Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *Eimeria* spp.

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ABSTRACT

The microbial diversity of wastewater used for irrigation and fertilization was assessed using specific polymerase chain reaction (PCR) assays to detect and genotype several pathogenic protists including *Cryptosporidium* spp., *Giardia duodenalis*, *Cyclospora* spp., *Eimeria* spp. and *Enterocytozoon bieneusi*. A total of 220 wastewater samples (110 raw, 110 treated) and 12 sludge samples were collected from 2005 to 2008 from 18 treatment plants located throughout Tunisia. Except for *Cyclospora*, which was detected only once, *E. bieneusi* (61%), *G. duodenalis* (28%), *Cryptosporidium* spp. (27%) and *Eimeria* spp. (45%) were frequently observed in wastewater and sludge. Sequencing of PCR products showed that *C. hominis*, *C. andersoni*, *G. duodenalis* sub-assemblage A-II and *E. bieneusi* genotypes D and IV were the most prevalent. An analysis of the distribution of 209 internal transcribed spacer sequences of *E. bieneusi* originating from wastewater at the 18 treatment plants showed a similar genetic diversity, regardless of the geographical location. The identification of these parasite species and genotypes and of host-specific *Eimeria* species indicates that the microbial quality of wastewater was impacted by humans, livestock and rodents. Given the public health risks that some of these parasites represent, guidelines on wastewater usage are needed to minimize human exposure to these pathogens.

Key words | *Cryptosporidium*, *Enterocytozoon bieneusi*, genotypes, *Giardia duodenalis*, Tunisia, wastewater

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INTRODUCTION

As a result of increased human consumption and irregular or diminishing rainfall, Tunisia is experiencing a growing shortage of water. To address this problem, the government is encouraging the reuse of treated wastewater (TWW). Water reuse for agriculture is increasing, with almost 30% of the total volume of TWW used to irrigate about 9,300 hectares (ONAS 2008). The reuse of sewage or sludge in agriculture may facilitate the dissemination of parasites and the contamination of source water via surface runoff, raising public health concerns (Rimhanen-Finne *et al.* 2004). It is therefore important to assess the safety and microbial quality of wastewater in Tunisia.

Tunisian regulations governing the use of TWW and sludge (NT 106.03 and 106.20, respectively; www.onas.nat.tn) define acceptable levels of specific chemicals, bacteria and helminth ova. However, there are no specifications for protists pathogenic to humans, such as *Cryptosporidium* and *Giardia*, which are responsible for large numbers of gastrointestinal infections (Karanis *et al.* 2007; Yoder & Beach 2010). Results of previous studies of wastewater and sludge

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in Tunisia showed a more frequent occurrence of *Giardia* and *Entamoeba* cysts than helminth ova (Ben Ayed *et al.* 2009, 2010). These observations are in agreement with the high prevalence of these parasites in humans. In the Tunis region, the prevalence of *Giardia duodenalis* and *Cryptosporidium* in diarrheic persons was found to be 46.2 and 8%, respectively (Bouratbine *et al.* 2000). Approximately 16% of immuno-compromised patients had cryptosporidiosis (Bouratbine *et al.* 2000). In contrast, the prevalence of helminth infections was estimated to be low; 0.03% for *Ascaris lumbricoides* (Chaker *et al.* 1995) and 10.3% for *Enterobius vermicularis* (Bouratbine *et al.* 2000).

Cryptosporidium and *Giardia* can cause severe gastrointestinal illnesses. *Cryptosporidium* infections have been associated with stunting and malnutrition in young children (Chekley *et al.* 1997, 1998). They also have an adverse impact on growth and survival of neonatal calves and on milk production (Estban & Anderson 1995; Olson *et al.* 2004).

Cyclospora cayetanensis is recognized as a food- and waterborne parasite, and is transmitted by environmentally robust oocysts (Ortega & Sanchez 2010). Most outbreaks of *Cyclospora* have been associated with food-borne transmission and have occurred in industrialized countries (Thompson *et al.* 2003; Yoder & Beach 2007); water has however been identified as a route of transmission in developing countries, particularly in South America and Nepal (Shields & Olson 2003). Although related to *Cyclospora*, *Eimeria* spp. commonly infect domestic and wild animals and are not pathogenic to humans. *Enterocytozoon bieneusi* is the most common microsporidian species infecting humans (Didier 1998), domestic animals, wild mammals and birds (Ghosh & Weiss 2009; Santin & Fayer 2011), and is transmitted through the fecal-oral route. Spores of this fungal pathogen have been detected in surface water (Sperfel *et al.* 1997; Dowd *et al.* 1998) and swimming pools (Fournier *et al.* 2002).

In the present study, polymerase chain reaction (PCR) was used to evaluate the occurrence and geographical distribution of *Cryptosporidium* spp., *Cyclospora.* spp., *Eimeria* spp., *G. duodenalis* and *E. bieneusi* in raw wastewater (RWW), TWW and sludge. Results of this survey document a high prevalence and genetic diversity of these parasites in wastewater and sludge in Tunisia.

MATERIALS AND METHODS

Study sites

A total of 110 samples of RWW, an equal number of TWW samples and 12 sludge samples (four dry and eight dehydrated) were collected monthly between 2005 and 2008 from 18 wastewater treatment plants located throughout Tunisia. Seven of the plants were located in the district of Tunis (Charguia, Chotrana, Cotière Nord, Grombalia, Kalaat el Andalous, Mornag and Sud Méliane), six in the costal and tourist region (Kélibia, Mahdia, Nabeul, Slimane, Sousse Nord and Sousse Sud), three in the southern part of the country (Gafsa, Sfax and Sidi Bouzid), one in the center of the country (Kairouan) and one in the Sahel region (Moknine) (Figure 1). The main characteristics and the treatment process for each plant are listed in Table 1. These plants were selected mainly based on: (1) the final destination of the effluents (surface or sea waters, agricultural reuse, green space irrigation, etc.); (2) prior evidence of intestinal parasites in the local population (Bouratbine *et al.* 2000; Fathallah *et al.* 2004); (3) treatment process (activated sludge, waste stabilization pond); and (4) diversified geographical representation.

Sample collection and processing

Samples of 5 L of RWW or TWW were collected over a 24 h period with an automatic sampler, whereas 100 g grab samples of sludge were collected. Upon arrival in the laboratory, wastewater samples were sedimented for 24 h at room temperature. The supernatant was removed and the sediment was centrifuged at $1{,}000 \times g$ for 15 min. The final volume of sediment was about 10 mL. About 2 mL of sediment was stored in 2.5% potassium dichromate solution at 4 °C for the detection of targeted protists by PCR. Prior to DNA extraction, suspended solids were washed in water by centrifugation to remove the potassium dichromate.

DNA extraction

DNA was extracted from 0.5 mL of wastewater sediment or 0.5 g of dry or dehydrated sludge using the FastDNA SPIN

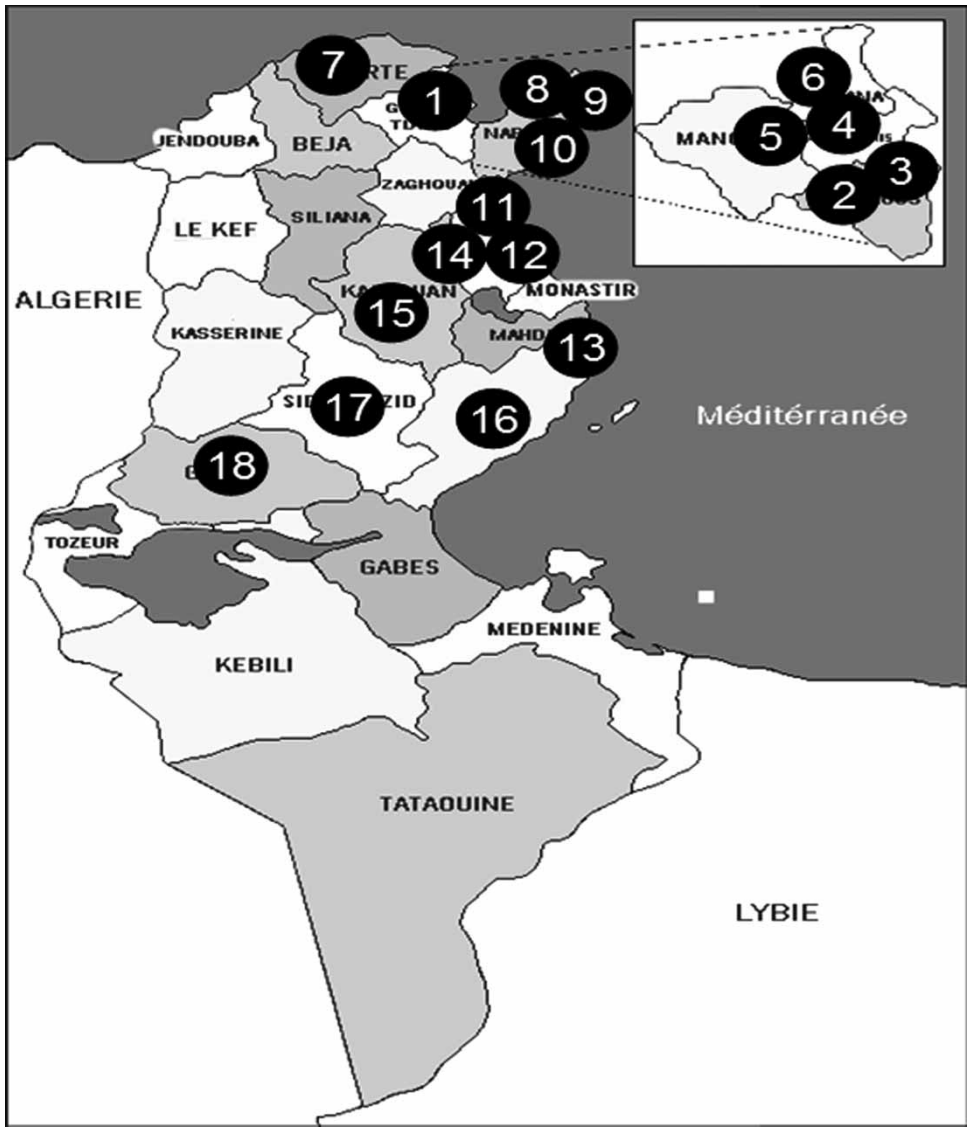

Figure 1 | Geographical location of 18 wastewater treatment plants. 1–6: Tunis metropolitan region; 7–13: coastal/tourist region; 14: Sahel region; 15: center; 16–18: south.

Kit for Soil (Q-Biogene, Irvine, CA) as described by Jiang *et al.* (2005). DNA was eluted into 100 μL of reagent-grade water and stored at –80 °C until analyzed by PCR. Each sample was analyzed at least five times using 2 μL of extracted DNA for each PCR target. The sample was considered positive if at least one reaction was positive. Following analysis with the nested PCR protocol described below, secondary PCR products were detected by gel electrophoresis on 1.5% agarose. Although a positive PCR outcome is not indicative of infectivity, the fact that DNA extracted from the precipitate was used supports the view that a positive PCR result indicates the presence of intact spores, oocysts and cysts.

Cryptosporidium detection, genotyping and subtyping

A fragment of approximately 830 bp of the small subunit (SSU) rRNA gene was amplified by nested PCR as described by Jiang *et al.* (2005). To identify the *C. parvum* and *C. hominis* subtypes, an approximately 850-bp product of the 60 kDa

Table 1 | Characteristics of treatment plants sampled in the study

		Treatment			
Plant	**Loc**	**Primary**	**Secondary**	**Disinfection**	**Final destination**
Charguia	DT	PD	AS	–	Irrigation, sea
Chotrana	DT				Irrigation, sea
Cotière Nord	DT	–	WSP	–	Irrigation, sea
Grombalia	DT	–	AS	–	Surface water
Kalaat El Andalous	TCA	–	WSP	–	Irrigation, sea
Kélibia	TCA	–	AS	UV	Surface water
Mahdia	TCA	–	WSP	UV	Irrigation, sea
Nabeul SE4	TCA	PD	AS	–	Surface water, recharge
Sousse Sud	TCA	PD	AS + BB	–	Surface water
Gafsa	SO	–	WSP	–	Irrigation
Sfax	SO	PD	AS	–	Irrigation, sea
Mornag	DT	PD	AS	UV	Irrigation, sea
Sud Méliane	DT	–	AS	–	Surface water, sea
Sidi Bouzid	SO	–	WSP	–	Surface water
Slimane I	TCA	–	AS	–	Irrigation, sea
Kairouan	C	–	AS	–	Irrigation, surface water, sea
Sousse Nord	TCA	PD	AS	–	Irrigation, sea
Moknine	SA	–	WSP	–	Irrigation, surface water

Loc: location; DT: District of Tunis; TCA: tourist and coastal area; C: central; N: north; SO: south; SA: Sahel; PD: primary decantation; AS: activated sludge; BB: bacterial bed; WSP: waste stabilization pond; UV: ultraviolet light.

glycoprotein (gp60) gene was amplified by nested PCR (Alves *et al.* 2003). The secondary PCR products were sequenced using the secondary PCR primers and an intermediary sequencing primer (5′-GAGATATATCTTGTTGCG-3′). The established subtype nomenclature (Sulaiman *et al.* 2005) was used to classify the gp60 subtypes.

Detection and genotyping of *G. duodenalis*

To detect and genotype *G. duodenalis*, a 530-bp fragment of the triosephosphate isomerase (tpi) gene was amplified by nested PCR (Sulaiman *et al.* 2003a). Likewise, to detect and genotype *E. bieneusi*, a fragment of about 392 bp of the ribosomal internal transcribed spacer (ITS) was amplified by nested PCR (Sulaiman *et al.* 2003b). The secondary PCR products of *G. duodenalis* and *E. bieneusi* were sequenced. The ITS genotypes of *E. bieneusi* were named according to the established nomenclature (Santin & Fayer 2009).

Detection and identification of *C. cayetanensis* and *Eimeria* spp.

To detect *C. cayetanensis*, a 294-bp fragment of the SSU rRNA gene was amplified by nested PCR (Ortega *et al.* 1993). Because these primers also amplify DNA of *Eimeria* spp., the identification of *C. cayetanensis* was confirmed by sequencing the secondary amplicons. *Eimeria* spp. were also targeted because they form monophyletic host-specific groups in sequence analyses, which was good for contamination source tracking.

DNA sequencing and sequence analysis

Secondary PCR products were purified using the Multiscreen PCR Plate (Millipore, Bedford, MA) and sequenced in both directions using the BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA).

To confirm the accuracy of the sequence and to detect multiple genotypes and subtypes in a sample, all positive PCR products from each sample were sequenced, except for *Cyclospora/Eimeria* for which only two positive products per sample were sequenced. The genetic diversity of *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Cyclospora/Eimeria* spp. was examined by aligning the nucleotide sequences with those found in GenBank using ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Genotypes were classified by comparing each sequence with reference sequences.

Meta-analysis of protist sequences

Given that the wastewater survey included treatment plants distributed over most of the country, protist species and genotypes were analyzed to identify geographical patterns in the occurrence and genetic diversity of different species and genotypes. Sequences were grouped into 15 taxa (*C. hominis*, *C. parvum*, *C. andersoni*, other *Cryptosporidium*, *G. duodenalis* A-I, *G. duodenalis* A-II, *G. duodenalis* B, other *Giardia*, *E. bieneusi* Peru8, *E. bieneusi* D, *E. bieneusi* IV, other *E. bieneusi*, ruminant *Eimeria*, poultry *Eimeria* and other *Eimeria*) and the presence/absence of each group in 111 samples tabulated. Pair-wise Jaccard distances (Jaccard 1908) between all pairs of taxa were calculated and taxa hierarchically clustered by similarity. The resulting tree was examined for the possible co-occurrence of protozoan species/genotypes with similar host range.

Parasite presence/absence data were analyzed for the co-occurrence of any *G. duodenalis* genotype and *Cryptosporidium* genotype, as well as the co-occurrence of *Cryptosporidium* and *E. bieneusi*, and *Cryptosporidium* species and *Eimeria* species. A Chi-square test was applied to test for any significant association between these species.

After eliminating ITS sequences with ambiguous positions, 209 *E. bieneusi* sequences were retained and numbered such that each unique sequence was assigned a different number ($n = 44$). Likewise, after removing sequences with ambiguous positions, 191 small-subunit rRNA sequences of *Eimeria/Cyclospora* were aligned and numbered such that each unique sequence ($n = 43$) was assigned an individual number. The diversity of the *E. bieneusi* ITS marker was compared to a previously published global collection of 135 ITS sequences (Widmer & Akiyoshi 2010) with individual-based rarefaction analysis (Gotelli & Colwell 2001) using the program EstimateS (http://viceroy.eeb.uconn.edu/estimates). Heatmaps representing *E. bieneusi* ITS genotype abundance were drawn with a Microsoft Excel macro downloaded from http://homepage.mac.com/yabyab/my.html.

The ITS sequence diversity of *E. bieneusi* was displayed as a phylogenetic tree using the Neighbor-Joining clustering method (Saitou & Nei 1987). Sequences from a diversified global collection ($n = 135$) and the present study ($n = 209$) were pooled, aligned using ClustalX and imported into Mega 4.0 software (Tamura *et al.* 2007). A bootstrap consensus tree was drawn based on 500 replicates. The percentage of replicate trees in which a specific cluster was found is indicated in the plot. Rank-abundance plots (Whittaker 1965) were drawn to visualize the diversity of *E. bieneusi* genotypes among nine plants that had provided a minimum of 10 complete ITS sequences, and a same number of plants from which a minimum of nine *Eimeria* SSU rRNA sequences were obtained. The aim of this analysis was to assess whether treatment plants differed with respect to the genetic diversity of parasites. These two parasites were selected because of their extensive genetic diversity. The evenness of *E. bieneusi* ITS diversity was quantified using Pielou's J' ratio (Pielou 1969). This measure expresses the relative evenness as compared to maximum evenness which is found when all sequences have the same abundance. Treatment plants ($n = 16$) contributing a minimum of four sequences were ranked from north to south to assess whether geography and environment would impact *E. bieneusi* genotype evenness.

Nucleotide sequence accession numbers

Unique nucleotide sequences were deposited in GenBank under accession numbers EU140501–EU140508 and JF909981–JF910012.

RESULTS

Frequency and distribution of *Cryptosporidium* spp. in wastewater and sludge

Based on PCR analysis of the SSU rRNA gene, 42 of 110 (38%) RWW, 14 of 110 (13%) TWW, and five of 12 (33%)

Table 2 | Parasites detected in raw and treated wastewater by treatment plant

Plant	Types of sample	# Sample	# Cry positive	# *Giardia* positive	# Eb positive	# Ei positive
Charguia	RWW/TWW	8/8	3/1	4/1	7/2	7/0
Chotrana	RWW/TWW/DeS	1/1/3	1/0/2	1/0/1	0/0/2	0/0/1
Cotière Nord	RWW/TWW	5/5	3/0	5/1	4/3	4/0
Grombalia	RWW/TWW	6/6	3/0	2/1	5/2	3/0
Kalaat El Andalous	RWW/TWW	28/28	7/5	8/2	24/12	22/2
Kélibia	RWW/TWW	4/4	0/0	1/1	3/1	2/0
Mahdia	RWW/TWW	4/4	3/1	0/0	3/2	2/0
Nabeul SE4	RWW/TWW/DeS	25/25/1	10/2/0	9/4/0	18/13/1	17/7
Sousse Sud	RWW/TWW/DeS	4/4/1	2/0/0	2/0/0	3/2/1	4/2
Gafsa	RWW/TWW	5/5	2/2	3/0	3/2	5/2
Sfax	RWW/TWW	6/6	0/0	3/1	4/3	4/1
Mornag	RWW/TWW/DrS	4/4/4	1/1/1	2/2/1	3/2/1	4/1/4
Sud Méliane	RWW/TWW/DeS	3/3/3	2/1/2	2/2/1	2/1/3	1/3/2
Sidi Bouzid	RWW/TWW	2/2	0/1	1/0	2/1	1/1
Slimane I	RWW/TWW	1/1	1/0	0/0	1/0	1/0
Kairouan	RWW/TWW	2/2	2/0	2/0	2/1	2/0
Sousse Nord	RWW/TWW	1/1	1/0	1/0	1/1	1/0
Moknine	RWW/TWW	1/1	1/0	1/0	1/1	1/0
TOTAL	RWW/TWW/sludge	110/110/12	42/14/5	47/15/3	86/49/8	81/19/7

Cry: *Cryptosporidium* spp; DeS: Dehydrated sludge; DrS: Dry sludge; Eb: *Enterocytozoon bieneusi*, Ei: *Eimeria*.

sludge samples were positive for *Cryptosporidium* spp. (Tables 2 and 3). Altogether, 16 of the 18 plants sampled were positive for *Cryptosporidium* spp., including RWW samples from 15 plants and TWW samples from eight plants (Table 2). Sequencing of PCR products revealed the presence of *C. parvum*, *C. muris*, *C. andersoni*, *C. hominis*, *C. ubiquitum*, rat genotype IV, an unknown *Cryptosporidium*, sheep genotype, *C. meleagridis* and avian genotype II (Table 4). With 47 positive PCR products from nine wastewater treatment plants, *C. andersoni* was the most prevalent species. Both the A and B copy of the gene, which differ by one T indel (Nagano *et al.* 2007), were found in RWW and TWW samples. The second-most common species was *C. hominis* (22 positive products), which was detected in RWW samples from 10 different plants and in one TWW sample collected from the plant of Charguia. In addition, 19 PCR products were positive for *C. parvum*, 11 for *C. muris*, six for *C. ubiquitum*, two for *C. meleagridis* and one each for the rat genotype IV, sheep genotype and a new genotype (Table 4). The sequence of the new *Cryptosporidium* genotype

Table 3 | Frequency of *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Eimeria* spp./*Cyclospora cayetanensis* in wastewater and sludge samples in Tunisia

			Cryptosporidium spp.		*Giardia duodenalis*		*Enterocytozoon bieneusi*		*Eimeria/Cyclospora* spp.	
Sample type	No. of plants sampled	Sample size	Positive plants	Positive samples	Positive plants	Positive samples	Positive plants	Positive samples	Positive plants	Positive samples
Raw wastewater	18	110	15	42	16	47	17	86	17	81
Treated wastewater	18	110	8	14	9	15	16	49	8	19
Dry sludge	1	8	1	1	1	1	1	1	1	4
Dehydrated sludge	4	4	2	4	2	2	4	7	2	3

Table 4 | *Cryptosporidium* species and genotypes in 232 wastewater and sludge samples

Species, genotype and gp60 subtype	No. of positive plants	No. of positive PCR products
Humans and ruminants		95
C. hominis (AF093491)	10	22
IaA26R3	1	1
IaA27R4	1	1
IdA14	2	3
C. parvum (AF093490)	10	19
IIaA15G2R1	1	1
IIaA17G2R1	1	1
IIaA18G3R1	1	1
IIaA20G2R1	1	1
IIaA21R1	1	2
IIaA21G2R1	2	2
IIcA5G3b	1	1
C. andersoni (AF093490)	9	47
Sheep genotype (AY898790)	1	1
C. ubiquitum (AF262328)	4	6
Rodents		12
C. muris (AF093498)	4	11
Rat genotype IV (AY737581)	1	1
Birds		3
C. meleagridis (AF112574)	1	2
Avian genotype II (DQ8853402)	2	4
New genotype	1	1

was similar to sequences of *C. baileyi* (DQ650339) and the avian genotype I (DQ885340) and could therefore be of avian origin. In 14 samples, more than one *Cryptosporidium* genotype was found; nine had two genotypes and five had three.

C. hominis and *C. parvum* gp60 subtypes

Fourteen of the *C. hominis*- or *C. parvum*-positive samples were successfully subtyped by sequence analysis of the gp60 gene (Table 4). This analysis showed the presence of the *C. hominis* gp60 subtype families Id (3 sequences of 1 subtype) and Ia (two sequences of two subtypes) and *C. parvum* subtype families IIa (eight sequences of six subtypes) and IIc (one sequence).

G. duodenalis genotypes

Sixty-five (28%) of 232 wastewater and sludge samples were positive for *G. duodenalis* by tpi PCR (Table 3), including RWW samples from 16 plants and TWW samples from nine plants (Table 2). For one collection each from Mornag and Kélibia, the TWW sample was positive despite a negative matching RWW sample as also observed with *Cryptosporidium*.

DNA sequence analysis of the PCR products revealed the presence of three known genotypes of *G. duodenalis* including assemblages A, B and E (Table 5). The most prevalent genotype was assemblage A, which was detected in 86 PCR products, followed by assemblage B in 12 PCR products. Assemblage E was detected in one sample (Table 5). Most of the assemblage A samples ($n = 84$ PCR products) had the A2 subtype; A1 was detected twice.

E. bieneusi genotypes

E. bieneusi was detected in 143 (62%) of 232 samples (Tables 2 and 3). In 2005, 61% of the samples were positive (5/7 RWW, 2/7 TWW, 1/2 dry sludge and 3/3 dehydrated sludge). In 2006, 44% of the samples showed the presence of *E. bieneusi* (38/57 RWW, 11/57 TWW and 4/5 dehydrated sludge). By 2007, *E. bieneusi* occurred in 80% of the samples (27/30 RWW and 21/30 TWW); in 2008 this species was present in all 16 RWW samples and in 94% (15/16) of TWW samples. A total of 86 RWW samples

Table 5 | *Giardia duodenalis* assemblages and genotypes in 232 wastewater and sludge samples

Genotype and subtype	No. of positive plants	No. of positive PCR products
Mammals (including humans)		14
A1 (L02120)	2	2
B (U564283)	4	10
B7 (EU140501)	1	1
B8 (EU140504)	1	1
Humans		84
A2 (U57897)	14	84
Livestock		1
E (EU189356)	1	1

from 17 plants and 49 TWW samples from 16 plants were positive for *E. bieneusi* (Tables 2 and 3).

Overall, 44 unique ITS sequences were identified (Figure 2). Genotypes D and IV were the most prevalent with 85 and 82 products from 15 and 16 plants, respectively. These genotypes were also found in 10 and 11 TWW samples, respectively. In order of decreasing frequency, the next genotypes were Peru8 and WL4/WL5. Previously unknown ITS genotypes were detected in 22 samples. These were found in raw and TWW samples collected in 2007 and 2008.

To analyze whether *E. bieneusi* ITS sequences were geographically clustered, genotype counts were normalized by plant and graphically represented as a heat map (Figure 2). The most prevalent genotypes appear as columns of dark grey and grey. The frequency of detection does not show any association with specific plants. Frequent genotypes were detected in most plants, and rare genotypes were geographically scattered. This analysis indicates no geographic partitioning.

A total of 16 PCR amplicons generated a cluster of sequences not reported previously (Figure 3). These sequences formed a branch within a large group of ITS sequences originating from human and animal hosts comprising the majority of known ITS sequences (Thellier & Breton 2008; Widmer & Akiyoshi 2010) and designated group I by Henriques-Gil *et al.* (2010). In a bootstrap analysis of 344 ITS sequences with 500 replicates, the cluster of 16 unique ITS sequences was present in 63% of the trees. The samples in which this sequence was found originated from Nabeul, Kalaat el Andalous, Mahdia, Kairouan and Sfax.

Noteworthy was the presence of three Tunisian ITS genotypes which cluster with a small, highly divergent group of three sequences previously identified in raccoons by Sulaiman *et al.* (2003b) and designated WL1, WL2, WL3. This cluster, designated as group III by Henriques-Gil *et al.* (Henriques *et al.* 2010) and group 4 by Thellier & Breton (2008), can therefore no longer be considered specific to raccoons as these animals do not live in North Africa. Consistent with the high prevalence of *E. bieneusi* positive PCR reactions, samples with more than one genotype were relatively common. A total of 35 samples from 14 plants had more than one genotype: 23 had two genotypes, eight had three and one had four different genotypes.

E. bieneusi genetic diversity

To compare the genetic diversity of *E. bieneusi* ITS sequences with a previously analyzed global collection of ITS genotypes (Widmer & Akiyoshi 2010), a rarefaction analysis was performed. From the 292 ITS sequences obtained, 209 that had no ambiguous nucleotide positions were included in the analysis. The sequence diversity of

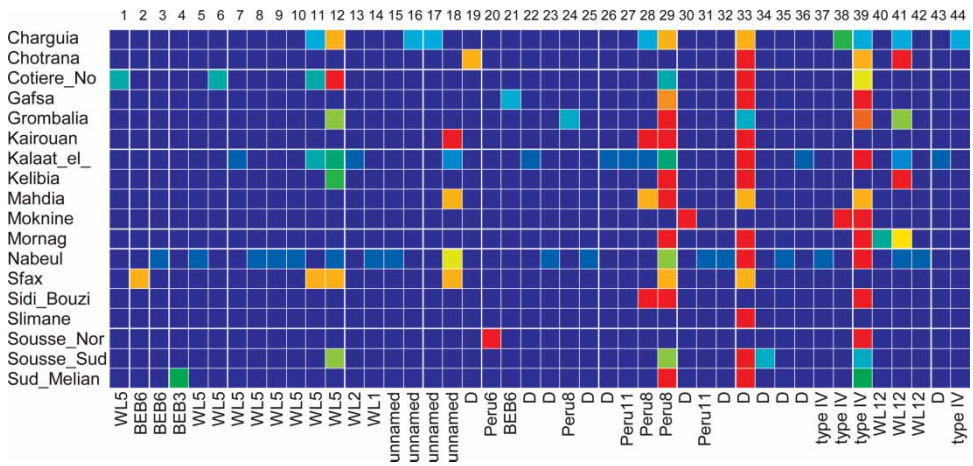

Figure 2 | Distribution of *E. bieneusi* ITS sequences in RWW and TWW collected from 18 treatment plants. A total of 44 unique ITS sequences were detected among 209 PCR amplicons. Raw ITS counts for each plant were normalized by row such that each cell represents the percentage of samples by treatment plant (rows add up to 100%). Light shading >= 12% and black = 0 (not detected); lighter shading indicates higher percentages. Note the homogeneous distribution of *E. bieneusi* genotypes among plants. The arbitrarily assigned genotype numbers are shown uppermost. ITS genotype designation according to Santin & Fayer (2009) is shown below. As multiple ITS sequences are ≥99% similar to a named genotype, the same genotype name may apply to different columns.

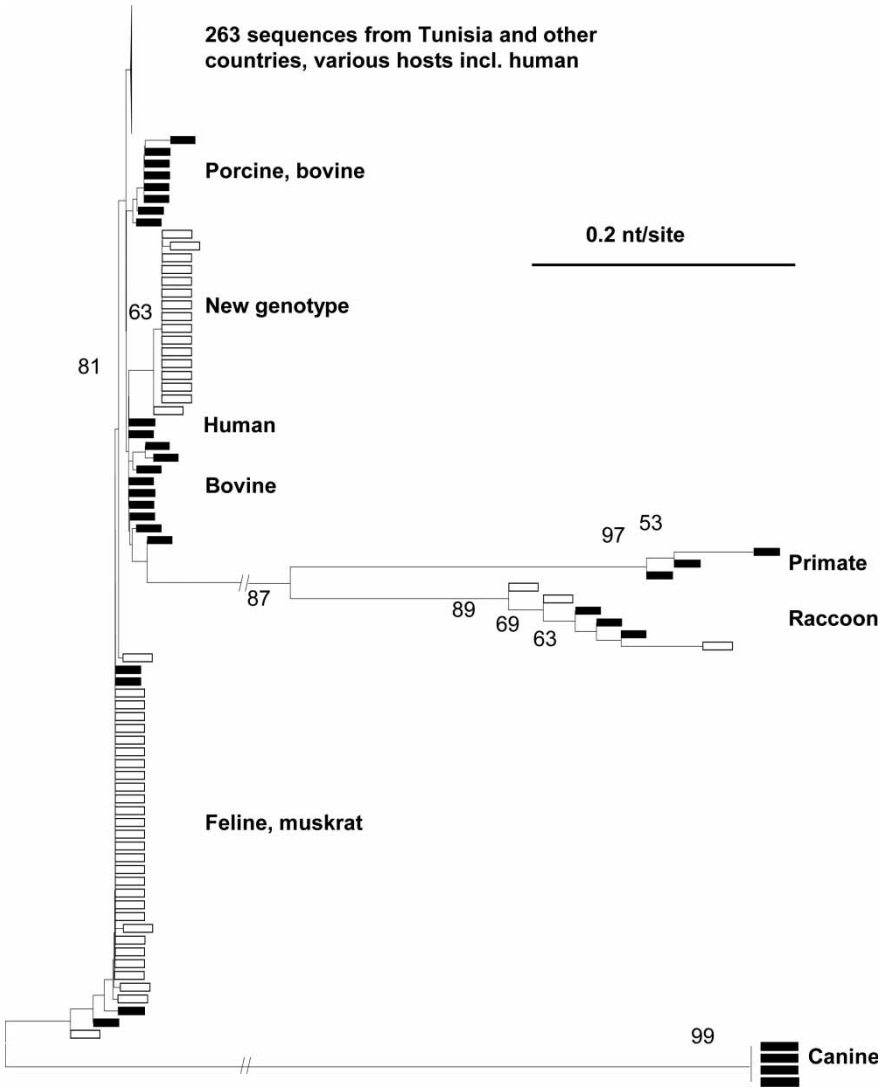

Figure 3 | Phylogenetic analysis of 344 *E. bieneusi* ITS sequences. Black and white bars represent samples from the global collection ($n = 135$) and from Tunisia ($n = 209$), respectively. Host species refer to isolates from the global collection. A large cluster of 263 related sequences from Tunisia and other countries is collapsed. Note the cluster of 16 ITS sequences not encountered previously. For branches observed >50% of the time in a bootstrap analysis with 500 replicates, the percentage of times the branch was observed is indicated. Scale: 0.2 nucleotide substitution per site.

the 209 sequences was 'rarefied' to a sample size of 135 to enable a direct comparison of the genetic diversity of the Tunisian and global ITS sequences. Based on this analysis, for a sample size of 135 the ITS diversity in Tunisia was estimated to be 29.2 genotypes with a 95% confidence interval of 23.0–35.4. This interval is less than half the observed global ITS diversity of 70 unique sequences. This finding is consistent with a more limited genetic diversity of *E. bieneusi* in Tunisian wastewater samples when compared to the global diversity.

PCR detection of *C. cayetanensis* and *Eimeria* spp.

Of the 232 samples analyzed for *C. cayetanensis*, 107 samples (45%) were PCR positive. Because the PCR also amplified DNA of *Eimeria* spp., amplicons were sequenced to identify the species. A total of 170 full-length sequences were obtained from PCR products originating from the 107 positive samples, including 81 RWW samples from 17 plants and 19 TWW samples from eight plants (Tables 2 and 3). *Cyclospora* DNA was detected in one RWW

Table 6 | *Eimeria* species and related parasites in 232 wastewater and sludge samples from Tunisia

Parasite[a]	No. of positive plants	No. of positive PCR products
Eimeria spp. from cattle		59
Eimeria alabamensis (AF291427)	34	52
Eimeria alabamensis-like (one SNP)	4	7
Eimeria spp. from chicken		15
E. tenella (U40264)	4	6
E. acervulina (U67115)	2	3
E. mitis (U40262)	1	1
E. tenella-like 1 (one SNP)	2	2
E. tenella-like 2 (two SNPs)	2	2
Unknown species from birds (EU044785)	1	1
Eimeria spp. from sheep		19
*E. ahsata (*AF338350)	3	5
E. crandallis (AF336339)	2	4
E. ahsata-like 1 (one SNP)	1	2
E. ahsata-like 2 (one SNP)	1	1
E. ahsata-like 3	1	1
E. ahsata-like 4	1	1
E. crandallis-like 1 (one SNP)	1	1
E. crandallis-like 2 (one SNP)	1	1
E. faurei-like 1 (one SNP comparing to AF345998)	1	1
E. faurei-like 2 (one SNP and two deletions)	2	2
Eimeria spp. from rodents		27
E. nieschulzi (U40263)	8	13
E. telekii (AF246717)	7	11
E. separate-like 1 (one SNP comparing to AF311643)	1	1
E. separate-like 2 (two SNPs comparing to AF311643)	2	2
Eimeria spp. from rabbits	3	3
E. magma or *E. media* (EF694016 or EF694013)	1	1
E. magna or E. media-like 1 (one SNP)	1	1
E. magna or E. media-like 2 (two SNPs)	1	1
Isospora spp. from birds	32	38
Isospora sp. (AY331571)	29	35
Isospora sp.-like 1	1	1
Isospora sp.-like *2*	2	2
Cyclospora cayetanensis-like (one SNP and one deletion comparing to AF111183)	1	1
New genotype 1	3	5
New genotype 2	1	2

[a]Forty-three unique sequences are assigned to 32 *Eimeria*, *Isospora* and *Cyclospora* species.

sample collected in Kalaat el Andalous on 26 March 2008. The sequence obtained had two nucleotide differences from *C. cayetanensis*. The other PCR amplicons originated mostly from *Eimeria alabamensis* and relates species infecting cattle (59 sequences), *E. tenella*, *E. acervulina*, *E. mitis* and their relatives from chicken (15 sequences), *E. ahsata*, *E. crandallis* and relatives from sheep (19 sequences), *E. nieschulzi*, *E. telekii* and their relatives from rodents (27 sequences), *E. magna* or *E. media* and their relatives infecting rabbits (three sequences), *Isospora* spp. from birds (38 sequences) and two unknown coccidia called new genotypes 1 (JF910008, JF910009 JF910011 and JF910012) and two (JF910004) (seven sequences) (Table 6).

Meta-analysis of protist sequences from wastewater

Using cluster analysis, no association between species with similar host range was found. For instance, *C. andersoni*, a parasite of cattle, did not cluster with bovine *Eimeria* species, and the anthroponotic species *C. hominis* did not cluster with *E. bieneusi* ITS genotypes found in humans, such as Peru8. The lack of detectable co-occurrence of parasites with similar host range indicates that parasites in different plants originate from multiple sources, including humans and livestock. The same set of presence/absence data was analyzed for the co-occurrence of *G. duodenalis* genotypes and *Cryptosporidium* genotypes, as well as the co-occurrence of *Cryptosporidium* and *E. bieneusi*, and *Cryptosporidium* species and *Eimeria* species. This analysis revealed a significant association among parasites infecting both humans and animals, i.e. *Cryptosporidium*, *G. duodenalis* and *E. bieneusi* (Bonferroni-adjusted $p = 0.03$), whereas the occurrence of animal parasites of the genus *Eimeria* was not associated with *Cryptosporidium* (Bonferroni-adjusted $p = 0.6$). Thus, although hierarchical clustering did not suggest the co-occurrence according to host range, a significant association between three zoonotic genera was found.

As mentioned above, in 209 *E. bieneusi* PCR products, 44 unique ITS sequences were identified. For *Eimeria*, 191 products were positive by PCR and 43 unique SSU rRNA sequences were found. This analysis revealed clear differences in *E. bieneusi* and *Eimeria* genetic diversity among treatment plants (data not shown). For instance, the *E. bieneusi* ITS sequences obtained from Charguia (District of Tunis) were the most diverse group, whereas those from Sousse Sud (Coastal area) and Sud Meliane (District of Tunis) were the least diverse. In the latter two plants, 62 and 60% of the ITS sequences, respectively, were identical. A similar wide range of genetic diversity among plants was observed in the SSU rRNA sequences of *Eimeria*. Interestingly, no correlation was observed between the diversity of the two parasites. Sousse Sud had the least diverse *E. bieneusi* population but the most diverse *Eimeria* population. Conversely, Charguia had the most diverse *E. bieneusi* population but the least diverse *Eimeria* population. This analysis could not be performed for other protists due to the relatively low genetic diversity.

The analysis of the evenness of *E. bieneusi* ITS genotypes revealed no north–south cline; plants with even ITS distribution were found in the north of the country, for instance Charguia ($J' = 0.78$), as well as in the center (Mahdia, $J' = 0.87$) and south (Sfax, $J' = 1.00$).

DISCUSSION

Results of the survey document a high prevalence and genetic diversity of pathogenic protists in RWW in Tunisia, and suggest that the various wastewater treatment processes adopted in Tunisia are insufficient in removing these parasites. As sedimentation was a key process in all treatment processes, the reduction in the occurrence of these protists was affected by the size of the environmental stage. As a result, a higher reduction in occurrence was achieved for the large *Eimeria* oocysts (76%) than for the small *E. bieneusi* spores (43%), with the medium-sized *Cryptosporidium* oocysts (66%) and *Giardia* cysts (68%) in between. Nevertheless, there were no apparent differences in the reduction of the occurrence of protists among different treatment practices. For example, the two plants that were sampled most frequently (Kalaat El Andalous and Nabeul SE4) had a similar reduction in the occurrence of *Cryptosporidium* spp., *G. duodenalis*, *E. bieneusi* and *Eimeria* spp., even though the former had no primary treatment and used waste stabilization pond as the secondary treatment, whereas the latter used decantation as the primary treatment and activated sludge as the secondary treatment

(Tables 1 and 2). These data should be useful in the development of measures to reduce the transmission of enteric pathogens and to improve the safety of discharge and reuse of wastewater and sludge.

The use of PCR for analyzing wastewater samples provided an overview of protists circulating in the population at the time of sampling. This information is relevant to understanding transmission cycles and the potential sources of infections affecting humans, livestock and other animals. This type of study could also provide a useful approach for collecting data on the occurrence of enteric diseases in different regions of the country. However, no information on the infectivity of waterborne parasite spores, cysts and oocysts was gained from the detection of DNA. The fact that solids from wastewater samples were sedimented and PCR was applied to DNA extracted from the solid fraction suggests that the DNA was enclosed in intact spores, cysts and oocysts.

The analysis of co-occurrence of parasites with similar host range gave inconsistent results. Hierarchical clustering showed no association, whereas a positive association among zoonotic parasites was found. This outcome could originate from the fact that for hierarchical clustering 15 species/genotypes were considered, whereas association was tested among *Giardia*, *Cryptosporidium*, *Eimeria* and *E. bieneusi* without differentiating between species and genotypes. The lack of consistent outcome of these analyses is in agreement with the wide distribution and frequent co-occurrence of protist species in different plants.

The large number of positive samples enabled an analysis of geographical distribution of species and genotypes. These analyses showed no obvious difference between plants or regions. Due to the large number of water samples positive for *E. bieneusi*, geographical trends were explored by analyzing the distribution of *E. bieneusi* ITS genotypes and, to some extent, *Eimeria* SSU rRNA genotypes. The lack of any geographical pattern is consistent with the relatively small distances between plants and the fact that wastewater is mostly impacted by human and livestock waste. Any geographical partition would be expected to be rapidly eliminated by human mobility and the presence of numerous slaughterhouses throughout the country.

The results of the rarefaction analysis of *E. bieneusi* ITS genotypes is consistent with a globally restricted distribution of certain ITS genotypes; that is, after adjustment for equal sampling, fewer *E. bieneusi* genotypes are present in Tunisia as compared to a global reference collection. This observation needs to be interpreted with caution. The diversity of the reference collection (Widmer & Akiyoshi 2010) might be higher than the real diversity because this collection is derived from the GenBank database. Investigators who deposit sequences from epidemiological surveys frequently only deposit one sequence representative of multiple identical sequences, and may not deposit a sequence that is already found in GenBank. Although this bias may be reduced by the fact that some investigators submit sequences regardless of what has already been submitted, the possibility that the global reference collection we used as a benchmark is more diverse than in reality needs to be considered.

Pathogens present in wastewater have the potential to be transmitted to humans, especially when water is reused for irrigation. In this study, wastewater was found to frequently carry pathogens of human and animal origin. The presence of species which do not infect humans such as *Eimeria* demonstrates that wastewater, even in urban areas, is contaminated with fecal matter of animal origin. Given the public health risk of these parasites to humans and farm animals, guidelines on the discharge and reuse of wastewater are needed to reduce the transmission potential and transport of these pathogens in Tunisia and other countries with limited water sources.

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