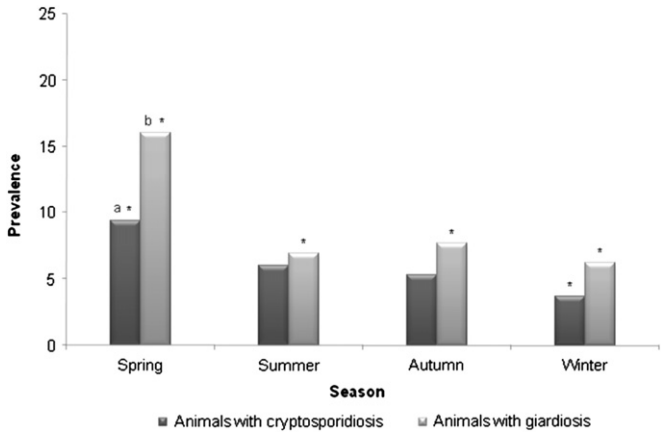

Fig. 2 – Seasonal prevalence of infection by *Cryptosporidium* spp. and *G. duodenalis* in dairy cattle farmed within the river basin. Means in the same season indicated with a different letter are significantly different. Mean prevalences in different seasons indicated with an asterisk are significantly different from each other.

according to age were: neonatal calves (23.8%; 20.0%); heifers (6.5%; 5.2%) and cows (8.2%; 4.4%). *Giardia duodenalis* was detected in 56 (16.0%) cattle in spring and in 24 (6.9%) animals in summer; in both seasons these animals belonged to 15 farms (83.3%). The prevalence according to age, in spring and summer was as follows: neonatal calves (21.4%; 23.3%), heifers (20.2%; 5.9%) and cows (11.9%; 4.9%), respectively. Cyst shedding was significantly higher ($P < 0.05$) in spring than in summer (5–7895 compared with 5–100 *G. duodenalis* cysts per gram of faeces) (Tables 1A,B).

3.2. *Prevalence and concentration of parasite forms of* Cryptosporidium *spp. and* G. duodenalis *in river water samples*

A total of 116 samples were collected, over a period of 1 year, from the main basin of the river Tambre (including 5 recreational areas); at the source and mouth of the 3 largest rivers and at the mouth of a secondary river. *Cryptosporidium* spp. oocysts were detected in 62/116 (53.4%) samples from 27/29 (93.1%) sampling points throughout the year, whereas

Table 1 – Prevalence and intensity of infection by *Cryptosporidium* spp. and *G. duodenalis* in dairy cattle during the spring (A), summer (B), autumn (C) and winter (D).

Animals (number)	Prevalence (%)	Oocysts per gram of faeces		Prevalence (%)	Cysts per gram of faeces	
		Min–Max	Mean		Min–Max	Mean
A						
Neonatal calves (42)	23.8[a]	1–4*	2.0*	21.4	14–7895	1423.7[b]
Heifers (124)	6.5	6–13	8.1	20.2	5–95	22.6
Cows (184)	8.2	5–60	17.9	11.9	5–82	14.7
B						
Neonatal calves (30)	20.0[a]	1–4*	2.5	23.3[a]	5–100	36.8
Heifers (135)	5.2	3–100	36.6	5.9	5–58	25.1
Cows (181)	4.4	6–84	24.1	4.9	6–59	23.6
C						
Neonatal calves (35)	37.2[a]	1–4*	2.5*	28.5[a]	10–250	111.7
Heifers (114)	4.4	2–8	5.8	5.3	6–21	11.2
Cows (172)	1.2	1–3	2.0	5.2	5–21	12.1
D						
Neonatal calves (32)	34.4[a]	1–4*	3.5*	9.4	10–40	21.6
Heifers (107)	0.0	0.0	0.0	5.6	6–9	7.3
Cows (160)	0.0	0.0	0.0	5.6	5–13	7.1

*The intensity of infection in this group was evaluated semiquantitatively according to the average number of oocysts in 50 randomly selected fields at 1000× magnification (0: absence of oocysts; 1: ≤1 oocyst per field; 2: 2–5 oocysts per field; 3: 6–10 oocysts per field; and 4: >10 oocysts per field).

a The prevalence was significantly higher ($P < 0.05$) than in heifers and cows.

b The intensity of infection was significantly higher ($P < 0.05$) than in heifers and cows.

G. duodenalis cysts were detected in 78/116 (67.2%) samples from 29/29 (100%) sampling points, in the different seasons (Table 2). No empty (i.e., without internal characteristics, ghosts), *Cryptosporidium* spp. oocysts or *G. duodenalis* cysts were found in positive water samples. The concentrations of parasites at the sampled points ranged from 2 to 1350 *Cryptosporidium* spp. oocysts per litre and from 2 to 722 *G. duodenalis* cysts per litre (Table 2), most of them DAPI + (91.0 and 93.0%, respectively). In the recreational river areas, the concentration of parasites ranged from 2 to 1200 oocysts per litre and from 2 to 400 cysts per litre, with highest numbers in spring, a time of year when many people use the areas (Table 2).

The distribution of results according to season revealed that at all sampling points, the highest numbers of (oo)cysts were found in spring and summer (Table 2). There was a significantly higher ($P < 0.05$) prevalence of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts in samples collected in spring and summer than in samples collected in autumn and winter. Taken together these 2 seasons when most parasitic forms were detected, the infective stages of both parasites were detected in 29/29 (100%) of the points samples. In spring, infective stages of *Cryptosporidium* spp. and *G. duodenalis* were detected in 26 (89.6%) and 27 (93.1%) samples, respectively. The concentrations ranged between 2 and 1200 oocysts per litre and between 2 and 400 cysts per litre, with no significant difference between these. In summer, oocysts and cysts were detected in 20 (68.9%) and 25 (86.2%) samples, respectively. The concentrations at the different sampling points were similar and ranged from 2 to 1350 oocysts per litre and from 2 to 722 cysts per litre. In spring, when many animals are grazed in pastures and fertilize the land, very high levels of contamination were registered for both *Cryptosporidium* spp.(1200 oocysts per litre) and *G. duodenalis* (400 cysts per litre) in 2 recreational areas (Table 2).

3.3. *Molecular identification of parasites in river water samples*

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. Samples that contained relatively high concentrations of nucleated (oo)cysts were selected for processing. Surface water samples were collected from 29 sampling points during spring and summer. At least 2 PCR products from each point

Table 2 – Detection of *Cryptosporidium* spp. and *G. duodenalis* in surface waters.

Sampling point*	Spring		Summer		Autumn		Winter	
	Oocysts per litre	Cysts per litre	Oocysts per litre	Cysts per litre	Oocysts per litre	Cysts per litre	Oocysts per litre	Cysts per litre
1	2	2	0	5	2	12	0	2
2	0	2	0	2	0	5	0	0
3	26	17	2	2	0	0	0	0
4	2	2	6	12	0	0	0	0
5	0	2	0	2	0	2	0	0
6	2	15	0	2	0	0	0	17
7	2	10	0	0	0	2	0	0
8	0	2	0	5	2	0	0	0
9	2	2	6	10	0	0	0	0
10	4	0	24	17	0	5	0	2
11	2	7	12	35	2	0	0	0
12	4	7	0	0	0	2	0	0
13	2	0	2	50	0	0	0	2
14	4	2	6	5	0	2	0	0
15	4	2	4	5	0	0	0	0
16	2	2	0	0	2	12	0	0
17	2	75	14	40	2	2	2	2
18	2	82	4	2	0	5	0	2
19	2	5	0	0	2	10	2	0
20	4	20	2	5	0	10	0	0
21	4	400	4	7	0	0	0	0
22	1200	10	2	2	2	0	2	0
23 (S^1)	6	2	88	125	2	0	0	0
24 (M^1)	2	12	1350	722	2	2	0	20
25 (S^2)	6	22	2	7	2	0	0	17
26 (M^2)	4	10	2	7	2	0	0	0
27 (S^3)	2	2	6	10	2	20	0	15
28 (M^3)	2	2	4	22	0	2	0	0
29 (M_s)	2	25	4	50	4	10	0	7

*1–17: points sampled in the main river; 18–22: recreational areas in the main river basin; (S) source or (M) mouth of the 3 largest rivers [1, 2 or 3]; (M_s) mouth of a secondary river.

were sequenced. Unfortunately, sequencing of the DNA of (oo)cysts from only 17 points was achieved.

Cryptosporidium parvum was detected in 7 (41.2%) samples of surface water, *C. andersoni* and *C. hominis* in 6 (35.3%) and 4 (23.5%) of the samples, respectively (Table 3). As regards *G. duodenalis*, cysts of assemblage A-I and A-II were detected in samples from 2 (11.8%) to 4 (23.5%) of the points, respectively. Nevertheless, in another 4 samples (23.5%) 2 assemblages (A-I + E) were detected, and in another 5 samples (29.4%), assemblages A-II + E were detected. Assemblage E only was detected in 2 water samples (11.8%).

The results obtained by sequencing and/or PCR-restriction fragment length polymorphism of the samples of (oo)cysts in the main river were similar to those identified in recreational areas, and at the source and mouth of the rivers. Nevertheless, *C. hominis* was the predominant species in the recreational areas and *C. andersoni* was mainly detected in the rivers (Table 3).

4. Discussion

The results of this study indicated that the Tambre river basin was highly contaminated with *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts (range = 2–1350 (oo)cysts per litre), which were viable and potentially infective to man and to domestic and wild ruminants. In outbreaks in Bradford (UK) and Milwaukee (USA), oocysts were detected in water samples at densities of less than 0.4 per litre (MacKenzie et al., 1994; Atherton et al., 1995). The range of concentrations of oocysts found in the present study is 5–3375 times higher than the 0.4 oocysts per litre known to have caused outbreaks. These data revealed the risk of a possible outbreak if people were to accidentally drink river water while bathing, playing or swimming. Moreover, the low infectious doses of both cryptosporidiosis and giardiosis would definitely facilitate the transmission of these parasites.

Table 3 – Identification of species and genotypes of *Cryptosporidium* spp. and *G. duodenalis* detected in surface waters.

Sampling point*	*Cryptosporidium* spp.	*G. duodenalis*
3*	*C. parvum*	A-I + E
4*, 6*, 13*, 18§	*C. parvum*	A-II + E
10*	*C. andersoni*	A-I + E
11*	*C. parvum*	A-I
17*	*C. andersoni*	A-II + E
20§	*C. hominis*	A-I + E
21§	*C. hominis*	E
22§	*C. hominis*	A-II
23 (S^1)	*C. andersoni*	A-II
24 (M^1)	*C. parvum*	A-I
25 (S^2)	*C. andersoni*	A-I + E
26 (M^2)	*C. andersoni*	A-II
28 (M^3)	*C. hominis*	A-II
29 (M_s)	*C. andersoni*	E

*Points sampled in the main river basin; §: recreational areas in the main river basin; (S) Source or (M) mouth of the 3 largest rivers [1, 2 or 3]; (M_s) mouth of a secondary river.

The Tambre river basin is an area of Galicia (NW, Spain) characterised by a high density of domestic animals, especially dairy cattle. The large number of farms, the large proportion of animals (neonatal calves, heifers and cows), and the large volume of faeces that they produce demonstrate the importance of dairy cattle as a potential source of environment contamination and as a possible reservoir of infectious parasites (Fayer et al., 2000b; Castro-Hermida et al., 2006). This study examined the prevalence and concentrations of *Cryptosporidium* spp. and *G. duodenalis* in 18 dairy herds located in the same hydrographical basin in which pumping areas for plants of treatment of drinking water are located close to recreational areas, river beaches and in some cases grazing areas, mainly for cattle. Throughout the year, *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts were detected in all dairy farms (100%). We believe that this prevalence reflects a serious situation, taking into account that the study was carried out on animals and farms selected at random. Moreover, as only 1 faecal sample was collected per animal, the prevalence data probably underestimates the actual number of infected animals.

Cryptosporidium spp. oocysts and *G. duodenalis* cysts have been demonstrated in runoff from agricultural areas (Slifko et al., 2000; Fayer, 2004), and some outbreaks of cryptosporidiosis and giardiosis in humans have been attributed to contamination of drinking and bathing water with such effluents. In the present study, both pathogens were detected in neonatal calves, heifers and cows. Throughout the year, the mean excretion of *Cryptosporidium* spp. oocysts in neonatal calves was 2 (2–5 oocysts per microscopic field at 1000× magnification) and the mean intensity of infection by *Cryptosporidium* spp. and *G. duodenalis* in the different age groups ranged between 2 and 100 oocysts per gram and between 5 and 7895 cysts per gram of faeces, respectively, figures that are similar to those of previous studies carried out in cattle (O'Handley et al., 1999; Ralston et al., 2003; Castro-Hermida et al., 2006). Cattle may excrete up to 40 kg of faeces in a day, so a high degree of contamination can occur in the environment around farms; this represents a hazard to human health when contamination occurs with zoonotic species.

Considering the seasonal prevalence of infection by *Cryptosporidium* spp. and *G. duodenalis* in dairy cattle in the hydrographical basin and independently of age, the prevalence of cryptosporidiosis and giardiosis was significantly higher in spring than in winter. Although some authors have observed an increase in the prevalence of these infections during certain seasons, in relation to high rainfall or the number of births, seasonal effects can only be correctly evaluated if the studies are repeated over several consecutive years. In a year-long study on the prevalence of neonatal bovine cryptosporidiosis in France, Lefay et al. (2000) found that the lowest levels of parasitization were found in July and August. Nevertheless, the absence of seasonality in the presence of both infections has been described by Wade et al. (2000) in a study of 109 dairy farms in 5 regions in the southeast of New York State. The absence of notable climatic changes in Galicia throughout the year as well as the lack of a particular calving season and the fact that neonatal dairy calves are permanently stabled may explain these results (Castro-Hermida et al., 2002).

Most farms surveyed in the present study had direct access to river water and there were no barriers (i.e., buffer zones, vegetation) to prevent contamination of the water. In addition, manure is frequently washed away from these areas along well-defined drainage paths during rainfall events, and heifers and cows typically have free access to nearby streams. In this scenario, both dairy cattle manure and grazing heifers and cows -especially in spring and summer- may contribute to contamination of the river basin with (oo)cysts. Therefore, in order to reduce the risk of human infection from cattle faeces, it is recommended that cattle farm owners apply good manure management practices. Direct access of cattle to watercourses should be controlled (e.g., rotational grazing, off-site watering, access ramps, fencing). Only decomposed manure should be used as fertilizer for fresh vegetable and fruit crops. These steps should be taken seriously so as to curb contamination of parasites from cattle farms into the river basin.

On the other hand, the analysis of 116 samples collected over a period of 1 year from the main basin of the river Tambre (including 5 recreational areas), the source and mouth of the 3 largest rivers and at the mouth of 1 secondary river, revealed *Cryptosporidium* spp. oocysts in 53.4% of the samples from 93.1% sampling points throughout the year, and *G. duodenalis* cysts in the 67.2% of the samples from 100% of the sampling points in the different seasons. In contrast, the occurrence that we report for river water samples is higher than reported in different countries or zones (LeChevallier et al., 1991; Ono et al., 2001; Briancesco and Bonadonna, 2005; Farizawati et al., 2005; Karanis et al., 2002, 2006). This contradiction can probably be explained by the underestimation of prevalence in other geographic zones because of the small numbers of samples analysed at one point. In this study, all sampling points were located in the same river basin, and monitoring was carried out during all 4 seasons of the year. The results of the examination of all the river water samples, along with the results obtained in dairy cattle confirm that infections by *Cryptosporidium* spp. and *G. duodenalis* are widespread throughout this region of Spain, with giardiosis being more widespread and occurring at greater intensity of infection than cryptosporidiosis. This is similar to findings reported for other countries (Fayer, 2004; Giovanni et al., 2006).

The high prevalence of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts in the Tambre river basin may be related to the fact that livestock farming, especially dairy cattle farming is predominant in the area. Nevertheless, uncontrolled contaminations via wild animals cannot be discounted (Ruecker et al., 2007). Moreover, the area includes wastewater treatment plants, which could contribute to the contamination of recreational river areas with *Cryptosporidium* spp. and *G. duodenalis* (Castro-Hermida et al., 2008).

The high mean concentrations of *Cryptosporidium* spp. oocysts (2–1350 oocysts per litre) and *G. duodenalis* cysts (2–722 cysts per litre) (of which 91.0 and 93.0%, respectively, were viable) in the river basin is a matter of public health concern. The infectious dose has been estimated to be as low as 10 (oo)cysts for *Cryptosporidium* spp.(Fayer et al., 2000a) and *G. duodenalis* (Adam, 2001). 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 or giardiosis 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 greatest numbers of (oo)cysts were found in spring and summer. The seasonality of these parasites in waters 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 high numbers of parasitic forms in river water in spring and summer coincide with the high rate of (oo)cyst shedding detected in cattle at these times of year. It is possible that the contamination in spring and summer is caused by agricultural-related activities (e.g., fertilization of cropped land with slurry). Since, at these times of year, many people use river beaches and recreational areas, especially during holiday periods, the monitoring of *Cryptosporidium* spp. and *G. duodenalis* must be intensified during spring and summer.

The species and genotypes present in river water samples were identified by means of a molecular assay. Unfortunately, the DNA of (oo)cysts from only 17 sampling points was finally able to be sequenced. This may have been because of 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 rather limited use with water samples containing low parasite loads.

Cryptosporidium hominis, formerly *C. parvum* genotype I or human, involved in the anthroponotic route of transmission, was the predominant species in the recreational areas, indicating a human source for this contamination that could be, at least in part, the origin of *C. parvum* contamination also detected. *Cryptosporidium andersoni*, which infects the bovine abomasum and is not know to infect animals other than cattle, was mainly detected in the rivers close to areas where dairy cattle farming predominates and that include several pastures and cultivated areas, which confirms the importance of dairy heifers and milking cows in the transmission of animal cryptosporidiosis in rural areas. Nevertheless, *C. parvum* was the most prevalent species in the river basin. *Cryptosporidium parvum* infects the small intestine, mainly of pre-weaned calves, although also human and other animals, often causing diarrhoeal disease (Castro-Hermida et al., 2002; Santín et al., 2004). As regards *G. duodenalis,* assemblages A-I (zoonotic), A-II (anthroponotic) and E (only infects livestock, cats and rats) were detected in the sampling area. The most commonly detected genotypes of *G. duodenalis*, i.e., assemblages A and B, have the widest host ranges, including humans and a variety of other animals, including cattle.

Assemblage E has been reported only in livestock (Monis et al., 2003; Trout et al., 2005). The present study demonstrates a high degree of contamination by *Cryptosporidium* spp. and *Giardia duodenalis* in the river basin studied. Moreover, cattle were an important source of contamination as can be deduced from the identification of species and assemblages. Indeed, in 13/29 (44.8%) of sampling points were identified *C. andersoni* and/or *G. duodenalis* assemblage E, which only infect livestock. We therefore recommend protection of watersheds used for potable water, and adequate and continuous monitoring of river water in order to control the spread of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts.

5. Conclusions

The present study demonstrates the importance of monitoring the presence of *Cryptosporidium* spp. and *G. duodenalis* in cattle, independently of the age of the animals, as well as in water destined for human consumption, for recreational purposes and in waterways used by the farmers (water channel, animal drinking water and drainage systems). Monitoring of both parasites is particularly important in stretches of rivers used as sources of drinking water, i.e., where water is pumped to drinking water treatment plants or to water storage deposits. Such monitoring should be carried out at least during spring and summer, as well in special situations, e.g., following floods, fire, uncontrolled release of manure or effluents from sewage treatment plants, in order to prevent infection by *Cryptosporidium* spp. and *G. duodenalis* and thus prevent risks to human and animal health. Finally, it is important that health authorities rethink the relevance of levels of contamination by *Cryptosporidium* spp. and *G. duodenalis* in surface waters and develop adequate countermeasures.

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