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# A year-long study of *Cryptosporidium* species and subtypes in recreational, drinking and wastewater from the central area of Spain

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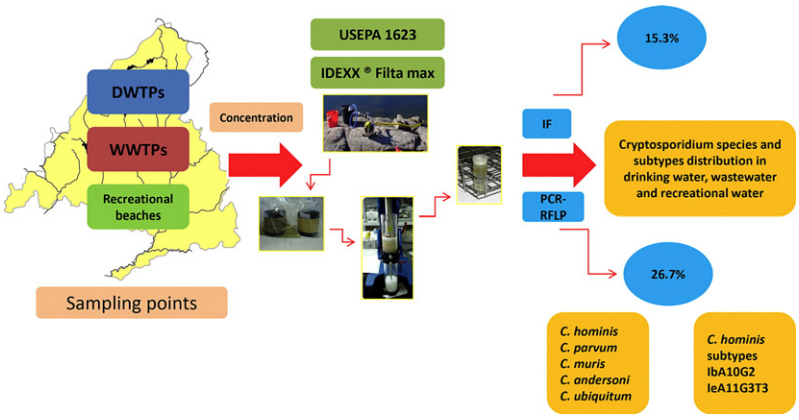
## HIGHLIGHTS

- First study on *Cryptosporidium* presence in water from the central area of Spain.
- *Cryptosporidium* species were identified at the molecular level.
- The IbA10G2 subtype closely associated to waterborne outbreaks was detected.
- The IeA11G3T3 subtype is described for the first time in water samples.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A year-long longitudinal study was undertaken to evaluate the presence of *Cryptosporidium* spp. in drinking water treatment plants (DWTPs), wastewater treatment plants (WWTPs) and freshwater bathing beaches (FBBs) from the central area of Spain. Water samples were collected according to USEPA Method 1623, and concentrated by the IDEXX Filta-Max® system. *Cryptosporidium* species were detected based on PCR-restriction fragment length polymorphism and sequence analyses of the ssuRNA gene. *C. hominis* and/or *C. parvum* isolates were subtyped by DNA sequencing of the Gp60 gene. Among 150 samples, 23 (15.3%) were positive by IFAT and 40 (26.7%) by PCR. *Cryptosporidium* spp. was more frequent in WWTPs (26.2 and 50.8%) and FBBs (12.5 and 17.5%) by IFAT and PCR respectively. Effluent waters from DWTPs were negative for this parasite suggesting that they are suitable for public use. Tertiary treatment in the WWTPs demonstrated a high removal efficiency of *Cryptosporidium* in the samples evaluated. *Cryptosporidium* species identified included *C. hominis*, *C. parvum*, *C. ubiquitum*, *C. andersoni* and *C. muris*. Subtyping analysis revealed *C. hominis* IbA10G2 and IeA11G3T3 alleles, which is the first report of the latter in water samples. *Cryptosporidium* highest frequency was observed in winter and spring. Our data provide information about the occurrence and diversity of *Cryptosporidium* in water of human use from the central area of Spain.



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## 1. Introduction

Waterborne parasitic protozoan infections have a worldwide distribution and are, in both developed and developing countries, a source for epidemic and endemic human diseases (Baldursson and Karanis, 2011). Interest in the contamination of water by enteric pathogenic protozoa has increased considerably during the past three decades and a number of human protozoan parasitic infections have been reported to be transmitted by the waterborne route (Baldursson and Karanis, 2011). *Cryptosporidium* species have emerged as major waterborne pathogens causing gastroenteritis in humans (Baldursson and Karanis, 2011). They are apicomplexa parasites that infect a variety of vertebrates including mammals, birds, reptiles, amphibians, and fish (Fayer, 2010). Currently, there are 26 valid *Cryptosporidium* species (Chalmers and Katzer, 2013). Six of them are associated as the most common important causes of human cryptosporidiosis: *C. hominis* and *C. parvum*, followed by *C. meleagridis* and occasionally *C. cuniculus*, *C. felis* and *C. canis* (Chalmers and Katzer, 2013). Cryptosporidiosis is commonly associated with enteritis and is usually characterized by acute, watery, or steatorrheic diarrhea (Putignani and Menichella, 2010). Asymptomatic infection can occur but in immunocompromised patients cryptosporidiosis may be severe, chronic, and life-threatening (Putignani and Menichella, 2010).

*Cryptosporidium* waterborne transmission is attributed to a number of factors that enable this parasite to survive in the environment and disseminate by water (Carmena, 2010). It has a life-cycle within a single host that excretes large numbers of immediately infective oocysts in faeces. Zoonotic transmission can occur, enhancing both the reservoir of infection and environmental contamination. *Cryptosporidium* also has small thick-walled oocysts with a low specific gravity, making them environmentally robust and facilitating their dissemination by water. Furthermore, their resistance to commonly used disinfectants in the water industry is well documented (Fayer, 2004). Outbreaks have been reported worldwide, which have been attributed to a combination of disinfectants resistance of oocysts and water treatment deficiencies that allow the passage of sufficient numbers of protozoa to cause illness (Baldursson and Karanis, 2011). Because of its waterborne transmission potential, *Cryptosporidium* has been included in the drinking water contaminant list of the U.S. Environmental Protection Agency-EPA.

Data regarding *Cryptosporidium* prevalence in the Spanish general population are scarce. There are several studies on immunocompetent population including adults and children (Clavel et al., 1996a, 1996b; Martin-Ampudia et al., 2012; Olivares et al., 2002), and also immunosuppressed individuals (Clavel et al., 1996a; Lopez-Velez et al., 1995; Navarro-i-Martinez et al., 2011). *Cryptosporidium* outbreaks have also been documented, most of them involving children (Artieda et al., 2012; Ortega et al., 2006; Rodriguez-Salinas et al., 2000) and travelers in contact with swimming pools (Galmes et al., 2003; Smerdon, 2000). Several studies have also described *Cryptosporidium* spp. in environmental samples, including drinking water (Carmena et al., 2007; Castro-Hermida et al., 2008b, 2010, 2011), wastewater (Alonso et al., 2011; Castro-Hermida et al., 2008a, 2010, 2011), recreational (Carmena et al., 2007; Castro-Hermida et al., 2008a, 2010) and surface waters (Castro-Hermida et al., 2009), sludge and biosolids (Alonso et al., 2011; Guzman et al., 2007; Reinoso and Becares, 2008). It is important to note that this protozoan has also been detected in animals including wild animals, cattle, pigs and dogs (Navarro-i-Martinez et al., 2011). All these data suggest a recurrent exposure of our population to *Cryptosporidium* and therefore imply that cryptosporidiosis could be a potential threat to public health in Spain.

Taking the above into account, and that to our knowledge there are no published data on *Cryptosporidium* occurrence in waters from the central area of Spain, this study was undertaken to evaluate the presence of *Cryptosporidium* during a 1-year survey in water samples from several drinking water treatment plants (DWTPs), wastewater treatment plants (WWTPs) and freshwater bathing beaches (FBBs) from the Autonomous Region of Madrid (Spain) (Fig. 1). *Cryptosporidium* species and subtypes were also identified.

## 2. Material and methods

### 2.1. Study area description

Autonomous Region of Madrid (ARM) is located at the centre of Iberian Peninsula, at the Castilian Central Plateau. It has a surface area of approximately 8028 km and a population of more than 6 million people, mostly concentrated at the metropolitan area of Madrid. ARM has a temperate continental Mediterranean climate with cold winters with temperatures sometimes dropping below 0 °C. The service, construction, and industry sectors are prominent in ARM commercial productive structure. Farm and livestock activities are less important. Currently, ARM has 13 drinking treatment plants (DWTPs) and 150 wastewater treatment plants (WWTPs) that provide service to the population. DWTPs supply system is mainly based on taking water from the seven rivers in the Sierra de Guadarrama Mountains: Alberche, Guadarrama-Aulencia, Guadalix, Manzanares, Lozoya, Jarama and Sorbe. Twenty-three WWTPs have implemented a tertiary treatment of the final effluents in their installations. Reclaimed waters generated by this treatment are suitable for public use like irrigation of green areas such as parks and golf courses. ARM has 5 fresh water bathing beaches (FBBs) officially registered, which are only used for recreational purposes, especially during the spring and summer time.

### 2.2. Sampling methodology

A year long-longitudinal study from autumn 2010 to summer 2011 was designed to evaluate and characterize *Cryptosporidium* in drinking water, wastewater and recreational water from ARM. Eleven sampling points that cover 5 river basins from this region were selected; including 3 DWTPs, 3 WWTPs, and 5 FBBs (Fig. 1). Sampling was done in duplicate in every season throughout the year. For DWTPs, up to 100 liters of untreated water (influent) and treated water (final effluent) was collected. For both WWTPs and FBBs, up to 50 liters of water was collected. In every WWTP, we analysed the influent and final effluents and also the reclaimed water. A total of 150 water samples were collected using a portable water pump connected to a foam filter module, following the manufacturer's instructions and the 1623 method used by the United States Environmental Protection Agency (USEPA) (EPA, 2005). Then, samples were concentrated using the IDEXX® Filta Max system according to manufacturer's instructions and the USEPA 1623 method. Recovery efficiency was determined by spiking 25 L of reagent water with *Cryptosporidium* oocysts according to the instructions of the USEPA 1623 method. This procedure was repeated four times. The mean oocyst recovery was 35.2 ± 7%, which meets the acceptance criteria described in this method. A total of 7 ml was finally eluted from each concentrated sample and fractioned for several analyses. Samples for molecular analysis were kept at −80 °C.

### 2.3. Immunofluorescence assay staining (IFAT) for recovered Cryptosporidium oocysts

Water samples were processed according to USEPA method 1623. *Cryptosporidium* oocysts were isolated by immunomagnetic separation (IMS) using the Dynal IMS procedure (Dynabeads® anti-*Cryptosporidium* kit; Invitrogen Dynal AS, Oslo Norway), and detected by epifluorescence and differential interference contrast microscopy after staining with fluoresceine isothiocyanate (FITC)-labelled monoclonal antibody (Aqua-Glo™ G/C Direct kit, New Orleans) and the nucleic acid dye 4′,6′-diamino-2-phenyl indole (DAPI) (Aqua-Glo™ G/C Direct kit, New Orleans). Oocyst concentration was calculated taking into account the mean recovery percentages of *Cryptosporidium* spp. oocysts obtained in our laboratory (35.2 ± 7%) and the volume of filtered water. Positive