Occurrence and genetic diversity of *Cryptosporidium* and *Giardia* in urban wastewater treatment plants in north-eastern Spain

Ana Ramo, Emilio Del Cacho, Caridad Sánchez-Acedo, Joaquín Quílez*

Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, 50013 Zaragoza, Spain

* Corresponding author: E-mail: jquilez@unizar.es (J. Quílez)

Key words: *Cryptosporidium*; *Giardia*; genetic diversity; wastewater; treated sludge; public health

Abstract

This study was designed to investigate the presence and removal efficiency of *Cryptosporidium* and *Giardia* in wastewater treatment plants at the 20 most populated towns in Aragón (north-eastern Spain). Samples of influent and effluent wastewater and dewatered sewage sludge were collected seasonally from 23 plants and processed according to USEPA Method 1623. All samples from raw and treated wastewater tested positive for *Giardia*, at an average concentration of 3,247 ± 2,039 cysts/l and 50 ± 28 cysts/l, respectively. *Cryptosporidium* was identified in most samples from both raw (85/92) and treated (78/92) wastewaters in a concentration significantly lower than *Giardia*, at both influent (96 ± 105 oocysts/l) and effluent samples (31 ± 70 oocysts/l) (P<0.001). The (oo)cyst counts peaked in summer in most plants. The removal efficiency was higher for *Giardia* (1.06-log to 2.34-log) than *Cryptosporidium* (0.35-log to 1.8-log). Overall, high removal efficiency values were found for *Giardia* after secondary treatment based on activated sludge, while tertiary treatment (microfiltration, chlorination and/or ultraviolet irradiation) was needed to achieve the greatest removal or inactivation of *Cryptosporidium*. Most samples of treated sludge were positive for *Giardia* (92/92) and *Cryptosporidium* (45/92), at an average concentration of 20-593 cysts/g and 2-44 oocyst/g, respectively. The molecular characterization of *Cryptosporidium* oocysts and *Giardia* cysts were attempted at the *SSU rRNA*/*GP60* and *bg/tpi* loci, respectively. *G. duodenalis* sub-assemblage AII was identified in all plants, with a large proportion of samples (15/47) harboring mixed assemblages (AII+B). Nine *Cryptosporidium* species and six subtypes were identified, with *C. parvum* IIaA15G2R1 being the most prevalent. The presence of significant numbers of (oo)cysts in samples of final effluents and treated sludge reveals the limited efficacy of conventional

treatments in removing (oo)cysts and highlights the potential environmental impact and public health risks associated with disposal and reclamation of wastewater.

1. Introduction

The environmental and public health risks associated with sewage disposal have focused attention on the importance of an efficient treatment of wastewater. Furthermore, reuse of reclaimed wastewater has emerged as a prominent option in the search for alternative sources of water (Mekala and Davidson, 2016). In addition to chemical contaminants, a wide range of bacteria, viruses, and parasites, pathogenic for humans and animals, end up in municipal sewage and should be reduced to acceptable levels (Montazeri et al. 2015). In addition to physical removal, potential pathogens can also be inactivated during wastewater treatment procedures. Wastewater management is, however, a challenging issue in the European Union, where legislation is fragmentary and in need of update, with some countries having more stringent regulations than those implemented by European Directives (Kelessidis and Stasinakis, 2012). The Urban Wastewater Treatment Directive 91/271/EC and the Sewage Sludge Directive 86/278/EC provide legal limits for physical and chemical parameters for the treatment of sewage effluents and sludge disposal in soil, respectively, but no pathogen standards are specified (CEC, 1986; CEC, 1991). The lack of pathogen standard protocols is greatly due to the inherent limitations of currently available methods to monitor these pathogens in water samples and to provide accurate, reliable and consistent concentration measures.

The major waterborne pathogens *Giardia duodenalis (*syn. *G. lamblia, G. intestinalis*) and *Cryptosporidium* spp. are among the most common parasites found in wastewater (Efstratiou et al., 2017). These intestinal protozoa are transmitted through environmentally-resistant cysts and oocysts, respectively, which are excreted in high numbers in the feces of infected hosts. The global emission of *Cryptosporidium* oocysts to surface waters has been estimated at 3×10^{17} oocysts per year, with comparable contributions from human wastewater and manure from livestock (Hofstra et al., 2013). Both *Giardia* and *Cryptosporidium*, particularly the latter, are resistant to chlorine-based disinfectants at the concentrations and exposure times commonly used in the water industry (Carmena, 2010). Additionally, many species and genotypes are infective to different livestock and companion animals, which may be a source for human infections and environmental contamination. At present, 31 *Cryptosporidium* species have been reported, although only two are responsible for the majority of human infections, including the anthroponotic species *C. hominis* and the zoonotic species *C. parvum* (Ryan et al., 2016). Subtyping at the highly polymorphic 60-kDa glycoprotein (*GP60*) gene, has enabled the identification of subtype families within *C. hominis* and *C. parvum*, as well as several subtypes within each family. Some of the *C. parvum* subtype families, such as IIa and IId, are responsible for zoonotic cryptosporidiosis, while other families, especially IIc, have so far only been found in humans (Xiao, 2010). Eight assemblages (A-H) and several sub-assemblages of *G. duodenalis* have been identified, but only two potentially zoonotic assemblages (A, B) are commonly found in humans (Ryan and Cacciò, 2013).

Studies conducted in some developed countries have reported the occurrence of *Giardia* and *Cryptosporidium* in raw wastewater, often at concentrations over 1,000 cysts/l and

10 oocysts/l, respectively (Cacciò et al., 2003; McCuin and Clancy, 2006; Cheng et al., 2009; Robertson et al., 2006; Lobo et al. 2009; Kitajima et al., 2014; Taran-Benshoshan et al., 2015). However, quantitative data have shown that conventional treatment processes are not designed to completely remove both protozoa from wastewater. Efficiencies of (oo)cyst removal varying from 75.3 to 100% for *Giardia* and 40 to 100% for *Cryptosporidium* have been reported (Nasser et al., 2012; Nasser, 2016). Moreover, several studies have demonstrated that commonly used bacterial indicators of the hygienic quality of water do not necessarily correlate with the concentration of these protozoa (Bonadonna et al., 2002; Keeley and Faulkner, 2008).

Spain accounts for the largest proportion of reused treated wastewater in Europe (500 Mm^3/yr out of 1,100 Mm^3/yr) and is among the greatest sewage sludge producers, with an annual production of 1,121,000 tons (Kelessidis and Stasinakis, 2012; BIO by Deloitte, 2015). In spite of this, the occurrence of *Giardia* and *Cryptosporidium* in Spanish wastewater treatment plants is not well documented and studies on the molecular characterization of isolates are limited. The scarcity of published data have shown that both protozoa are found in relatively high concentrations in wastewater, reclaimed water, sewage sludge, and even in fresh salad products, revealing the need to include them in regulations on urban wastewater reuse (Montemayor et al., 2005; Guzmán et al., 2007; Castro-Hermida, 2008, 2010; Galván et al., 2014; Amorós et al., 2010; 2016). However, no requirements are mentioned in current Spanish legislation, which only establishes certain limits for *Escherichia coli* and intestinal nematodes (Royal Decree 1620/2007). In this study, samples of raw wastewater, treated effluent and treated sewage sludge were seasonally investigated for the presence of *Giardia* and *Cryptosporidium* in municipal wastewater treatment plants in north-eastern Spain, in

order to assess the occurrence, concentration and genetic diversity of both protozoa, and the reduction of pathogen load through different wastewater treatments.

2. Material and methods

2.1. Sample collection and processing

Over the period 2013-2015, samples were collected from 23 urban wastewater treatment plants located in the 20 most populated towns in Aragón (north-eastern Spain) (Figure 1). This geographical area (42°56′ to 39°51′ N, 2°10′ O to 0°46′ E) is primarily agricultural, with an important ovine farming activity and an increasing industrial activity. These plants serve local settlements ranging from 5,000 to over 660,000 inhabitants and treat wastewater from nearly 1 million people, which represents over 75% of the total population in Aragón. Only three plants served a population over 20,000. In addition to human wastewater, most facilities received industrial waste and seven plants also treated waste from slaughterhouses and/or farms. Most plants discharged the final effluents into rivers, although reclaimed water from three plants was also used to irrigate public parks, residential lawns, for street sweeping, or agricultural irrigation (Table 1). All facilities had biological reactor systems based on activated sludge and extended aeration with oxygen to improve the digestion of organic material by aerobic bacteria. Three plants used an Orbal® oxidation ditch process based on aerobic and anaerobic water depuration. Eleven plants had a Carrousel type reactor with canal configuration and vertical and superficial diffusers, typically used in low and medium load plants. Nine plants used biological reactors based on big and deep tanks with static diffusers commonly used in plants with high load. Only six plants used

primary sedimentation and ten plants applied tertiary treatment, mostly based on chlorination, with two facilities using a combination of microfiltration and ultraviolet irradiation.

Samples of untreated influent and final effluent were collected from each wastewater treatment facility at four different times, each sampling time matching a different season (spring, summer, autumn, and winter). The holding times of each step in the treatment were taken into account during sampling, in order to examine the same wastewater at both points in the process. A sample of dewatered sewage sludge was also collected at each sampling time and kept for further analysis. Turbidity of influent and effluent samples was measured with a portable turbidimeter model HI93703 (Hanna Instruments, Spain) and the results were expressed in nephelometric turbidity units (NTU). The turbidity removal efficiency achieved by each plant was calculated using the following equation:

Turbidity removal efficiency (%) = [(turbidity influent –turbidity effluent) / (turbidity influent)] × 100

2.2. Detection of Giardia cysts and Cryptosporidium oocysts

A total of 184 samples of wastewater and 92 samples of treated sludge were analysed for the presence of *Cryptosporidium* oocysts and *Giardia* cysts. Wastewater samples were processed according to the U.S. Environmental Protection Agency Method 1623 (USEPA, 2005). Briefly, samples of untreated influent (10 litres) and treated effluent (50 litres) were filtered through Filta-Max filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) at recommended flow rates using a motorized pump. The filters

were transported to the laboratory in labelled and sealed plastic bags, and stored at 4°C. Elution procedures were carried out within 24 hours after collection with the Filta-Max Manual System (IDEXX Laboratories, Inc.), according to the manufacturer's instructions. After centrifugation at 1,500 × *g* for 15 min, the supernatant was aspirated to 20 ml and 10 ml of the resuspended pellet was subjected to immunomagnetic separation (IMS), with the remaining sample being kept frozen for future studies. Sewage sludge samples were subjected to a biphasic (water/ethyl acetate) sedimentation method using Mini Parasep tubes according to the manufacturer's instructions (Diasys, Berkshire, UK). Briefly, two grams of sludge were mixed with 8 ml of distilled water in 15 ml tubes containing glass beads, and vortexed for a minimum of 10 sec. The tubes were then centrifuged at 1,200 × *g* for 3 min and the supernatant discarded. The pellet was resuspended in 2 ml of ethyl acetate and 4 ml of distilled water and centrifuged at 1,200 × *g* for 5 min. The supernatant was discarded and the pellet resuspended in 5 ml of distilled water prior to being processed by IMS.

(Oo)cysts in concentrated samples of wastewater and sewage sludge were further purified from other particulates using the Dynal IMS procedure (Dynabeads GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway) according to the manufacturer's instructions. IMS-purified (oo)cysts were stained on well slides by fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Crypto/Giardia Cel, Cellabs Pty Ltd, Australia). The internal structure of (oo)cysts was confirmed by staining with the nuclear fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Slides were examined using an epifluorescence microscope and (oo)cysts showing typical, confirmatory features (size, internal contents, fluorescence) were enumerated and numbers extrapolated to concentrations of

parasite per litre of wastewater and gram of sludge. Positive and negative staining controls were routinely included. All calculations for quantification of (oo)cysts in wastewater samples were adjusted taking into account the recovery efficiency of *Giardia* and *Cryptosporidium* reported in our laboratory and the volume of water filtered.

The potential viability of (oo)cysts was estimated by staining with the vital dye propidium iodide (PI) (Sigma-Aldrich, USA). Stock solution was prepared by dissolving PI in distilled water (1mg/ml). A volume of 10 µl of PI working solution was added to each well and incubated at room temperature in the dark for 1 minute. The (oo)cysts were counted according to whether they were PI-positive (permeable and presumably dead), PI-negative, DAPI-positive (impermeable and presumably viable), or PI-negative, DAPI-negative (impermeable and viable after further trigger) (Jenkins et al., 1997; Thiriat et al., 1998).

The recovery efficiency of the method was determined by seeding 10 litres of distilled water with different turbidity values (0, 20, 50, 100, 300, 700 NTU) with a known number of (oo)cysts according to the instructions of the USEPA 1623 method. Manually enumerated (oo)cysts stained with FITC-labelled antibodies were used due to cost restrictions. This procedure was repeated three times for each turbidity value. The mean recovery efficiency was 37.2 ± 18.5% for *Giardia* and 17.9 ± 5.2% for *Cryptosporidium*, which meets the acceptance criteria described in this method (USEPA, 2005).

The removal efficiency of (oo)cysts by each plant and sampling time was calculated as follows:

226 Log removal = Log influent concentration – Log final effluent concentration

227

228 *2.3. DNA extraction and molecular characterization*

229

230 Both *Giardia* and *Cryptosporidium* positive samples were subjected to molecular
231 characterization. DNA was extracted from IMS-purified (oo)cysts using the QIAamp
232 DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's
233 instructions, incorporating an initial step of three freeze-thaw cycles (freezing in liquid
234 nitrogen for 5 min and heating at 100ºC for 1 min) in the protocol. Chelex 100 (Sigma-
235 Aldrich, Madrid, Spain) was added to DNA samples to a final concentration of 5% w/v
236 and incubated at 56ºC for 8 min and 96ºC for 20 min. Bovine serum albumin (BSA)
237 (Sigma-Aldrich, Madrid, Spain) was added to every PCR reaction to a final
238 concentration of 20 μg/μl to overcome PCR-inhibitory substances in wastewater and
239 sewage samples.

240

241 *Cryptosporidium* species were identified using a previously described PCR protocol and
242 sequence analyses of the small-subunit ribosomal RNA (*SSU rRNA*) gene locus (Xiao et
243 al., 2001). Samples that contained *C. parvum* or *C. hominis* were further subtyped by
244 DNA sequencing of the *GP60* gene following the protocol described by Alves et al.
245 (2003). The presence of mixed *Cryptosporidium* species was determined using a
246 species-specific multiplex genotyping real-time PCR targeting the *SSU rRNA* and *Lib13*
247 loci (Hadfield et al., 2011). Previously described PCR protocols and sequence analyses
248 of β-giardin (*bg*) and triose phosphate isomerase (*tpi*) genes were used for specific
249 assemblage and sub-assemblage identification of *G. duodenalis* positive specimens
250 (Sulaiman et al., 2003; Lalle et al., 2005). At least three replicates of the PCR were

conducted on each wastewater sample testing negative at any locus of *G. duodenalis* or *Cryptosporidium* spp. Successfully amplified PCR products were subjected to bi-directional sequencing on a 3500xL Genetic Analyser (Applied Biosystems®, Life Technologies) according to the manufacturer's instructions. The sense and antisense strand sequences were aligned and edited using Bioedit version 7.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and consensus sequences analysed using BLASTN searches of the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Cryptosporidium* alleles at *GP60* locus were named according to the nomenclature proposed by Sulaiman et al. (2005). Phylogenetic trees were inferred using FastTree 2 (Price et al. 2010). A Python script for the automatization of the estimation and display processes was created using MEvoLib and Biopython libraries (Cock et al., 2009; Álvarez-Jarreta and Ruiz-Pesini, 2016). Representatives of all of the *Cryptosporidium* and *Giardia* sequences generated during this study have been deposited in GenBank under the accession numbers KY483958 – KY483978 (*Giardia*) and KY483979- KY483988 (*Cryptosporidium*).

2.4. Statistical analysis

The Chi-squared or two-tailed Fisher's exact tests, as appropriate, were used to evaluate differences in the seasonal pattern of *Giardia* and *Cryptosporidium* positive samples. The nonparametric Kruskal-Wallis test was used to evaluate differences in the (oo)cyst counts between influent and effluent water and between seasons. Analyses were performed using the SPSS statistical package for Windows version 18 (SPSS Inc.). Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Occurrence of Giardia and Cryptosporidium

All samples from wastewater treatment plants tested positive for *Giardia* cysts, at an average concentration of 3,247 ± 2,039 cysts/l in the influent samples and 50 ± 28 cysts/l in the effluent samples (Table 2). The highest numbers of cysts in the raw influents were found in summer in most plants (15/23), although differences were only statistically significant when compared to autumn ($P<0.05$). *Cryptosporidium* oocysts were identified in all samples from winter and summer, but some plants tested negative for this protozoan in spring and autumn, either in the raw influent (2 and 5 plants, respectively) or the final effluent (6 and 8 plants, respectively). *Cryptosporidium* oocysts were identified in a concentration significantly lower than *Giardia* cysts, in any season and both in influent (96 ± 105 oocysts/l) and effluent samples (31 ± 70 oocysts/l) ($P<0.001$) (Table 3). Oocyst counts in the influent samples peaked in summer in most plants (14/23), and the mean oocyst concentration was significantly higher in summer than in other seasons ($P<0.05$). Most of the facilities recording the highest *Cryptosporidium* concentration in the raw influent received wastes from farms and/or slaughterhouses in addition to domestic or industrial sewage (Tables 1, 3). Overall, those sewage treatment plants serving the three most populated towns had higher concentrations of *Giardia* cysts in raw sewage. In contrast, the size of the population served was not correlated to the concentration of *Cryptosporidium* oocysts. The mean turbidity of the raw influent samples ranged from 16 to 344 NTU and was not associated with the origin of the wastewater or the concentration of (oo)cysts. Most

300 plants achieved more than 95% turbidity removal efficiency, and turbidity values below
301 5 NTU were detected in the final effluent of most facilities (Table 1).
302
303 Wastewater treatments did not reduce the number of positive plants, but significantly
304 reduced the (oo)cyst counts in the final effluents in all seasons for both *Giardia*
305 (P<0.001) and *Cryptosporidium* (P<0.05). Treatment processes were more efficient for
306 the removal of *Giardia* than *Cryptosporidium* in all plants. The removal efficiency of
307 *Giardia* cysts ranged from 1.06-log to 2.34-log, and only three facilities had values
308 under 1.5-log. The overall removal of *Cryptosporidium* oocysts ranged from 0.35-log to
309 1.8-log, with thirteen plants showing mean removal efficiencies lower than 1-log and
310 two plants showing negative values. Nevertheless, high parasite counts were still
311 detected in the effluent of some plants (mean values up to 134 cysts/l and 301
312 oocysts/l). The removal efficiency of studied parasites was not correlated to the
313 turbidity of raw sewage. Overall, a combination of secondary and tertiary treatments
314 was needed to achieve a high removal efficiency of *Cryptosporidium* oocysts, while
315 those facilities using only activated sludge processes were less efficient in removing this
316 protozoan. In contrast, the removal efficiency of *Giardia* was not associated with a
317 particular combination of wastewater treatments, and five of the seven most efficient
318 plants in removing this protozoan used only a secondary treatment based on activated
319 sludge (Tables 1–3).
320
321 Comparison of (oo)cyst viability between the raw influents and treated effluents
322 demonstrated a reduction in the average concentration of potentially viable *Giardia*
323 cysts (72-83% *versus* 52-74%) and *Cryptosporidium* oocysts (73-97% *versus* 61-93%),
324 although differences were not statistically significant. All samples from treated sewage

sludge were positive for *Giardia*, at average concentrations ranging from 20 to 593 cysts/g. *Cryptosporidium* oocysts were also identified in sewage sludge from most plants (20/23) and samples (45/92), at average concentrations between 2 and 44 oocyst/g (Tables 2 and 3). The mean potential viability of (oo)cysts in sewage samples, as estimated by staining with the vital dyes DAPI and PI, exceeded 60% in most positive samples.

3.2. Molecular characterization

Successful amplification of the partial *bg* and *tpi* genes was achieved in 42 (45.6%) and 44 (47.8%) of the 92 *Giardia* positive samples, respectively, with 47 samples being successfully typed based on the results of one or both of the latter loci. Sequence alignment analyses of the PCR-products with appropriate reference sequences from GenBank revealed the presence of the *G. duodenalis* assemblages AII (28 samples), assemblage B (3 samples) and mixed assemblages AII + B (15 samples) and B+E (1 sample) (Table 4). Only 23 of the 39 samples for which sequence information was available at both loci were allocated to the same assemblage/sub-assemblage. The remaining samples were interpreted as a mixture of templates (Table 4) (Suppl. S1 Table).

At the *bg* locus, all but three samples were typed as sub-assemblage AII. These specimens formed three discrete groups in the phylogenetic tree differing from each other by one single-nucleotide polymorphism (SNP) and showing 100% identity to the reference sequences AY072723, AY072724, and KT310377, respectively. An additional sample typed as sub-assemblage AII was a novel subtype differing by two

SNPs with the above-mentioned sequences. This subtype was deposited in GenBank under accession number KY483961. Two isolates that differed by two SNPs were characterized as *G. duodenalis* assemblage B and one specimen was typed as assemblage E. Eight samples amplified at the *bg* locus exhibited a mixture of templates, as demonstrated by overlapping nucleotides at specific positions in the chromatograms, and were allocated each to two different sub-assemblage AII groups in the phylogenetic tree (Fig. 2) (Suppl. S1 Table).

A higher degree of genetic diversity was seen based on typing of the *tpi* gene. The sub-assemblage AII was also the most commonly found at this locus, with 23 samples forming a single group identical to the reference sequence AF069557, while three specimens were novel genotypes. These three variants were submitted to GenBank under accession numbers KY483967, KY483969, and KY483970. The remaining isolates (n: 17) were typed as assemblage B and were mostly allocated into two distinct clusters identical to the reference sequences AF069560 and AF069561, respectively. Five novel *G. duodenalis* assemblage B isolates each represented by a single isolate were also identified. These novel subtypes were deposited in GenBank under accession numbers KY483971 to KY483973, and KY483975 to KY483976. The presence of overlapping nucleotide peaks indicative of mixed templates at the *tpi* locus was seen in a single specimen typed as assemblage B (Fig. 3) (Suppl. S1 Table).

A total of 49 of the 87 *Cryptosporidium*-positive samples were successfully amplified and identified at the species level based on sequencing of the *SSU rRNA* locus and/or a species-specific multiplexed real-time PCR assay (Table 5). Nine *Cryptosporidium* species (*C. parvum*, *C. hominis*, *C. cuniculus*, *C. ubiquitum*, *C. galli*, *C. canis*, *C.*

375 *andersoni, C. muris, C. suis*) were identified. *C. parvum* (33 samples) and *C. hominis*
376 (15 samples) were the most frequently found, with the remaining species being detected
377 in only 1-2 samples, including one species not previously reported in humans or animals
378 in Spain (*C. galli*). Two concurrent *Cryptosporidium* species were found in 11 samples.
379 *GP60* products of the expected size were generated for 21 samples, showing the
380 presence of five *C. parvum* (IIaA15G2R1, IIaA16G2R1, IIaA18G3R1, IIdA21G1,
381 IIdA22G1) and one *C. hominis* (IbA10G2) subtypes. Subtype IIaA15G2R1 was the
382 most prevalent, with the remaining subtypes being found in only 1-3 specimens. All
383 sequences identified in this study showed 100% identity with *Cryptosporidium* spp.
384 sequences previously deposited in GenBank, including one currently unnamed species
385 homologous to the *Cryptosporidium* sp. sequence with accession number AY737585
386 from water samples in New York.
387
388 **4. Discussion**
389
390 The current study has shown the ubiquity of *Giardia* and *Cryptosporidium* in
391 wastewater treatment works in the north-east of Spain, revealing that raw sewage is
392 highly contaminated with both protozoa. *Giardia* cysts were identified in the totality of
393 samples and *Cryptosporidium* oocysts were found in most of them, at an average
394 concentration over 3,000 cysts/l and 50 oocysts/l, respectively. The mean recovery
395 efficiency of the detection method was lower for *Cryptosporidium* (17.9 ± 5.2%) than
396 *Giardia* (37.2 ± 18.5%), which is in agreement with previous studies and has been
397 attributed to the smaller size of the former protozoan, with debris and organic material
398 of sewage trapping and hampering staining and visualization of oocysts (Taran-
399 Benshoshan et al., 2015). Nevertheless, these values were considered acceptable for the

USEPA method 1623 (USEPA, 2005). *Giardia* cysts were detected in the influent wastewater at higher concentration than *Cryptosporidium* oocysts in all facilities and seasons, a finding which could be attributed to a lower prevalence of cryptosporidiosis in local populations, in accordance with earlier studies in Spain and other countries (Navarro-i-Martínez et al., 2011; Carmena et al., 2012; Nasser et al., 2012; Nasser, 2016). In Europe, the prevalence of giardiasis and cryptosporidiosis in 2012 were 5.4 and 3.2 cases per 100,000 population, respectively, although case rate in children younger than five years increased to 11.6 and 10.5-13.8 per 100,000 population, respectively (ECDC, 2014).

Previous studies in Europe and other regions have highlighted the contamination by *Cryptosporidium* and *Giardia* in wastewater (Cacciò et al., 2003; McCuin and Clancy, 2006; Cheng et al., 2009; Robertson et al., 2006; Lobo et al., 2009; Kitajima et al., 2014; Spanakos et al., 2015; Taran-Benshoshan et al., 2015; Ma et al., 2016). In northern and central Spain, most samples of sewage were positive for *Giardia* (98-100%) and *Cryptosporidium* (47-100%), at average concentrations of 89-8,305 cysts/l and 6-350 oocysts/l (Montemayor et al., 2005; Castro-Hermida et al., 2008, 2010; Galván et al., 2014). No clear patterns of seasonality were observed, since the highest frequencies were reported in spring and autumn in the north-east (Montemayor et al., 2005), spring and summer in the north-west (Castro-Hermida et al., 2008), and winter and spring in the central area (Galván et al., 2014). In the current study, both parasites were prevalent throughout the year, but (oo)cysts counts peaked in summer in most plants, which is consistent with the peak in the number of human cases of cryptosporidiosis reported in summer in Spain (Semenza and Nichols, 2007). Nevertheless, a recent study in the same geographical area showed that the percentage

425 of drinking water plants positive to *Cryptosporidium* and *Giardia* peaked in winter,
426 which indicates that the concentration of these pathogens in surface water and
427 wastewater has no uniform seasonal pattern (Ramo et al., 2017).
428
429 The high occurrence and concentration of (oo)cysts in raw sewage also indicates that
430 human infections by both protozoa are much more common than the diagnostic data and
431 available official figures indicate, given that most facilities treated wastewater from
432 urban and industrial origin. In Spain, giardiasis and cryptosporidiosis have been
433 nationally notifiable diseases since 2015, but routine testing is not always carried out
434 and many diagnosed cases are not notified (Martín-Ampudia et al., 2012). A total of
435 1,483 human cases of giardiasis and 324 cases of cryptosporidiosis were reported in the
436 Spanish National Centre for Epidemiology in 2014, with only 126 and 10 cases being
437 notified in Aragón, respectively (NCE, 2016). However, studies conducted for several
438 years in patients with gastrointestinal symptoms showed that *Cryptosporidium* infection
439 is much more prevalent in the latter region, with a mean infection rate of 1.93%, and
440 values up to 6.2% in children aged 1-3 years (Clavel et al., 1996).
441
442 The amount of parasites in the influent sewage has been linked to the size of the
443 population served, a trend that was also observed in this study, although only for
444 *Giardia* (Lim et al., 2007). The potential contribution of livestock to the
445 *Cryptosporidium* contamination of wastewater should also be noted, since those plants
446 receiving wastes from farms and/or slaughterhouses recorded the highest oocyst counts,
447 which is in agreement with the high prevalence of this protozoan in livestock in this
448 geographical area (Quílez et al., 2008a,b). In contrast, the (oo)cyst concentration was
449 not associated with the turbidity of either the influent sewage or treated effluents, in

450 accordance with other authors indicating the unsuitability of turbidity as an indicator
451 for these parasites in wastewater (Bonadonna et al., 2002; Keeley and Faulkner, 2008).
452
453 Wastewater treatment facilities were effective in removing most parasites, as
454 demonstrated by a significant reduction of the (oo)cyst concentration in the final
455 effluents and a decrease in their potential viability. Cysts were removed more efficiently
456 than oocysts and high removal values were found for *Giardia* by secondary treatment,
457 while tertiary treatment was needed to achieve the greatest removal of *Cryptosporidium*.
458 Two plants exhibited negative removal efficiencies for the latter protozoan, a finding
459 which others have related to several factors, such as the small volume of raw sewage
460 filtered because of filter clogging, the higher recovery efficiency with the cleaner
461 effluent samples, or the uneven distribution of parasites in the sample matrices
462 (Robertson et al., 2006; Castro-Hermida et al., 2008; Nasser, 2016). Nevertheless,
463 relatively high numbers of potentially viable (oo)cysts were still detected in the final
464 effluents, with mean concentrations of over 20 cysts/l and 1 oocysts/l in all but one
465 plant, which reveals that discharge from sewage treatment facilities is an important
466 point source of *Giardia* cysts and *Cryptosporidium* oocysts in the environment. Most
467 plants in this study discharged into rivers, and reclaimed water of three plants was used
468 for irrigation of public spaces or crops, which may be of public significance (Amorós et
469 al. 2010). A significant concentration of potentially viable (oo)cysts was also detected
470 in dewatered sludge, which reflects the resistance of transmissive stages to different
471 depuration treatments and shows the public health and veterinary risks associated with
472 the application of treated sludge on agricultural and livestock grazing lands (Guzmán et
473 al., 2007).
474

These findings are in agreement with previous studies showing that conventional wastewater treatment processes have a limited efficacy at removing both protozoa, especially *Cryptosporidium*. Some common technologies such as activated sludge, high-rate sand filtration, and chlorine disinfection are not completely effective for the removal or inactivation of *Cryptosporidium* (Taran-Benshoshan et al., 2015; Nasser et al., 2016). Up to one-third of oocysts can survive after secondary wastewater treatment and advanced physical treatments such as membrane ultrafiltration are required to further reduction of *Cryptosporidium* for reuse purposes (Cheng et al., 2009; Fu et al., 2010). Treatment processes are much more efficient for the removal of *Giardia,* which can be reduced in one to two orders of magnitude after activated sludge treatment, although additional methods such as high-sand filtration or ultrafiltration are required for an optimal removal of cysts (Kitajima et al. 2014, Taran-Benshoshan et al., 2015; Nasser et al., 2012).

The molecular analyses of positive samples unraveled a considerable genetic diversity of *Giardia* and *Cryptosporidium* in wastewater and provided some indication on source hosts. The percentage of PCR-positive samples was similar for *Giardia* (51.1%) and *Cryptosporidium* (56.3%), revealing a relatively low sensitivity for the PCR assays which has been attributed to the presence of PCR inhibitors, the uneven distribution of (oo)cysts in sample concentrates, or the smaller proportion of water concentrate used for PCR versus immunofluorescence microscopy (Robertson et al., 2006; Lobo et al., 2009; Ma et al., 2016). It is worth mentioning that most PCR-negative samples were found in spring and autumn for both *Giardia* (34/45) and *Cryptosporidium* (29/38), a finding which may be due to the transfer of PCR-inhibitory substances such as humic or fulvic

499 acids from soils to the water collection areas as a consequence of seasonal rainfall
500 events (Artemyev, 2012).
501
502 Alignment analysis of *Giardia*-positive samples with reference sequences from
503 GenBank showed the presence of multiple genetic variants allocated to the potentially
504 zoonotic assemblages A and/or B. The sub-assemblage AII was identified in all plants
505 and was by far the most prevalent genotype. A large proportion of samples harbored
506 mixed *G. duodenalis* assemblages (AII+B, B+E), as inferred from discrepant results
507 between PCR analyses at both genes. The non-concordance in typing results has been
508 explained by the preferential amplification of certain assemblages at a particular locus,
509 which highlights the relevance of adopting a multilocus approach for genotyping this
510 parasite (Ryan and Cacciò, 2013). In this study, phylogenetic analysis at the *bg* gene
511 assigned most samples to the sub-assemblage AII, which formed three distinct
512 subgroups with highly conserved nucleotide sequences differing by only one SNP. In
513 contrast, a higher diversity was found based on sequencing at the *tpi* gene, which
514 allocated almost half of samples to the assemblage B and allowed the detection of up to
515 eight novel genetic variants within assemblages AII and B.
516
517 The occurrence of mixed *Giardia* genotypes has been frequently detected in both
518 human or animals hosts, and is also commonly found in wastewater, as may be expected
519 in samples which presumably contains cysts from a variety of sources (Ryan and
520 Cacció, 2013). Moreover, chromatograms of some samples showed several double
521 peaks at different positions, which were also consistent with a mixture of different
522 genotypes (Ryan and Cacció, 2013). The accurate identification was possible when the

523 contributing genotypes belonged to the same assemblage, because in this case, there are
524 very few nucleotide differences between the two templates (Lalle et al., 2005).
525
526 *G. duodenalis* assemblages A and B have been reported in humans and animals, but
527 sub-assemblages appear to differ in host preference, with some being more common in
528 humans (AII) and other more prevalent in animals (AI) (Ryan and Cacciò, 2013). In
529 Spain, the *G. duodenalis* assemblage B has been reported as the most common in
530 human populations and municipal wastewater, followed by sub-assemblage AII, which
531 suggests that transmission is mainly anthroponotic (Goñi et al., 2010; Mateo et al, 2014;
532 de Lucio et al., 2015). The livestock-specific assemblage E, which is the most prevalent
533 in livestock and drinking water treatment facilities in the north-west of Spain, was
534 found in a single plant receiving sewage from a slaughterhouse in this study, which
535 indicates that livestock is a minor contributor of this assemblage in our geographical
536 area (Castro-Hermida et al., 2015).
537
538 Previous studies in human populations have shown that *Cryptosporidium* transmission
539 is mostly anthroponotic in Spain, with the predominance of *C. hominis* over *C. parvum*
540 and the occasional description of other species such as *C. meleagridis*, *C. felis*, *C.*
541 *ubiquitum* or *C. cuniculus* (Cieloszyk et al., 2012; de Lucio et al., 2016; Segura et al.,
542 2015; Martínez-Ruiz et al, 2016). A similar conclusion was reported in two studies
543 conducted in this geographical area, where more than 63% and 93% of isolates were
544 identified as *C. hominis*, respectively, although *C. parvum* was more common than *C.*
545 *hominis* in children from rural areas (Llorente et al., 2007; Ramo et al., 2015). In the
546 current study, a total of nine *Cryptosporidium* species were identified, including some
547 minor species of zoonotic origin which have been sporadically associated with human

relatively high numbers of potentially viable (oo)cysts in the final effluents and dewatered sewage sludge is a consequence of the limited efficacy of conventional treatments in removing (oo)cysts. These observations raise concerns regarding the environmental impact and public health risks associated with disposal and reuse of treated wastewater and highlight the need of updating legislation, including both pathogenic organisms in regulations for wastewater reclamation.

Acknowledgments

We thank Jorge Álvarez Jarreta (Faculty of Informatics Engineering, University of Zaragoza, Spain) and Dr. Agustín Estrada (Faculty of Veterinary Sciences, University of Zaragoza, Spain) for technical assistance with phylogenetic analyses and figures. This study was financially supported by funds from Spanish (AGL2012- 32138) and regional (GA-LC-043/2011; DGA-B82) research programs, and the European Regional Development Fund.

References

Álvarez-Jarreta, J., Ruiz-Pesini, E. 2016. MEvoLib v1.0: the first molecular evolution library for Python. BMC Bioinformatics. 17: 436. doi**:** 10.1186/s12859-016-1303-3.

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F. 2003. Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J. Clin. Microbiol. 41: 2744–2747. doi: 10.1128/JCM.41.6.2744-2747.2003

598 Amorós, I., Alonso, J.L., Cuesta, G. 2010. *Cryptosporidium* oocysts and *Giardia* cysts
599 on salad products irrigated with contaminated water. J. Food. Prot. 73: 1138-1140
600
601 Amorós, I., Moreno, Y., Reyes, M., Moreno-Mesonero, L., Alonso, J.L. 2016.
602 Prevalence of *Cryptosporidium* oocysts and *Giardia* cysts in raw and treated sewage
603 sludges. Environ. Technol. 37:22. doi: 10.1080/09593330.2016.1168486
604
605 Appelbee, A.J., Thompson, R.C.A., Olson, M.E. 2005. *Giardia* and *Cryptosporidium* in
606 mammalian wildlife - current status and future trends. Trends Parasitol. 21:370-376.
607 doi: 10.1016/j.pt.2005.06.004
608
609 Artemyev, V.E. 2012. Geochemistry of organic matter in rive-sea systems. Springer
610 Science & Business Media. 190 pp.
611
612 Baldursson, S., Karanis, P., 2011. Waterborne transmission of protozoan parasites:
613 Review of worldwide outbreaks - An update 2004-2010. Water Res. 45, 6603–6614.
614 doi.org/10.1016/j.watres.2011.10.013
615
616 BIO by Deloitte. 2015. Optimising water reuse in the EU – Final report prepared for the
617 European Commission (DG ENV), Part I. In collaboration with ICF and Cranfield
618 University.
619
620 Bonadonna, L., Briancesco, R., Ottaviani, M., Veschetti, E. 2002. Ocurrence of
621 *Cryptosporidium* oocysts in sewage effluents and correlation with microbial, chemical
622 and physical water variables. Environ. Monit. Assess. 75:241–252

623

624 Cacciò, S.M., De Giacomo, M., Aulicino, F.A, Pozio, E. 2003. *Giardia* cysts in
625 wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69: 3393–3398. doi:
626 10.1128/AEM.69.6.3393-3398.2003

627

628 Carmena, D., 2010. Waterborne transmission of *Cryptosporidium* and *Giardia*:
629 detection, surveillance and implications for publich health. In: Mendez-Vilas A, (Ed.).
630 Current Research, Technology and Education Topicas in Applied Microbiology and
631 Microbial Biotechnology. Formatex microbiology series, vol 2. Formatex Research
632 Center, Badajoz, Spain. pp. 3–14.

633

634 Carmena, D., Cardona, G.A., Sánchez-Serrano, L.P. 2012. Current situation of *Giardia*
635 infection in Spain: implications for public health. World. J. Clin. Infect. Dis. 2:1–12.
636 doi/10.5495/wjcid.v2.i1.1

637

638 Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia
639 Da Costa, J.M., Mezo, M. 2008. Contribution of treated wastewater to the
640 contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia*
641 *duodenalis*. Water Res. 42: 3528–3538. doi: 10.1016/j.watres.2008.05.001

642

643 Castro-Hermida, J.A., García-Presedo, I., González-warleta, M., Mezo, M. 2010.
644 *Cryptosporidium* and *Giardia* detection in water bodies of Galicia, Spain. Water Res.
645 44:5887–5896. doi: 10.1016/j.watres.2010.07.010

646

647 Castro-Hermida, J.A., González-Warleta, M., Mezo, M. 2015. *Cryptosporidium* spp.
648 and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: The
649 need for safe drinking water. Int. J. Hyg. Environ. Health 218:132–138
650
651 CEC (Council of the European Communities). 1986. Council Directive 86/278/EEC of
652 12 June 1986 on the protection of the environment, and in particular of the soil, when
653 sewage sludge is used in agriculture. Off. J. Eur. Union L. 181: 6–12
654
655 CEC (Council of the European Communities). 1991. Council directive 91/271/EEC of
656 21 May 1991 concerning urban wastewater treatment. Off. J. Eur. Union L. 135: 40–52
657 (Official Journal of the European Communities)
658
659 Chalmers, R.M., Katzer, F. 2013. Looking for *Cryptosporidium*: the application of
660 advances in detection and diagnosis. Trends Parasitol. 29:237–251
661 doi:10.1016/j.pt.2013.03.001.
662
663 Cheng, H.-W.A., Lucy, F.E., Graczyk, T.K., Broaders, M.A., Tamang, L., Connolly, M.
664 2009. Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and
665 *Giardia duodenalis* cysts during secondary wastewater treatments. Parasitol. Res.
666 105:689–696
667
668 Cieloszyk, J., Goñi, P., García, A., Remacha, M.A., Sánchez, E., Clavel, A. 2012. Two
669 cases of zoonotic cryptosporidiosis in Spain by the unusual species *Cryptosporidium*
670 *ubiquitum* and *Cryptosporidium felis*. Enf. Infec. Microbiol. Clin. 30:549–551
671

672 Clavel, A., Olivares, J.L., Fleta, J., Castillo, J., Varea, M., Ramos, F.J., Arnal, A.C.,
673 Quílez, J. 1996. Seasonality of cryptosporidiosis in children. Eur. J. Clin. Microbiol.
674 Infect. Dis. 15: 77–79.
675
676 Cock, P.J., Antao, T., Chang, J.T., Chapman, B.A., Cox, C.J., Dalke, A., Friedberg, I.,
677 Hamelryck, T., Kauff, F., Wilczynski, B., de Hoon, M.J. 2009. Biopython: freely
678 available Python tools for computational molecular biology and bioinformatics.
679 Bioinformatics. 25: 1422–1423. doi: 10.1093/bioinformatics/btp163
680
681 De Lucio, A., Martínez-Ruiz, R., Merino, F.J., Bailo, B., Aguilera, M., Fuentes, I.,
682 Carmena, D. 2015. Molecular Genotyping of *Giardia duodenalis* Isolates from
683 Symptomatic Individuals Attending Two Major Public Hospitals in Madrid, Spain.
684 PLoS ONE. 10: e0143981. http://dx.doi.org/10.1371/journal.pone.0143981
685
686 De Lucio, A., Amor-Aramendia, A., Bailo, B., Saugar, J.M., Anegagrie, M.; Arroyo, A.,
687 Loez-Quintana, B., Zewdie, D., Ayehubizu, Z., Yizengaw, E., et al. 2016. Prevalence
688 and genetic diversity of *Giardia duodenalis* and *Cryptosporidium* spp. among school
689 children in a rural area of the Amhara region, north-west Ethiopia. PLoS ONE. 11.
690 e0159992. doi: 10.1371/journal.pone.0159992
691
692 ECDC (European Centre for Disease Prevention, Control). 2014. Annual
693 epidemiological Report 2014—Food and Waterborne Diseases and Zoonoses. ECDC,
694 Stockholm.
695

696 Efstratiou, A., Ongerth, J.E., Karanis, P. 2017. Waterborne transmission of protozoan
697 parasites: Review of worldwide outbreaks–An update 2011-2015. Water Res. 114:14–
698 22. doi: 10.1016/j.watres.2017.01.036
699
700 Fu, C.Y., Xie, X., Huang, J.J., Zhang, T., Wu, Q.Y., Chen, J.N., Hu, H.Y. 2010.
701 Monitoring and evaluation of removal of pathogens at municipal wastewater treatment
702 plants. Water Sci. Technol. 61:1589–1599.
703
704 Galván, A.L., Magnet, A., Izquierdo, F., Fernández-Vadillo, C., Peralta, R.H., Angulo,
705 S., Fenoy, S., del Aguila, C. 2014. A year-long study of *Cryptosporidium* species and
706 subtypes in recreational, drinking and wastewater from the central area of Spain. Sci
707 Total Environ. 468–469: 368–375. http://dx.doi.org/10.1016/j.scitotenv.2013.08.053
708
709 Goñi, P., Aldana, D.E., Clavel, A., Seral, C., Remacha, M.A., Castillo, F.J. 2010.
710 Prevalence of *Giardia duodenalis* assemblage B in humans in Zaragoza and León,
711 Spain. Enferm. Infecc. Microbiol. Clin. 28: 710–712.
712
713 Guzmán, C., Jofre, J., Montemayor, M., Lucena, F. 2007. Occurrence and levels of
714 indicators and selected pathogens in different sludges and biosolids. J. Appl. Microbiol.
715 103: 2420–9.
716
717 Hadfield, S.J., Robinson, G., Elwin, K., Chalmers, R.M. 2011. Detection and
718 differentiation of *Cryptosporidium* spp. In human clinical samples by use of real-time
719 PCR. J. Clin. Microbiol. 49: 918–924.
720

721 Hofstra, N., Bouwman, A.F., Beusen, A.H., Medema, G.J. 2013. Exploring global
722 *Cryptosporidium* emissions to surface water. Sci. Total. Environ. 442:10–19. doi:
723 10.1016/j.scitotenv.2012.10.013.
724
725 Jenkins, M.B., Anguish, L.J., Bowman, D.D.,Walker,M.J., Ghiorse,W.C., 1997.
726 Assessment of a dye permeability assay for determination of inactivation rates of
727 *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 63, 3844–3850.
728
729 Keeley, A., Faulkner, B.R. 2008. Influence of land use and watershed characterization
730 protozoa contamination in a potential drinking water resources reservoir. Water Res. 42:
731 2803–2813.
732
733 Kelessidis, A., Stasinakis, A.S. 2012. Comparative study of the methods used for
734 treatment and final disposal of sewage sludge in European countries. Waste. Manag.
735 32:1186–1895
736
737 Kitajima, M., Haramoto, E., Iker, B.C., Gerba, C.P. 2014. Occurrence of
738 *Cryptosporidium*, *Giardia,* and *Cyclospora* in influent and effluent water at wastewater
739 treatment plants in Arizona. Sci. Total Environ. 484:129–136. doi:
740 10.1016/j.scitotenv.2014.03.036
741
742 Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S. M. 2005. Genetic
743 heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia*
744 *duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35:
745 207–213. http://dx.doi.org/10.1016/j.ijpara.2004.10.022

746

747 Lim, Y.A., Wan-Hafiz, W.I., Nissapatorn, V. 2007. Reduction of *Cryptosporidium* and
748 *Giardia* by sewage treatment processes. Trop. Biomed. 24:95–104

749

750 Llorente, M.T., Clavel, A., Goñi, M.P., Varea, M., Seral, C., Becerril, R., Suárez, L.,
751 Gómez-Lus, R. 2007. Genetic characterization of *Cryptosporidium* species from
752 humans in Spain. Parasitol. Int. 56:201–205

753

754 Lobo, M.L., Xiao, L., Antunes, F., Matos, O. 2009: Occurrence of *Cryptosporidium* and
755 *Giardia* genotypes and subtypes in raw and treated water in Portugal. Lett. Appl.
756 Microbiol. 48: 732–737. doi: 10.1111/j.1472-765X.2009.02605.x

757

758 Ma, J., Feng, Y., Hu, Y., Villegas, E.N., Xiao, L. 2016. Human infective potential of
759 *Cryptosporidium* spp., *Giardia duodena/is* and *Enterocytozoon bieneusi* in urban
760 wastewater treatment plant effluents. J. Water Health. 14: 411–423. doi:
761 10.2166/wh.2016.192

762

763 Martín-Ampudia, M., Mariscal, A., Lopez-Gigosos, R.M., Mora, L., Fernandez-
764 Crehuet, J. 2012. Under-notification of cryptosporidiosis by routine clinical and
765 laboratory practices among non-hospitalised children with acute diarrhoea in Southern
766 Spain. Infection. 40: 113–119. doi.org/10.1007/s15010-011-0188-3

767

768 Martínez-Ruiz, R., de Lucio, A., Fuentes, I., Carmena, D. 2016. Autochthonous
769 *Cryptosporidium cuniculus* infection in Spain: First report in a symptomatic paediatric

patient from Madrid. Enf. Infec. Microbiol. Clin. 8:532–534. doi: 10.1016/j.eimc.2015.11.012

Mateo, M., Mateo. M., Montoya, A., Bailo, B., Saugar, J.M., Aguilera, M., Fuentes, I., Carmena, D. 2014. Detection and molecular characterization of *Giardia duodenalis* in children attending day care centers in Majadahonda, Madrid, Central Spain. Medicine. 93:e75

McCuin, R.M., Clancy, J.L. 2006. Occurrence of *Cryptosporidium* oocysts in US wastewaters. J. Water Health. 44:437–52. doi.org/10.2166/wh.2006.019.

Mekala, G.D., Davidson, B. 2016. A review of literature on the factors affecting wastewater treatment and recycling across a broad spectrum of economic stages of development. Water Policy. 18: 217–234. doi: 10.2166/wp.2015.191

Montazeri, N., Goettert, D., Achberger, E.C., Johnon, C., Prinyawiwatkul, W., Janes, M.E. 2015.Pathogenic enteric viruses and microbial indicators during secondary treatment of municipal wastewater. Appl. Environ. Microbiol. 81:6436–6446. doi:10.1128/AEM.01218-15.

Montemayor, M., Valero, F., Jofre, J., Lucena, F.. 2005. Occurrence of *Cryptosporidium* spp. oocysts in raw and treated sewage and river water in north-eastern Spain. J. Appl. Microbiol. 99:1455–1462. doi.org/10.1111/j.1365-2672.2005.02737.x

795 Navarro-i-Martínez, L., Del Águila, C., Bornay-Llinares, F.J. 2011. *Cryptosporidium*: a
796 genus in revisión. The situation in Spain. Enferm. Infecc. Microbiol. Clin. 29:135–143.
797 doi: 10.1016/j.eimc.2010.12.002
798
799 Nasser, A.M., Vaizel-Ohayon, D., Aharoni, A., Revhun, M. 2012. Prevalence and fate
800 of giardia cysts in wastewater treatment plants. J.Appl.Microbiol. 113:477–484. doi:
801 10.1111/j.1365-2672.2012.05335.x.
802
803 Nasser, A.M. 2016. Removal of *Cryptosporidium* by wastewater treatment processes: a
804 review. J. Water Health. 14:1–13. doi: 10.2166/wh.2015.131.
805
806 NCE (National Centre for Epidemiology), 2016. CIBER Epidemiología y Salud Pública
807 (CIBERESP). Instituto de Salud Carlos III. Resultados de la Vigilancia Epidemiológica
808 de las enfermedades transmisibles. Informe anual 2014. Madrid, 2016.
809 http://www.isciii.es/ISCIII/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-
810 alertas/fd-enfermedades/pdf_2016/RENAVE_INFORME_ANUAL_2014.pdf
811
812 Price, M.N., Dehal, P.S., Arkin, A.P. 2010. FastTree 2 – Approximately Maximum-
813 Likelihood Trees for Large Alignments Morgan N. PLoS One. 5: e9490.
814 doi: 10.1371/journal.pone.0009490
815
816 Quílez, J., Torres, E., Chalmers, R.M., Robinson, G., Del Cacho, E., Sánchez-Acedo, C.
817 2008a. *Cryptosporidium* species and subtype analysis from dairy calves in Spain.
818 Parasitology. 135: 1613–1620. doi.org/10.1017/S0031182008005088
819

820 Quílez, J., Torres, E., Chalmers, R.M., Hadfield, S.J., del Cacho, E., Sánchez-Acedo, C.
821 2008b. *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. Appl.
822 Environ. Microbiol. 74:6026–6031.
823
824 Ramo, A., Quílez, J., Vergara-Castiblanco, C., Monteagudo, L., del Cacho, E., Clavel,
825 A. 2015. Multilocus typing and population structure of Cryptosporidium from children
826 in Zaragoza, Spain. Infect. Genet. Evol. 31:190–197.
827 doi.org/10.1016/j.meegid.2015.01.023
828
829 Ramo, A., del Cacho, E., Sánchez-Acedo, C., Quílez, J. 2017. Ocurrence of
830 *Cryptosporidium* and *Giardia* in raw and finished drinking water in north-eastern Spain.
831 Sci. Total Environ. 580: 1007-1013 doi: 10.1016/j.scitotenv.2016.12.055
832
833 Robertson, L.J., Hermansen, L., Gjerde, B.K. 2006. Occurrence of *Cryptosporidium*
834 oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72: 5297–
835 5303. doi: 10.1128/AEM.00464-06
836
837 Royal Decree 1620/2007, February 7th, by which juridical regimen for treated
838 wastewater reutilization is established. (Real Decreto 1620/2007 de 7 de diciembre, por
839 el que se establece el régimen jurídico de la reutilización de las aguas depuradas).
840 https://www.boe.es/diario_boe/txt.php?id=BOE-A-2007-21092
841
842 Ryan, U., Cacciò, S.M. 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43:943-
843 956. doi: 10.1016/j.ijpara.2013.06.001
844

845 Ryan, U., Zahedi, A., Paparini, A. 2016. *Cryptosporidium* in humans and animals – a
846 one health approach to prophylaxis. Parasite Immunol. 38:535–547.
847 doi:10.1111/pim.12350
848
849 Segura, R., Prim, N., Montemayor, M., Valls, M.E:, Muñoz, C.2015. Predominant
850 virulent IbA10G2 Subtype of *Cryptosporidiumm hominis* in human isolates in
851 Barcelona: a five-year study. PLos ONE. 10:e0121753.
852 doi:10.1371/journal.pone.0121753
853
854 Semenza, J.C., Nichols, G. 2007. Cryptosporidiosis surveillance and water-borne
855 outbreaks in Europe. Euro. Surveill. 12, pii: 711
856
857 Spanakos, G., Biba, A., Mavridou, A., Karanis, P. 2015. Ocurrence of *Cryptosporidium*
858 and *Giardia* in recycled waters used for irrigation and first description of
859 *Cryptosporidium parvum* and *C. muris* in Greece. Parasitol. Res. 114: 1803-1810
860
861 Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R:H., Trout, J.M., Schantz, P.M., Das, P.,
862 Lai, A.A., Xiao, L.H. 2003. Triosephosphate isomerase gene characterization and
863 potential zoonotic transmission of *Giardia duodenalis*. Emerg.Infect. Dis. 9:1444–1452
864
865 Sulaiman, I.M., Hira, P.R., Zhou, L., Al-Ali, F.M., Al-Shelahi, F.A., Shweiki, H.M.,
866 Iqbal, J., Khalid, N., Xiao, L. 2005. Unique endemicity of cryptosporidiosis in children
867 in Kuwait. J. Clin. Microbiol. 43:2805–2809.
868

869 Taran-Benshoshan, M., Ofer, N., Dalit, V.O., Aharoni, A., Revhun, M., Nitzan, Y.,
870 Nasser, A.M. 2015. *Cryptosporidium* and *Giardia* removal by secondary and tertiary
871 wastewater treatment. J. Environ. Sci. Health A. Tox. Hazard Subs. Environ. Eng.
872 50:1265–1273. doi: 10.1080/10934529.2015.1055152
873
874 Thiriat, L., Sidaner, F., Schwartzbrod, J. 1998. Determination of *Giardia* cyst viability
875 in environmental and faecal samples by immunofluorescence, fluorogenic dye staining
876 and differential interference contrast microscopy. Lett. Appl. Microbiol. 26:237-242
877
878 USEPA (U.S. Enviromental Protection Agency), 2005. Method 1623: *Cryptosporidium*
879 and *Giardia* in Water by Filtration/IMS/FA. Office of Water, U.S. Environmental
880 Protection Agency, Washington, D.C. https://www.epa.gov/sites/production/files/2015-
881 07/documents/epa-1623.pdf. Accessed 20 July 2016.
882
883 Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., Lal, A. 2001. Molecular
884 characterization of *Cryptosporidium* oocysts in samples of raw surfacewater and
885 wastewater. Appl. Environ. Microbiol. 67: 1097–1101. doi: 10.1128/AEM.67.3.1097-
886 1101.2001
887
888 Xiao, L. 2010. Molecular epidemiology of cryptosporidiosis: an update. Exp. Parasitol.
889 124:80–89. doi:10.1016/j.exppara.2009.03.018
890
891
892
893
894

Figure 1. Geographic location of wastewater treatment facilities (WWTF) sampled in Aragón (north-eastern Spain). The water origin, water destination, and type of water treatment for each plant are indicated in Table 1.

Figure 2. Phylogenetic relationships of the isolates examined in the current study and different *G. duodenalis* assemblages/sub-assemblages at the *β-giardin* locus, as inferred by neighbour-joining analysis of the nucleotide sequence covering a 427-bp region of the gene (positions 134 to 557 of GenBank accession number AY072724). Bootstrap values over 50% from 1,000 pseudo-replicates are indicated at the left of the supported node. Samples with overlapping nucleotide peaks indicative of mixed templates were allocated to the corresponding assemblage/sub-assemblage. Isolates analysed in this study are indicated with the code ED, and reference sequences are indicated with the GenBank accession number.

Figure 3. Phylogenetic relationships of the isolates examined in the current study and different *G. duodenalis* assemblages/sub-assemblages at the triose phosphate isomerase (*tpi*) locus, as inferred by neighbour-joining analysis of the nucleotide sequence covering a 448-bp region of the gene (positions 31 to 478 of GenBank accession number AF069557). Bootstrap values over 50% from 1,000 pseudo-replicates are indicated at the left of the supported node. Samples with overlapping nucleotide peaks indicative of mixed templates were allocated to the corresponding assemblage/sub-assemblage. Isolates analysed in this study are indicated with the code ED, and reference sequences are indicated with the GenBank accession number.

Table 1
Click here to download Table: Table 1.docx

Table 1. Main features of municipal wastewater treatment plants at the most populated towns in Aragón (north-eastern Spain). Average values and ranges (minimum and maximum) in the turbidity and turbidity removal efficiency are indicated. Values of turbidity are expressed in nephelometric turbidity units (NTU).

Plant[a]	Wastewater origin	Wastewater destination	Wastewater treatment	Turbidity (NTU)		Turbidity removal efficiency (%)
				Influent	Effluent	
1	U + I + S	River	T0 + T1 + T2c + T3a	116 (81-164)	38 (8-94)	67
2	U + I *	River	T0 + T1 + T2c + T3a	134 (40-242)	16 (4 -31)	87.8
3	I	River (autumn, winter), park irrigation (spring, summer)	T0 + T2a + T3bc	344 (106-665)	6 (3-12)	98.2
4	U + I	River	T0 + T2b + T3a	64 (49-93)	5 (0-14)	91.8
5	U	River	T0 + T1 + T2c	59 (38-70)	2 (0-6)	97
6	U + I	River	T0 + T2a	52 (24-80)	2 (0-7)	95.5
7	U + I + F	River	T0 + T2a	62 (11-159)	3 (0.3-9)	95.7
8	U + I	River	T0 + T2c + T3a	230 (18-736)	0.2 (0-0.6)	99.9
9	U + I	River	T0 + T2a	74 (10-132)	1 (0-3)	98.2
10	U	River	T0 + T2a	36 (8 -70)	0	100
11	U + I + S	River	T0 + T1 + T2c	74 (57-91)	0.6 (0-1)	99.1
12	U + I + F	Crops irrigation	T0 + T2a + T3bc	112 (89-148)	4 (1-8)	96.8
13	U + I + S	River	T0 + T2b	70 (5-122)	4 (0-9)	94.7
14	U + I + S + F	River	T0 + T1 + T2c + T3a	83 (53-100)	5 (1- 9)	94
15	U + I *	River	T0 + T2a	35 (24.5-45)	0.03 (0-0.1)	99.9
16	U	River	T0 + T2a	90 (53-155)	0.8 (0-2)	99.9
17	U	River	T0 + T2b	16 (9-33)	6 (0-22)	63.6
18	U	River	T0 + T2a	51 (44-55)	0.3 (0-1)	99.4
19	U + I	River	T0 + t2a	127 (30-327)	1 (0-4)	99.3
20	U + I + S	River	T0 + T2a + T3a	99 (37-200)	2 (2-5)	98

21	U + I	River (chlorination)/ Recycled water (filtration)**	T0 + T1 + T2c + T3a/T3b	108 (22-243)	1 (0-5)	99.8
22	U + I	River	T0 + T2c	62 (19-148)	0.2 (0-1)	99.6
23	U	River	T0 + T2c + T3a	204 (140-294)	0.4 (0-1)	99.8

U: urban, I: industrial, S: slaughterhouse, F: farms

T0: preliminary treatment consisted in screening and grit separation, oil, grease and fat removal

T1: primary sedimentation

T2: secondary treatment based on activated sludge (a: Carrousel, b: Orbal®, c: static diffusers) with extended aireation and secondary sedimentation

T3: tertiary treatment based on chlorination (a), microfiltration (b) or UV light (c)

* Most wastewater (90%) was from urban origin

** Reclaimed water used for irrigation of residential lawns or street sweeping

Table 2
Click here to download Table: Table 2.docx

Table 2. *Giardia* cyst counts [arithmetic mean and ranges (minimum and maximum)] and removal efficiency (removal log) of cysts in wastewater treatment plants in north-eastern Spain. Samples from influent and effluent wastewater and sewage sludge were collected four times from each plant, each sampling time matching with a different season.

Plant	Influent		Effluent		Removal log	Sewage sludge
	Positive season[a]	Cysts / litre	Positive season[a]	Cysts / litre		Cysts/gram
1	All	6189 (1740–14223)	All	46 (5–145)	2.13	91 (8–180)
2	All	6703 (288–18088)	All	134 (54–323)	1.70	593 (104–1560)
3	All	605 (204–1248)	All	38 (8–78)	1.20	51 (30–78)
4	All	2305 (1910–3257)	All	56 (22–121)	1.61	217 (64–520)
5	All	2534 (38–3601)	All	35 (8–70)	1.86	475 (12–1242)
6	All	3203 (3–8459)	All	65 (54–86)	1.69	119 (10–186)
7	All	3946 (675–6681)	All	75 (19–172)	1.72	79 (66–100)
8	All	293 (86–549)	All	19 (11–22)	1.19	20 (10–38)
9	All	3354 (1915–6974)	All	38 (11–86)	1.94	103 (22–210)
10	All	764 (97–1522)	All	24 (1–48)	1.50	111 (36–176)
11	All	3384 (1299–8257)	All	22 (3–46)	2.19	23 (10–46)
12	All	1885 (363–554)	All	49 (3–105)	1.59	63 (34–98)
13	All	2189 (13–3825)	All	27 (5–48)	1.91	47 (14–84)
14	All	4422 (465–9360)	All	91 (30–167)	1.68	341 (68–890)
15	All	3607 (1867–6471)	All	48 (3–151)	1.87	368 (40–1226)
16	All	5866 (234–10845)	All	81 (11–161)	1.86	74 (12–154)
17	All	3486 (2031–4761)	All	30 (13–78)	2.07	220 (154–304)
18	All	2587 (1797–3297)	All	48 (13–129)	1.73	41 (16–80)
19	All	6606 (5646–7816)	All	30 (5–67)	2.34	111 (18–164)

20	All	438 (102–398)	All	38 (16–86)	1.06	67 (26-102)
21	All	7093 (2665–9833)	All	97 (8–266)	1.86	508 (74-1094)
22	All	1420 (452–3271)	All	27 (8–56)	1.72	130 (58-190)
23	All	1810 (643–3410)	All	38 (8–67)	1.68	77 (44-142)

[a] All: samples from all four seasons were positive

Table 3
Click here to download Table: Table 3.docx

Table 3. *Cryptosporidium* oocyst counts [arithmetic mean and ranges (minimum and maximum)] and removal efficiency (removal log) of oocysts in wastewater treatment plants in north-eastern Spain. Samples from influent and effluent wastewater and sewage sludge were collected four times from each plant, each sampling time matching with a different season.

Plant	Influent		Effluent		Removal log	Sewage sludge
	Positive season[a]	Oocysts / litre	Positive season[a]	Oocysts / litre		Oocysts/gram
1	All	128 (17–362)	All	2 (0.5–6)	1.80	9 (8–10)
2	All	134 (22–445)	All	8 (0.5–17)	1.22	10 (2–18)
3	W, SM, SP	28 (22–33)	W, SM, SP	2 (1–3)	1.14	0
4	All	78 (22 –206)	All	6 (1–12)	1.11	9 (2–20)
5	All	22 (17–28)	W, SM, A	2 (1–3)	1.04	6 (4–8)
6	All	32 (2–83)	W, SM, A	34 (6–78)	− 0.02	15 (2–36)
7	All	456 (83–1041)	All	200 (11–573)	0.35	36 (2–60)
8	All	6 (6–7)	W, SM	0.5 (0.5–0.8)	1.07	4
9	W, SM, A	50 (17–61)	All	11 (2–39)	0.65	2
10	W, SM, SP	45 (6–111)	W, SM, SP	6 (0.5–17)	0.87	44 (12–76)
11	W, SM, SP	89 (6–228)	W, SM, SP	39 (0.5–117)	0.35	14
12	All	223 (11–857)	W, SM, SP	11 (0.5–33)	1.30	44 (12–76)
13	All	256 (11–929)	All	301 (45–1157)	− 0.07	9 (4–14)
14	All	72 (22–990)	All	17 (2–57)	0.62	5 (4–8)
15	All	72 (11–211)	W, SM, A	28 (2–57)	0.41	6 (4–8)
16	W, SM	256 (250–262)	W, SM	6 (6–11)	1.63	2
17	All	39 (11–89)	All	11 (2–28)	0.54	8 (2–16)
18	All	39 (11–100)	W, SM, A	8 (0.5–22)	0.68	4
19	All	45 (33–56)	All	6 (0.5–13)	0.87	0

20	W, SM, SP	22 (11–39)	All	4 (2–6)	0.74	0
21	All	56 (22–106)	All	19 (0.5–47)	0.46	8 (4–10)
22	All	33 (17–72)	W, SM, SP	3 (1–6)	1.04	3 (2–4)
23	All	28 (6–56)	W, SM, SP	1 (0.5–2)	1.44	4

[a] SP: Spring; SM: summer; A: autumn; W: Winter

Table 4. ~~Frequency~~ Number of assemblages/sub-assemblages ~~among~~ within *G. duodenalis* isolates from wastewater samples determined by genotyping at the β-giardin (*bg*) and triose phosphate isomerase (*tpi*) genes.

Assemblage / Sub-assemblage	Number of isolates		
	bg	***tpi***	**Combined results**
AII	39	27	28
B	2	17	3
AII+B	-	-	15
E	1	-	-
B + E	-	-	1

Table 5
Click here to download Table: Table 5.docx

Table 5. Seasonal distribution of *Cryptosporidium* species and subtypes in wastewater samples. The molecular characterization was based on sequencing of SSU rRNA and GP60 genes, and a species-specific multiplex genotyping real-time PCR targeting the SSU rRNA and Lib13 loci.

	Nº plants (n = 23)			
***Cryptosporidium* spp.**	**Winter**	**Summer**	**Autumn**	**Spring**
C. parvum	7	9	4	5
C. hominis	2	7		
C. cuniculus	1			
C. ubiquitum	2			
C. galli			1	
C. parvum + *C. hominis*	1	3	1	
C. parvum + *Cryptosporidium* sp.*	1			
C. parvum + *C. canis*	1			
C. parvum + *C. andersoni*		1		
C. hominis + *C. muris*	1			
C. canis + *C. suis*	1			
C. galli + *C. andersoni*			1	
Not identified	6	3	12	17
***C. parvum* / *C.hominis* GP60 subtypes**				
IIaA15G2R1	3	6	1	2
IIaA16G2R1			2	1
IIaA18G3R1		1		
IIdA21G1	1			
IIdA22G1	1			
IbA10G2	2	1		

* 100% sequence identity to *Cryptosporidium* AY737585 from GenBank

Figure 1

Click here to download high resolution image

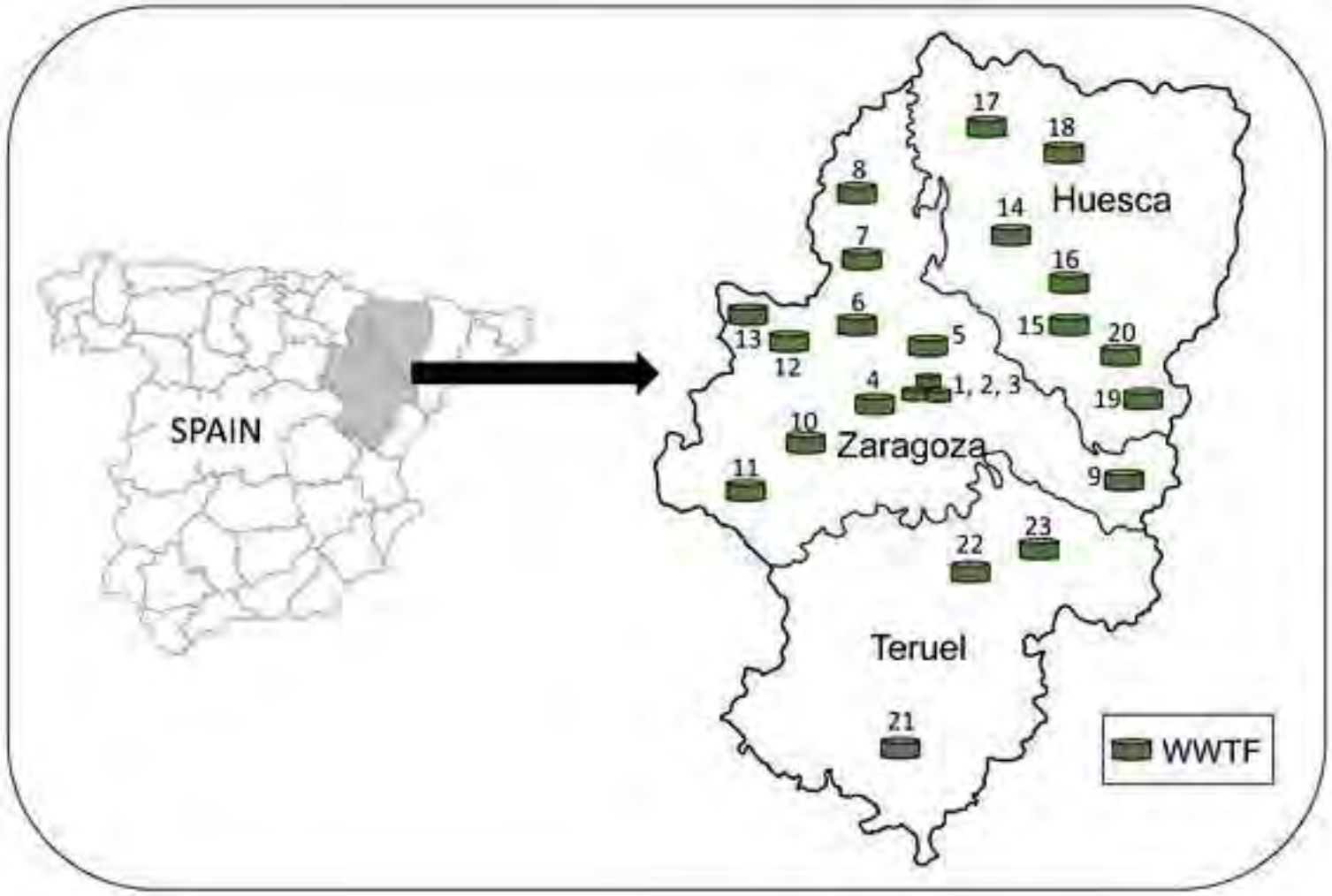

Figure 2

Click here to download high resolution image

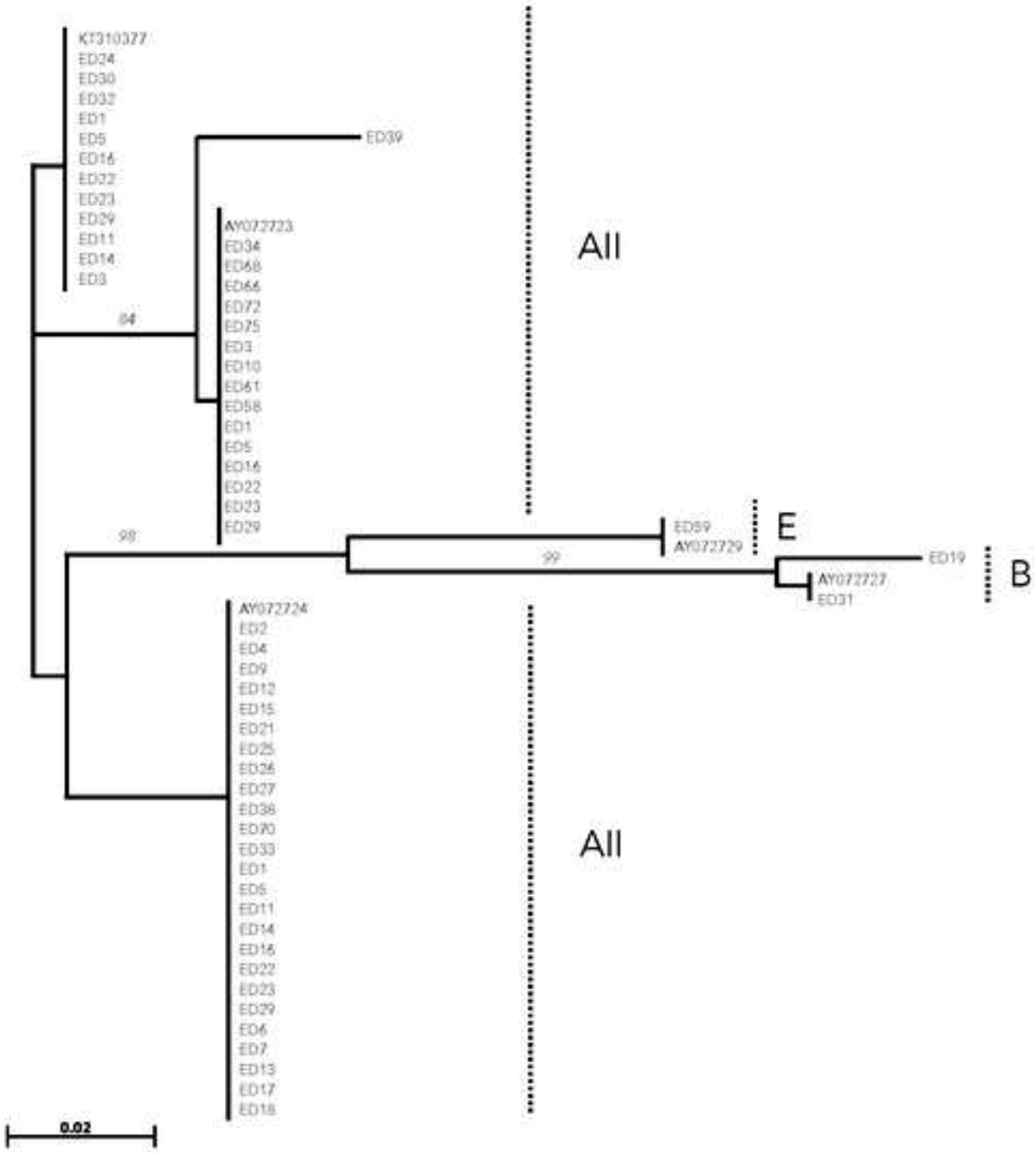

Figure 3

Click here to download high resolution image