ITALIAN JOURNAL OF PUBLIC HEALTH

Molecular characterization of Giardia duodenalis cysts in the Oreto River (Sicily, Southern Italy)

Maria Antonella Di Benedetto, Florinda Di Piazza, Carmelo Massimo Maida, Nino Romano

Dipartimento di Scienze per la Promozione della Salute, Università degli Studi di Palermo, Italia

Correspondence to: Maria Antonella Di Benedetto, Dipartimento di Scienze per la Promozione della Salute, Via del Vespro 133, 90127 Palermo, Italy. E-mail: dibenedetto@igiene.unipa.it

Abstract

Background: The presence of *Giardia* was investigated along the Oreto river between January 2008 and May 2009 with the aim of understanding the source of contamination by molecular typing of cysts.

Methods: A total of 38 water samples (10 collected from the river mouth, 24 from the whole Oreto basin and 4 sewage samples from the Monreale treatment plant) were processed. In addition, 22 faecal samples of livestock living close to the Oreto area, were analyzed. The presence of *Giardia* was determined by immunofluorescence assay and their genetic characterization was achieved by a nested PCR assay targeting the triosephosphate isomerase gene.

Results: All water samples from the river mouth were positive for *Giardia*, even if the concentration of cysts fluctuated considerably among sampling occasions. Our investigation showed that the *Vadduneddu* and *Altofonte* torrents, two influents of the river, were the principal sources of contamination. Moreover, the genotypes of *Vadduneddu* torrent were the same as those detected in human wastewater taken from the activated sludge plant of Monreale city. Assemblages A and B were found in water samples with a predominance of Assemblage A, subtype AII. Assemblage E was only found in a single calf isolate.

Conclusions: The data show that the high cyst counts regularly detected in the Oreto river are due to contamination with wastewater of human origin. This finding is relevant for public health, particularly because river water is used for agricultural purposes.

Key words: Giardia, Oreto River, contamination

Introduction

Giardia is an ubiquitous enteric parasite that infects humans and many other animals. It produces environmentally resistant cysts that are voided in the faeces and are transmitted directly or indirectly via water or food [1]. The immune status of the host appears to influence the susceptibility to the infection, as well as the severity of clinical signs [2].

G. duodenalis is a widespread cause of gastrointestinal disease, primarily by the ingestion of contaminated water and is believed to be responsible for 2.5 million diarrhoea-associated deaths and nutritional deficiencies in children in developing countries [3]. Epidemiological surveys have indicated that the most important sources for human infection are contaminated drinking and recreational water, food, household animals and infected people [4]. Moreover, in recent years, waterborne outbreaks of giardiasis are a public health problem in developed nations also [5, 6].

In a previous study [7], we demonstrated that the Oreto river, which cuts through Palermo city (Sicily) and is used for agricultural purposes (only in the past for drinking water), was contaminated by *Giardia* and *Cryptosporidium*. *Giardia* cysts were found in river water in high concentrations for all the samples collected during one year. Because the molecular characterization of the cysts was not performed on that occasion, the present study was carried out to include genetic characterization of *Giardia* cysts and to identify the possible source of river contamination.

Methods

Area and study design

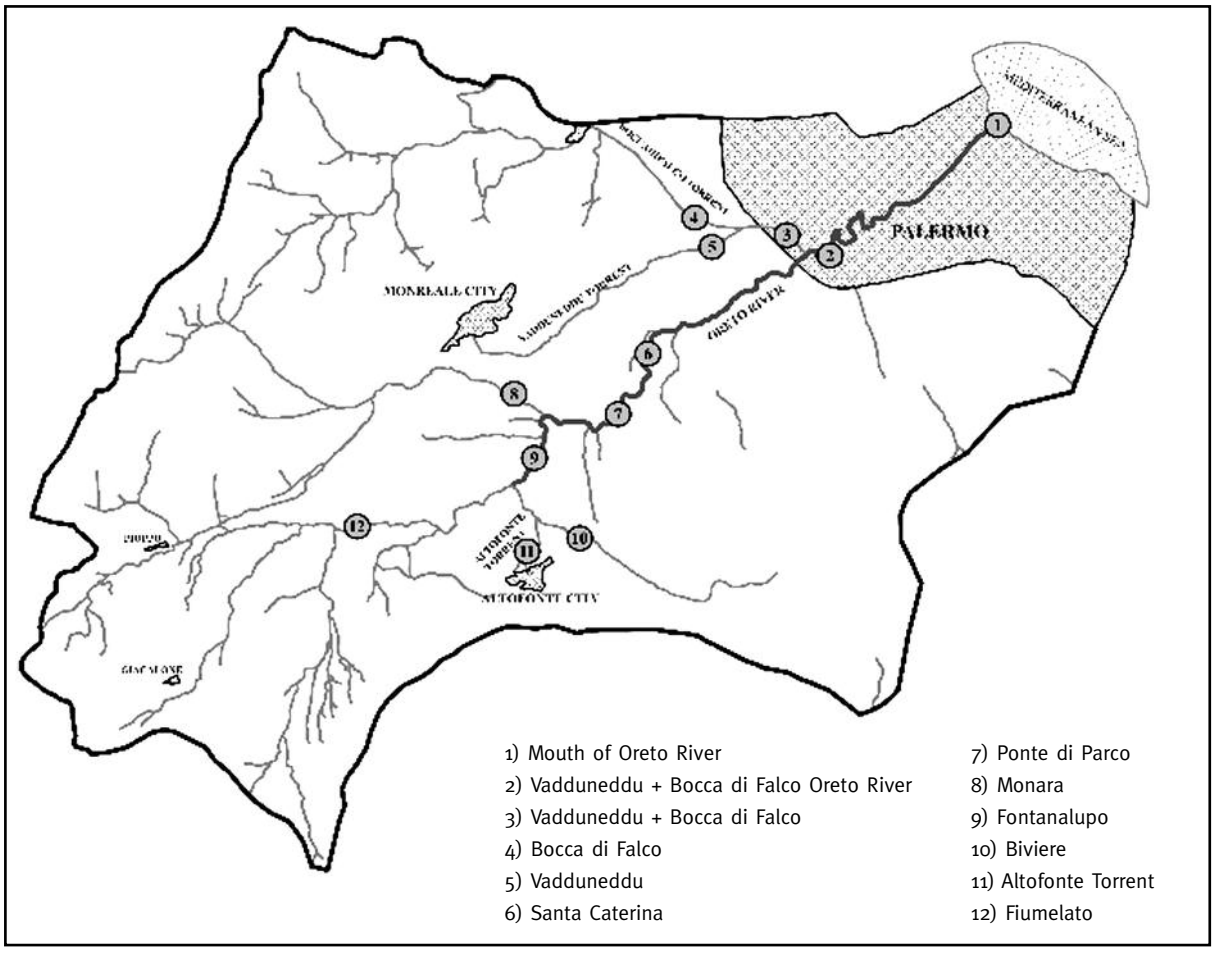
The Oreto river is 21 km long and crosses the city of Palermo before flowing into the Mediterranean sea in front of the city. It receives waters from several small influents. One of them, the Vadduneddu torrent, gets wastewater from an activated sludge plant of Monreale city and another one, the Altofonte torrent, gets untreated sewage from a small town (Figure. 1). In order to

identify the possible source of the contamination of the Oreto river, we carried out our study in the whole Oreto river basin, from the mouth of the river to the its source. Moreover, we included animal faecal samples coming from three farms near the river in order to evaluate the circulation of different genotypes of *Giardia* in the area of the Oreto basin.

Water sample collection and processing

A total of 38 water samples were processed between January 2008 and May 2009. During a preliminary study (from January to October 2008), 10 samples of water per month were collected from the mouth of the river (site n.1, Figure 1). Subsequently, a total of 24 samples were taken from the whole Oreto basin (from site n.1 to site n. 12), 12 samples collected in December 2008 and 12 in May 2009, respectively. Moreover, 4 sewage samples were also collected from the Monreale treatment plant (2 before and 2 after treatment) whose effluents are discharged in one of the important tributaries (called Vadduneddu) of the Oreto river. The main physicochemical parameters such as atmospheric temperature (environmental thermometer mod."Giant"-PBI International), turbidity (turbidimeter mod. 2100 - HACH Ultra Analytics) and pH (pH metro PP-20 - Sartorius) were recorded at each sampling. Because of the different nature of samples, arbitrarily we first used the bacterial indicator of faecal contamination *Escherichia coli*, determined by *Standard Methods for the Examination of Water and Wastewater* [8]. This method was applied to compare water samples to raw wastewater (WW) when the count of *E. coli* colonies was greater than 1,0 x 10^2 cfu/100 ml (sites n. 1, 2, 3, 4, 5, 11) and to assimilate surface water (SW) when *E. coli* was present in concentration lower than 1,0 x 102 cfu/100 ml (sites n. 6, 7, 8, 9, 10, 12) in accordance with Italian law for the surface water [9]. Then, water samples (10 L of SW) were processed as reported in a previous study [7] in accordance with the ICR protozoan method for the detecting *Giardia* cysts and *Cryptosporidium* oocysts in water [10]. Waste Water (WW) samples (200 ml) were processed by centrifugation at 1100 x g for 10 minutes. Six aliquots of 50 µl of eluted pellets (SW and WW samples) were examined by

Figure 1. The Oreto river basin with the indication of river water sampling sites (n. 1 – 12).



immunofluorescence assay (IFA) (Merifluor G/C Combo, Meridian Diagnostic, Cincinnati, Ohio), according to the manufacturer's instructions, and stained with 4',6-diamino-2-phenylindolo (DAPI). Sample examination was conducted using a microscope (Leitz Diaplan) at 400x magnifications configured for protozoan analysis. In addition, cysts on each slide well were enumerated and the mean number of replicates was used to calculate the concentration in the original sample. *Giardia* cysts were defined as objects with characteristic apple-green fluorescence, typical for Giardia size and shapes, distinct DAPI-stained nuclei and lack of atypical morphological characteristics.

Animal faecal samples collection and processing

Over the same period of time, 22 faecal samples from cattle, goats and sheep from 3 farms along the Oreto area were collected. Each sample was transported to the laboratory in a plastic specimen cup, within 4 hours of collection. All faeces samples were processed by a sucrose flotation method described by Olson et al. [11]. *Giardia* cysts were identified microscopically by immunofluorescence as previously described.

DNA extraction and PCR amplification

DNA was extracted according to the method described by da Silva [12].The amplification of the triose phosphate isomerase (TPI) gene was performed using an already described nested PCR protocol [13].The PCR products were analyzed by 1% agarose gel electrophoresis and visualized after ethidium bromide staining.

DNA sequencing

The secondary PCR products were purified using Microcon PCR centrifugal filter devices (Millipore Corp., Bedford, MA) and sequenced on an ABI 3100 automated sequencer (Perkin Elmer). Sequence accuracy was confirmed by sequencing an independent PCR product on each strand. Multiple alignments were performed using the computer software package Clustal X [14]. Published *Giardia* TPI nucleotide sequences (AI, AII, BIII, BIV, E GenBank accession numbers: L02120, U57897, AF069561, L02116 and AY655705, respectively) were aligned with the homologous sequences determined in the present work.

DNA cloning

The *tpi* gene PCR products that had double peaks in the chromatogram were cloned into TOPO TA vector (Invitrogen Corporation, Carlsbad, USA), transferred into One Shot chemically competent *E. coli* and then sequenced to verify the presence of genetically different cysts in the same sample. For each reaction 10 colonies were selected and sequenced.

Nucleotide sequence accession numbers

The new TPI sequences obtained in this study have been deposited in the GenBank (accession numbers: from FJ465157 to FJ465161).

Results

Water samples

The results from the water samples examined are shown in Table 1. All water samples collected from the mouth were positive to *Giardia* cysts using IF microscopy and PCR (Table 1a). Cyst concentration varied from 1,40 x 10^3 to 1,31 x 10^4 cysts/l. The lowest and highest number of cysts/l were detected in the samples taken soon after torrential rain (pH 8.3; turbidity 37,3 NTU) and five days of heat wave in Palermo city (atmospheric temperatures ranged from 30°C to 45°C; pH 8.0; turbidity 9,5 NTU), respectively.

Sequence analysis of a TPI gene fragment showed the presence of two different *G. duodenalis* assemblages, A and B. Five water samples were positive for assemblage A, two for assemblage B and three for both assemblages. Within assemblage A, the sequences were identical to subtype AII (GenBank accession n.U57897), except in one case where a single nucleotide substitution was detected (GenBank accession n. FJ465157). On the contrary, three novel sequences were detected among assemblage B isolates, two of which (GenBank accession n. FJ465158, FJ465159) were present in a sample collected after heavy rain.The variability of subtypes found in river mouth samples prompted us to determine the possible source of contamination and the distribution of the different genotypes by two cross sectional investigations carried out from the river's mouth to the source of the Oreto river. Therefore, we analysed 12 samples collected in December 2008 from the rivers' mouth, its course and the outlets of two important tributaries (Vaddunedu and Boccadifalco) of the Oreto river (sites n.1, 2, 3, 4, 5, 6 - Figure 1) as well as several sites up stream of the river (sites n. 7, 8, 9, 10, 11, 12 – Figure 1).The cyst concentration increased (8.7 x 10^3/l) at the outlet of the two influents (site n. 2) then decreased again (5.9 x 10^3/l) at the mouth of the river (site n. 1) (Table 1b). The concentration of Giardia cysts in the Oreto river seemed to be due to the contribution of Boccadifalco torrent (1.2 x

Table 1. Occurrence and molecular characterization of *Giardia* cysts detected in 10 water samples coming from the Oreto's mouth (1.a), 24 water samples coming from the Oreto's basin (1.b) and in 4 wastewater samples coming from the Monreale treatment plant (1.c).

1.a Oreto mouth

Sampling sites[a]	*Giardia* positive/ total samples	Nr. cysts /L	Assemblage	Subgenotype
January 2008– October 2008 (10 samples)				
Site N.1	10/10	Range from 1.40×10^3 to 1.31×10^4	A (n=5)	AII (n=4) and new A sequence (n=1) (FJ465157)[b]
			B (n=2)	new B sequence (n=1) (FJ465161)[b]; B sequence not identified[c] (n=1)
			A+B (n=3)	A+B (n=1) identified as AII (n=1) and new B sequences (n=2) (FJ465158, FJ465159)[b]; A+B (n=2) N.I.[c]

1.b Oreto basin

Sampling sites[a]	**First sampling, December 2008** (12 samples)		**Second sampling, May 2009** (12 samples)	
	No. cysts/L	Assemblage (subtype)	No. cysts/L	Assemblage (subtype)
Site N. 1	5.9×10^3	A (II)	4.5×10^3	A+B (subtypes not identified)[c]
Site N. 2	8.7×10^3	A (II)	6.0×10^3	A+B (subtypes not identified)[c]
Site N. 3	2.6×10^4	A (II)	4.0×10^3	A+B (subtypes not identified)[c]
Site N. 4	1.2×10^2	A (II)	1.5×10^3	A (II)
Site N. 5	1.3×10^4	A (II)	2.6×10^3	B (new sequence FJ465160)[b]
Site N. 6	6.3×10^1	A (II)	1.8×10^1	N.A.
Site N. 7	1.2×10^2	A (II)	1.5×10^1	A (II)
Site N. 8	0.5×10^1	N.A.[d]	0.2×10^1	N.A.[d]
Site N. 9	0.2×10^1	N.A.[d]	0.4×10^1	N.A.[d]
Site N. 10	1.0×10^1	N.A.[d]	1.6×10^1	N.A.[d]
Site N. 11	3.0×10^2	A (II)	6.4×10^3	A (II)
Site N. 12	0	/	0	/

1.c The Monreale treatment plant

	First sampling (2 samples) (December 2008)			**Second sampling (2 samples)** (May 2009)		
	No. cysts/L	Removal efficiency	Assemblage (subtype)	No. cysts/L	Removal efficiency	Assemblage (subtype)
Raw wastewater	5.5×10^4		A+B (subtypes not identified)[c]	3.0×10^4		B+A (subtypes not identified)[c]
Treated wastewater	1.6×10^4	71%	A+B[e] (AII and B sequence (n=1) (FJ465162)[b, f]	6.0×10^3	80%	B+A[g] (B sequence (n=1) (FJ465160)[b] and AII)

[a] See Figure 1

[b] GenBank accession number

[c] Subtypes weren't identified although the sample was processed by cloning

[d] DNA not Amplified

[e] Assemblage A was quantitatively predominant in comparison to Assemblage B (datum observed by DNA cloning)

[f] Subtype previously identified in human faeces in Palermo city (data non published)

[g] Assemblage B was quantitatively predominant in comparison to Assemblage A (datum observed by DNA cloning)

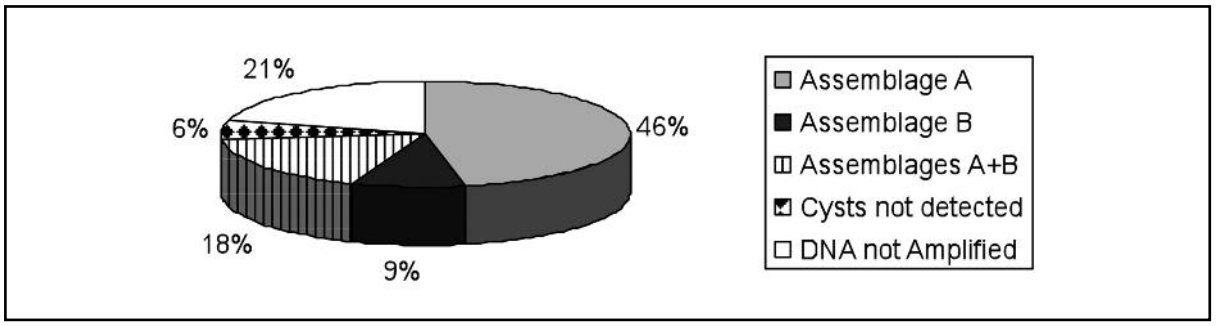
10^2/l) (site n. 4) and especially to the contribution of Vadduneddu torrent (1.3 x 10^4/l) (site n. 5).The results indeed show that the concentration of cysts was low (63/l) upstream from the influents of the Vadduneddu and the Boccadifalco into the main river (site n. 6). Subtype AII was detected in all sites analysed.

Since only subtype A (II) was detected in all samples, a second cross-sectional study was carried out in Spring (May 2009), aiming to explain why the presence of different genotypes (A, B, A+B) were found during the preliminary phase of the study from the mouth of the river. In this second study, again the cyst concentration decreased from the mouth of the river up to its source; the highest concentration of cysts was again found in the Boccadifalco (site n. 4) (1,5x10^3/l) and in the Vadduneddu (site n. 5) (2.6x10^3/l) torrents and, in this occasion, also in Altofonte torrent (site n.11) (6.4x10^3/l), while the other sites had low or no concentration of cysts the inlet and the outlet of the treatment plant, although at different concentrations. Assemblage A, subtype AII, was quantitatively predominant in December in comparison to assemblage B. On the contrary, assemblage B (GenBank accession n. FJ465160) was predominant in May. These predominant assemblages were also detected in the Vadduneddu torrent sampled on December 2008 (AII) and May 2009 (assemblage B GenBank accession n. FJ465160), respectively.

Faecal samples

Giardia cysts were found by IFA in 7 stools out of 22 samples (31,8%) obtained from one goat, one sheep, four calves and one cow. Only one sample, coming from a sucking calf, resulted positive to PCR. Molecular sequencing of this sample revealed the presence of *G. duodenalis* assemblage E and the sequence was identical to an already published sequence (GenBank accession n. EF654692) (data not shown).

Figure 2. The prevalence of *Giardia duodenalis* genotypes in the Oreto river.



(Table 1b). Genotyping of these Giardia isolates revealed different assemblages along the Oreto basin: AII in Altofonte and Boccadifalco torrents and in Ponte di Parco; a new sequence of Assemblage B (GenBank accession n. FJ465160) in Vadduneddu torrent; both assemblages A and B in the mouth of the river. In summary, as shown in Figure 2, assemblage A cyst types were detected in a high proportion of water river samples (46%) compared to assemblage B (9%); mixed contaminations (A + B) were detected in 18 % of the specimens. Amplification of Giardia DNA was not obtained in 21% of samples and 6% of the river waters did not reveal the presence of Giardia cysts.

To establish whether the subtypes of *Giardia*, detected in Vadduneddu torrent, came from treated human sewage, four wastewater samples (two before and two after treatment) were taken from the treatment plant of Monreale city on December 2008 and May 2009. Table 1c shows that both assemblages A and B were recovered at

Discussion

Giardia cysts were found in all samples collected at the mouth of the Oreto river during the whole period of the study. However, the cyst concentration varied during the different months in which the samples were collected. Indeed, the mouth showed the highest concentration of *Giardia* cysts in summer (July 2008), after a period of hot weather, which caused a lowering of the river level, thus increasing the amount of cysts per unit of volume twofold (from 6560 to 13100 in nine days, data not shown). The influence of weather conditions on cyst concentration has been reported by other authors, although data are not unequivocal [15, 16]. Weather conditions may also influence the occurrence of *Giardia* genotypes. Three different subtypes were detected (AII and two new sequences of assemblage B) in one sample collected in coincidence of heavy rainfall, indicating that

precipitation might enhance the entry of these cysts into the river presumably through an increased water influx from its torrents.

The Oreto river is heavily contaminated by *G. duodenalis* cysts of human origin, as suggested by genotype analysis of human sewage taken from the Monreale treatment plant. Indeed, the river flows through urban areas and receives urban treated and untreated domestic wastewater from three of its influents (Vaddunedu, Boccadifalco and Altofonte). For the small volume of samples, the concentration of cysts is relatively high, thus indicating that the river and its tributaries are highly impacted with wastewater discharged.

The occurrence of *Giardia* of human origin in natural waters should be of public health concern moreover if the zoonotic potential of some assemblages of *G. duodenalis* is considered.

Since only Assemblages A and B were found in water samples, but not Assemblage E, the contribution of livestock to the Oreto river contamination seems negligible, or may not reach detectable levels. Our study has also shown the occurrence of several assemblages and subtypes of *Giardia* cysts at different points of the Oreto river. However, a single genotype is not always associated with a particular point of release, but could vary over time. For example, the Vaddunedu was the main source of A genotype contamination in December 2008, and five months later of the B genotype. This depends on different concentrations of both A and B assemblages present in the outlet of treatment plant of Monreale city at different observation times. However, further molecular investigations will be needed to determine the origin (human or animal) of Giardia cysts in the Oreto river basin.

During our survey, Assemblage A predominated, being found in 22/34 river samples. This result confirms previous findings in wastewater and surface water in Italy where the zoonotic Assemblage A is prevalent both in humans and in animals [15, 17, 18]. In particular, in Southern Italy, *Giardia* cysts were found in 16 out of 21 samples of treated wastewater and in seven out of 21 samples from downstream water channels; molecular analysis identified 15 isolates as Assemblage A and two as Assemblage B [19].

Moreover, all Assemblage B sequences were new, demonstrating more genetic variability among isolates of this genotype compared to Assemblage A (Table 2). It is likely that Assemblage A is more conserved and evolves more slowly than Assemblage B. Alternatively, AII may be more infectious to human that other *Giardia* subtypes [20].

In conclusion, our results show that the Oreto river is commonly contaminated by high number of cysts of Assemblage A, particularly of subtype AII. This finding underlines the importance of improving the treatment of wastewater originating from the city of Monreale and to treat sewage originating from others urban centres. It was recently awarded the tender to improve the efficiency of the sewage treatment plant in the territory of Monreale. The *"Agenzia dell'acqua siciliana"* has funded an allocation of 3 million 300 thousand euro to make the plant safer. An intervention has been planned for several parts of the plant (mechanical parts, settling tanks and all

Table 2. Variation in the *tpi* nucleotide sequences of *Giardia duodenalis* assemblage A and B subtypes.

Assemblage A	*tpi*		
Isolate (code)	129	183	399
AI reference sequence (L02120)	T	G	C
AII reference sequence (U57897)	C	.	T
Mouth (FJ465157) [a]	.	A	.

Assemblage B	*tpi*															
Isolate (code)	39	45	51	71	91	110	157	162	165	168	192	210	297	315	342	429
BIII reference sequence (AF069561)	A	T	G	A	C	T	G	G	C	C	A	G	A	G	G	G
BIV reference sequence (L02116)	.	.	.	.	T	.	.	.	T	T	.	A	.	.	.	A
Mouth (FJ465158) [a]	.	C	.	G	.	C	.	A	.	.	G	.	.	.	.	.
Mouth (FJ465159) [a]	.	.	A	.	T	.	.	A	.	.	G	.	G	.	.	.
Mouth (FJ465161) [a]	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Site n.5 and outlet of Monreale treatment plant (FJ465160) [b]	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.

[a] Sample taken during the sampling of Oreto mouth
[b] Sample taken during the sampling of Oreto basin

conduits) in order to improve the capability of depuration of human sewage coming from Monreale city. In recent years, reductions in annual rainfall in Sicily and increased human consumption of water have caused a shortage of freshwater resources, forcing an increased need to reclaim the few resources present in our territory. For this reason, an important, yet difficult, goal will be to reduce contamination by *Giardia* cysts in local water resources.

References

1) Adam RD. Biology of Giardia lamblia. Clin Microbiol Rev 2001; 14: 447-75.
2) Dwivedi KK, Prasad G, Saini S, Mahajan S, Lal S, Baveja UK. Enteric opportunistic parasites among HIV infected individuals: associated risk factors and immune status. Jpn J Infect Dis 2007; 60: 76-81.
3) WHO (World Health Organization). Intestinal parasites control: burden and trends. Geneva: Division of Control of Tropical Diseases, 1998.
4) Cacciò SM, Thompson RCA, McLauchlin J, Smith HV. Unravelling Cryptosporidium and Giardia epidemiology. Trends Parasitol 2005; 21: 430-7.
5) Karanis P, Kourenti C, Smith H. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health 2007; 5: 1-38.
6) Robertson L, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl Environ Microbiol 2006; 72: 2212-7.
7) Di Benedetto MA, Di Piazza F, Maida CM, Firenze A, Oliveri R. Occurrence of Giardia and Cryptosporidium in wastewater, surface water and ground water samples in Palermo (Sicily). Ann Ig 2005; 17: 367-75.
8) APHA (American Public Health Association). Standard Methods for the Examination of Water and Wastewater 20th edition. Baltimore, Maryland: United Book Press, Inc. American Public Health Association/American Water Works Association/Water Environment Federation, 1998
9) Decreto Legislativo n. 152. "Norme in materia ambientale". Gazzetta Ufficiale n. 88 del 14 aprile 2006 - Supplemento Ordinario n. 96.
10) U.S.E.P.A (U.S. Environmental Protection Agency). ICR protozoan method for detecting Giardia cysts and Cryptosporidium oocysts in water by a fluorescent antibody procedure. Washington, District of Columbia: U.S.E.P.A. Office of Ground Water and Drinking Water, 1995.
11) Olson ME, Guselle NJ, O'Handley RM et al. Giardia and Cryptosporidium in dairy calves in British Columbia. Can Vet J 1997; 38: 703-6.
12) da Silva AJ, Bornay-Llinares, FJ, Moura INS, Slemenda SB, Tuttle JL, Pieniazek NJ. Fast and reliable extraction of protozoan parasite DNA from faecal specimens. Mol Diagn 1999; 4: 57-64.
13) Sulaiman IM, Fayer R, Bern C et al. Triosephosphate isomerase gene characterization and potential zoonotic transmission of multispecies Giardia duodenalis. Emerg Infect Dis 2003; 9: 1444-52.
14) Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 24: 4876-82.
15) Cacciò SM, De Giacomo M, Aulicino FA, Pozio E. Giardia cysts in wastewater treatment plants in Italy. Appl Environ Microbiol 2003; 69: 3393-8.
16) Robertson L, Gjerde BK. Occurrence of Cryptosporidium oocysts and Giardia cysts in raw waters in Norway. Scand J Public Health 2001; 29: 200-7.
17) Giangaspero A, Berrilli F, Brandonisio O. Giardia and Cryptosporidium and public health: the epidemiological scenario from the Italian perspective. Parasitol Res 2007; 5: 1169- 82.
18) Lonigro A, Pollice A, Spinelli R et al. Giardia cysts and Cryptosporidium oocysts in membrane-filtered municipal wastewater used for irrigation. Appl Environ Microbiol 2006; 72: 7916-8.
19) Giangaspero A, Cirillo R, Lacasella V et al. Giardia and Cryptosporidium in inflowing water and harvested shellfish in a Lagoon in Southern Italy. Parasitol Int 2009; 58: 12-7.
20) Sulaiman IM, Jiang J, Singh A, Xiao L. Distribution of Giardia duodenalis genotypes and sub-genotypes in raw urban wastewater in Milwaukee, Wisconsin. Appl Env Microbiol 2004; 70: 3776-80.