Molecular detection of Cyclospora in water, soil, vegetables and humans in southern Italy signals a need for improved monitoring by health authorities

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Highlights

- Cyclospora cayetanensis is an important food- and water-borne human enteric pathogen.

- Cyclospora detected in well water and treated water used for irrigation

- Cyclospora detected in soil and fresh plant produce

- Contaminated water, soil and vegetables (mainly fennel) might be a source of human infection.

Abstract

To date, in Europe, there is scant information on the occurrence of Cyclospora in water from treatment plants and in humans, and no data are available on soil or fresh plant products. Here, we undertook the first molecular survey of Cyclospora in multiple biological matrices collected from the Apulia region of southern Italy. Samples of irrigation water from four municipal treatment plants, eight different types of vegetables or fruit (cucumber, lettuce, fennel, celery, tomato, melon, endive and chicory) and soil from the same farms on which these plants were grown, as well as faecal samples from humans living in the same region were tested by qPCR-coupled single-strand conformation polymorphism (SSCP) analysis and DNA sequencing. Cyclospora was detected in 15.5% of all 213 samples tested. Specifically, this protist was detected in (i) treated water (21.3% of 94 samples), well water (6.2% of 16), but not drinking water (0% of 3); (ii) soil (11.8% of 51 samples) and vegetables (12.2% of 49), with the highest prevalence (18.7%) on

fennel; and (iii) human stools (27.5% of 40 samples). In environmental and food samples, Cyclospora was detected mainly in autumn and was significantly more prevalent in the faeces from humans of 40–50 years of age. This is the first comprehensive molecular survey of Cyclospora in environmental, food and human faecal samples in Europe. These data suggest that irrigation water, soil and vegetables might be contaminated by Cyclospora cayetanensis, which might represent a source of infection to humans in the study area and calls for monitoring by health authorities.

Keywords

Cyclospora cayetanensis, qPCR, SSCP, Water, Soil, Vegetable, Human Food safety

1. Introduction

A number of water- and food-borne protistan parasites, including Giardia, Cryptosporidium spp., Toxoplasma gondii, Entamoeba histolytica and Cyclospora, are of major public health concern, particularly in young, old and/or immuno-compromised or -suppressed people in both developed and developing countries (Chacin-Bonilla, 2010a, Fletcher et al., 2012, Ortega and Sanchez, 2010). Of these protozoans, Cyclospora cayetanensis (Apicomplexa, Eimeriidae) has received relatively little attention in Europe as a food- and water-borne pathogen, in spite of its reported significance in other industrialized countries (Baldursson and Karanis, 2011, CDC, 2013, Insulander et al., 2010). In humans, this protist is transmitted via the ingestion of water or food contaminated by oocysts and is responsible for cyclosporosis, an enteric disease associated with symptoms including diarrhoea, abdominal cramps and nausea (Ortega et al., 1993, Ortega et al., 1994). Although Cyclospora spp. have been recorded in various animals, including non-human primates, cattle, dogs, rodents and birds (Ortega and Sanchez, 2010), only humans are recognised to harbour C. cayetanensis.

The oocysts of Cyclospora can survive in water at 4 °C for 2 months and 37 °C for 7 days (Ortega et al., 1998, Smith et al., 1997). It has been established that water facilitates both the development and transmission of coccidial oocysts (Mansfield and Gajadhar, 2004), which are resistant to routine chemical disinfectants and to some sanitisation approaches employed in water-treatment plants, irrigation systems and swimming pools (Ortega et al., 1998, Ortega and Sanchez, 2010). The dissemination of oocysts in water, soil and unprocessed foods, such as fruits and vegetables, is facilitated by the small size, low specific gravity of their infective stages and relatively high infectivity (Chacin-Bonilla, 2010a, Dixon et al., 2005, Sterling and Ortega, 1999). Oocysts of C. cayetanensis have been detected in drinking water, waste- and recreational water as well as on vegetables in developed and developing countries (Chacin-Bonilla, 2010a, Ortega and Sanchez, 2010). Outbreaks of cyclosporosis in humans have been reported mostly from North America (CDC, 2013, Dixon et al., 2013, Baldursson and Karanis, 2011, Kozak et al., 2013), where infection sources have been linked to contaminated tap, river and municipal waters, watercress and fresh produce, such as soft fruits (usually raspberries) (Herwaldt, 2000, Ortega and Sanchez, 2010) and leafy vegetables (e.g., basil, mesclun, lettuce and spinach) (Hall et al., 2012, Dixon et al., 2013, Ortega and Sanchez, 2010). Soil is also suspected to play a role as a source of human infection/disease (Chacin-Bonilla et al., 2007, Chacin-Bonilla, 2008, Koumans et al., 1998, Mansfield and Gajadhar, 2004), but, to date, there are no studies of this biological matrix.

In contrast to the USA (http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm), European legislation (EEC Directive 98/83) does not list Cyclospora as a contaminant of drinking water, and the responsibility for its detection is left to the initiatives of national/local health authorities. In Europe, data on the occurrence

of C. cayetanensis in water from treatment plants are very scant compared with some other protists, such as Giardia and Cryptosporidium. Indeed, the only published record of C. cayetanensis contamination of several kinds of water (drinking, wastewater and river water) is from Spain (Galvan et al., 2013), whereas no data are available for either soil or fresh plant products. In addition, there are very few case reports of Cyclospora infection in humans in Europe (Chacin-Bonilla, 2010a, Ortega and Sanchez, 2010). Clearly, investigations of Cyclospora in water, soil and vegetables are needed to start to understand the epidemiology of cyclosporosis and its potential public health significance. For this reason, we undertook here the first molecular survey in Italy of Cyclospora in multiple biological matrices (irrigation water, soil, vegetables and human stool samples) from the region of Apulia.

2. Materials and methods

2.1. Sample collection, preparation and storage

2.1.1. Environmental and food samples

During a two-year study (spring 2012 to winter 2014), we collected samples of well water, drinking water and water treated using various physicochemical and biological methods from municipal treatment plants located at four different sites (A, B, C and D)1 in Apulia, Italy, and used to irrigate eight different vegetable or fruit crops planted in succession (cucumber, lettuce, fennel, celery, tomato, melon, endive and chicory).

At each irrigation session time (depending on the kind of crop and on weather conditions), 100 l of water were collected and filtered through a yarn-wound cartridge filter (pore-size: 1 µm; maximal pressure: 3 bar, maximal flux speed: 6 l/min (G.F., Italy). The filter was opened and washed three times, with a volume of 1 l of buffered detergent solution containing phosphate-buffered saline (PBS), 0.1% Tween-80 (cat. no P1754, Sigma Aldrich), 0.1% sodium dodecyl sulphate (SDS) (cat. No. L6026, Sigma Aldrich) and 0.005% antifoam B emulsion (cat. no. A5757, Sigma Aldrich). The three washes were concentrated by centrifugation (1800 × g for 15 min), and the water sediment was subjected to Percoll-sucrose (specific gravity: 1.10) flotation.

Upon harvesting of each vegetable crop, pooled soil samples (1 kg) were collected from under the water dripper from each plot using a hand-made cylindrical steel sampling column (100 mm h/45 mm diameter) to a depth of 10 cm, and vegetables or fruits (5 kg each) were yielded by hand. All samples (cartridge filters, soil and vegetables) were kept at + 4 °C in plastic bags and transported to the Soil Chemistry Laboratory in the Department of Agricultural and Environmental Science of the University of Bari, Italy, where they were processed within 48 h of sampling.

Soil and vegetable samples were processed essentially as described previously (Kuczynska and Shelton, 1999, Robertson and Gjerde, 2001). In brief, soil samples (10 g) were individually suspended in 50 ml of buffered detergent solution, vortexed for 10 min, and the suspension filtered through double gauze. After washing of the gauze with 50 ml of buffered detergent solution, the filtrate was centrifuged at 500 × g for 10 min. Then, the pellet was overlaid on Percoll-sucrose and centrifuged for 10 min, 1800 × g at 4 °C. For vegetable samples, external leaves of lettuce, fennel or celery (100 g of each), or the external part of cucumber, melon or tomato (1 kg of each) was washed three times with buffered detergent solution. The washing water was centrifuged at 1800 × g for 15 min, and the pellet subjected to Percoll-sucrose flotation.

2.1.2. Human faecal samples

Human stool samples (n = 40) submitted to the main Hospital in Apulia region (Azienda Ospedaliera Policlinico, Bari, Italy; period 2009 to 2011) were tested in this study. Subjects were 25 males and 15 females of 1–81 years of age; nine of them were immigrants and 31 were native Italians; 19 had diarrhoea and 21 did not (Table 1. Supplementary data). The stool samples were transported frozen to the Parasitology Laboratory, in the Department of Agriculture Science, Food and Environment of the University of Foggia, Italy.

2.2. Isolation of genomic DNA, quantitative (q)PCR and melting curve analysis

Genomic DNA was isolated from water, soil, vegetable and faecal samples using the Nucleospin tissue/stool kit (Macherey-Nagel, Netherlands), according to the manufacturer's instructions. The extracted DNA was eluted in 50 µl of distilled water, quantified using a Qubit 2.0 fluorometer and subjected to qPCR.

The qPCR and melting curve analysis were performed in a CFX-96 Real Time Instrument (BioRad, Italy), as described previously (Marangi et al., 2015). In brief, PCR was performed in 20 µl (final volume) containing EvaGreen® Reagent (BioRad, Italy), 0.5 µM of each primer CCITS2-F (5′-GCAGTCACAGGAGGCATATATCC-3′) and CCITS2-R (5′-ATGAGAGACCTCACAGCCAAAC-3′) designed to a region (116 bp) within ITS-2 of C. cayetanensis (see Lalonde and Gajadhar, 2008). Approximately 50–100 ng of either genomic DNA, cloned ITS-2 rDNA (0.5 pg; reference, positive-control) or water (negative control) in 5 µl was added to the reaction. Cycling conditions were: initial denaturation at 98 °C for 2 min, followed by 35 cycles at 98 °C for 5 s, and 59 °C for 15 s. Fluorescence data were collected at the end of each cycle as a single acquisition. Melting curve analysis was performed at the end of each PCR run (70 °C to 95 °C at 0.5 °C/5 s). Each sample was analysed in duplicate, and the amplification cycle threshold (Ct) and melting temperature (Tm) values were calculated. The diagnostic Tm peak for Cyclospora was 84.5 °C. The criteria used to define a test-positive sample were: (a) a detectable amplification curve, (b) a Tm value of ± 0.5 °C with reference to the Tm value of plasmid control, and (c) a dF/dT fluorescence value of > 2.

Raw data were normalized by applying curve-scaling to a line of best fit, so that the highest fluorescence value was 100 and the lowest was zero (standard normalized melt curve). Then, the curves were differentiated, and a composite median curve was constructed using the median fluorescence values for each sample. The melting traces for each sample were subtracted from this composite median curve in order to draw a residual plot (difference graph). The number of DNA copies per µl was calculated by relating the Ct mean value of each sample to a standard curve for the plasmid control, and the number of oocysts was calculated, assuming that an oocyst contains 15 copies of rDNA, depending on the stage of sporulation (Varma et al., 2003).

2.3. Single-strand conformation polymorphism (SSCP) analysis and sequencing

All amplicons were subjected to single-strand conformation polymorphism (SSCP) analysis using protocol B (Gasser et al., 2006). Amplicons representing distinct banding profiles were selected and treated with exonuclease I and shrimp alkaline phosphatase (Fermentas), according to the manufacturer's instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) using the primer CCITS2-R. The quality of each sequence was assessed based on the corresponding electropherogram using the programme BioEdit; the sequence tags determined (~ 100 bp)

were compared with known reference sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Statistical analysis

For the various matrices (water, soil, vegetable/fruit and stool), prevalence was defined as the number of samples that tested positive for C. cayetanensis using the molecular assay / number of samples examined × 100, using a 95% confidence interval (95% CI). For environmental samples, prevalences were compared among different sampling sites and matrices as well as for the same type of matrix among different collection sites, among various matrices from the same site, and among sampling seasons. Humans were categorised according to age (10-year intervals); data for human samples were compared according to gender, age group, country of origin (Italian vs. non-Italian), and presence or absence of diarrhoea. Comparisons were carried out using Chi-square test or Fisher's exact test. Differences were recorded as significant ($P < 0.05$) and highly significant ($P < 0.01$). Odds ratios using a 95% CI were also calculated as a measure of association.

3. Results

3.1. Environmental and food samples

Of a total of 213 tested samples [i.e., well (n = 16), treated (94) and drinking (3) water samples used for irrigation, soil (51) and vegetable/fruit (49) samples], 33 (15.5%) were qPCR test-positive for Cyclospora (Table 1). Cyclospora DNA was detected in treated water (21.3% of 94), and in well water (6.2% of 16), but not in drinking water (0% of 3). The prevalence in water from site C (35.9%) was significantly higher than at other sites (Table 1). Cyclospora DNA was also detected in soil samples (11.8% of 51) and in vegetables (12.2% of 49). The prevalence of Cyclospora in soil irrigated with water from treatment plant C (35.9%) was significantly higher than that at other sites. Among the eight crops (of fennel, tomato, cucumber and celery), the highest (individual) prevalence (18.7%) was recorded on fennel irrigated with water from treatment plant D (Table 1). The number of Cyclospora oocysts estimated in qPCR test-positive samples was predicted to range from 67 to 526 (water), from 230 to 661 (soil) and from 74 to 493 (vegetables) per 5 µl of genomic DNA. In environmental and food samples, the highest prevalence was seen in autumn (20.4%) and the lowest (10.7%) in summer (Table 2).

3.2. Human faecal samples

Of 40 faecal samples, 11 (27.5%) of those that tested negative for common intestinal parasites by conventional coproscopy (data not shown) were qPCR test-positive for Cyclospora, and five of them (5/11, 45.4%) originated from people with diarrhoea. Cyclospora was significantly more prevalent in patients of 40–50 years of age (P value = 0.0245, odd ratio = 7.22; 95% CI = 1.34–38.92), but no statistically significant differences were found between females and males, Italians and immigrants, or between people with diarrhoea and those without (Table 1; Supplementary data). The number of Cyclospora oocysts per gram of faeces in qPCR test-positive samples was predicted to range from 84 to 476. All 44 ITS-2 amplicons produced in qPCR were subjected to SSCP analysis. Two distinct profiles were displayed; profile 1 represented 43 amplicons (from water, soil, vegetables and stool samples); profile 2 represented one amplicon (from a human faecal sample). The sequence tag representing profile 1 differed by one nucleotide from that of profile 2, and had 100% and 99% identity to a reference sequence with accession no. AF301386, respectively. For all samples studied, the agreement between qPCR and SSCP results was perfect (K-statistic = 1).

4. Discussion

This is the first comprehensive molecular survey of Cyclospora in environmental, food and faecal samples from humans in Europe. Cyclospora DNA was specifically amplified from these samples using primers designed to ITS-2 of C. cayetanensis (Lalonde and Gajadhar, 2008). Cyclospora DNA was shown to be highly prevalent (21.3%) in treated water and well water (6%) usually used for irrigation, as well as soil (11.8%), vegetables (18.7%) and human stool (27.5%) samples; the numbers of oocyst equivalents in test-positive samples varied between 67 and 661 per 5 µl of genomic DNA. Given that the degree of nucleotide sequence variation in ITS-2 both within and among recognised species of Cyclospora has not yet been rigorously assessed, we have not assigned a particular species name to Cyclospora detected here in water, soil or vegetable samples using PCR-based methods.

The detection of Cyclospora DNA in several water sources, including drinking water, has possible public health implications, provided that viable, infective oocysts are present in the water. Contaminated drinking water has been responsible for previous cyclosporosis outbreaks in humans in countries including USA and Nepal (reviewed by Ortega and Sanchez, 2010). In Europe, Cyclospora has been recorded previously in various water sources only in two countries. Specifically, in Spain, this protist was found in drinking, river water and wastewater at a prevalence of 6%, 13% and 2%, respectively (Galvan et al., 2013). Cyclospora DNA has also been detected in tap water samples from the toilets on Italian trains (Giangaspero et al., 2015). In addition, C. cayetanensis has been detected or inferred to occur in wastewater in countries endemic for cyclosporosis, including Nepal (Sherchand et al., 1999), Peru (Sturbaum et al., 1998), Tunisia (Ben Ayed et al., 2012) and the USA (Kitajima et al., 2014). Moreover, Cyclospora has also been detected in water used for the irrigation or processing of vegetables in Vietnam (Tram et al., 2008) and Cambodia (Vuong et al., 2007).

Although contaminated soil is recognised as a possible source of human infection, particularly when living or hygiene standards are low (Chacin-Bonilla, 2010b), presently, there is no other published information on the presence of Cyclospora in soil. In the present study, 6 of 51 (11.8%) soil samples irrigated with well water or treated water were shown to be contaminated with Cyclospora DNA. The extent of wastewater treatment would determine whether oocysts remain intact and viable. Here, it seems reasonable to assume that the most advanced technologies used in treatment plants A and B (i.e. membrane ultrafiltration; GDF plus UV radiation) prevented the passage of oocysts, so that contamination could be attributed to the presence of Cyclospora DNA. On the other hand, oocysts and/or DNA might have been present as a consequence of a failure of water treatment systems to remove them completely. The use of apparently inadequate, traditional water treatment techniques in some plants (i.e. disinfection/flocculation/sedimentation; flocculation systems; activated sludge and chlorination system; lagoon treatment system) seems to explain the high level of Cyclospora DNA in soil irrigated with water from plant C and on vegetables irrigated with water from plant D, and high levels of Escherichia coli and total coliforms (139,000 and 660,500 CFU/100 ml (data not shown), respectively, far exceeding the thresholds permitted in the EU). Clearly, further research is warranted to assess the significance of well water, wastewater and soil contamination in relation to the dissemination of Cyclospora infections or cyclosporosis in humans and/or animals.

The detection of Cyclospora DNA on vegetables is likely to be of considerable public health concern, depending on the specific identity of any oocysts present, their viability and infectivity to humans. Numerous studies have already shown considerable levels of Cyclospora contamination on fresh produce (spinach, lettuce, cabbage, basil, mustard leaves and various herbs) on sale at agricultural markets, with prevalences of up to 8% in Asia (Cambodia, Nepal) (Sherchand et al., 1999, Vuong et al., 2007), North Africa

(Egypt, Ghana) (Duedu et al., 2014, Hassan et al., 2012) and South America (Peru, Costa Rica) (Calvo et al., 2004, Ortega et al., 1997). More recently, Cyclospora has been detected even on ready-to-eat packaged leafy greens in Canada (Dixon et al., 2013). However, there are no previous data for Cyclospora contamination on vegetables for Europe, although there is one report of an outbreak in Germany involving 34 people associated with contaminated butterhead lettuce (imported from France) and mixed lettuce, dill, parsley and green onions (imported from Southern Italy, including the same area in which the present study was carried out), likely linked to contaminated irrigation water and/or soil (Doller et al., 2002).

In the current investigation, the variation in prevalence of Cyclospora contamination on vegetables may relate to differences in plant morphology. For instance, cucumbers (Cucumis sativus) have a relatively uneven surface that might allow Cyclospora oocysts to attach to the surface when irrigated with contaminated water. By contrast, tomatoes have a smooth surface, which may tend to reduce the rate of oocyst adhesion. Irrespective, the present data indicate that contaminated vegetables should be considered as a potential source of C. cayetanensis, particularly when untreated water is used for the irrigation of vegetables or preparation prior to packaging. The statistically higher prevalence in autumn of Cyclospora on vegetables (18.7%) and in environmental samples (20.4%) might relate to the relatively low (6–15 °C) temperatures and higher rainfall (52–97 mm) during this season in Apulia, a Mediterranean area with hot, dry summers (http://www.ilmeteo.it/portale/medie-climatiche/Puglia). The use of contaminated wastewater to irrigate vegetables in the dry months might be the reason for greater contamination with Cyclospora in summer. Regarding environmental samples, only a few studies have detected Cyclospora and assessed seasonal variation in prevalence and/or distribution. Other studies have reported the presence of Cyclospora in treated drinking water from tanks (el-Karamany et al., 2005) and in treated, piped water (Elshazly et al., 2007) throughout the year. By contrast, in Spain, a study of different drinking water, treated water and river water (Galvan et al., 2013) revealed that Cyclospora tends to be present in spring, but this finding was not supported statistically. As Cyclospora has been detected morphologically and/or molecularly in domestic and wild birds, mice, rats, dogs, cattle, primates and even invertebrates such as cockroaches, beetles and millipedes (Ortega and Sanchez, 2010), it is possible that such animals might also contribute to the spread of Cyclospora oocysts via water and the environment.

In the present study, the percentage of test-positive samples (27.5%) from humans was quite high. Although the present sample set might have been somewhat biased, it seems that the prevalence of cyclosporosis remains underestimated. Nonetheless, autochthonous cases of this disease have been recorded in Italy (Maggi et al., 1995, Masucci et al., 2008, Masucci et al., 2011, Scaglia et al., 1994). The higher number of test-positive samples found in the 40–50 age group concurs with a previous study conducted in this country (Masucci et al., 2011) and might relate to the greater tendency for this age group to eat raw vegetables. Since it is particularly difficult to remove all of the soil from between internal leaves of fennel, for example, this vegetable appears to present a relatively high risk for contamination. Based on the findings of the present study, other Cyclospora-contaminated fresh produce and drinking water may also represent possible sources of infection to humans in and around the study areas. However, the small number of human stool samples examined prevents any detailed conclusions regarding transmission or seasonal occurrence of Cyclospora infection or cyclosporosis in humans.

Previous studies have also detected Cyclospora in humans in various parts of the world, with prevalences of up to 41% in developing countries (Chacin-Bonilla, 2010a, Ortega and Sanchez, 2010). Isolated cyclosporosis cases, mainly involving travellers returning from endemic countries or outbreaks, have been recorded in European countries, including France, Germany, Greece, Sweden, Ireland, Netherlands, Spain, Sweden,

Turkey, the UK (Insulander et al., 2010, Ortega and Sanchez, 2010) and Italy, where Cyclospora has been reported to occur in travellers (Caramello et al., 1995, Drenaggi et al., 1998, Masucci et al., 2011) as well as in immuno-compromised (Maggi et al., 1995, Scaglia et al., 1994) or immuno-competent residents (Masucci et al., 2008).

In conclusion, we believe that this is the first detailed study in Europe of Cyclospora in several biological matrices in a particular region of Italy. Our data suggest that irrigation water, soil and vegetables might be contaminated by C. cayetanensis oocysts, which might represent a source of infection to humans in the geographical area of Apulia. The estimated numbers of oocyst equivalents found in soil and vegetables in this study, together with evidence of a high viability of Cyclospora oocysts usually found in water (Ortega et al., 1998, Smith et al., 1997) and the low infectious dose of this protist (Dixon et al., 2005), give some cause for concern. Future investigations are warranted to confirm the specific status of Cyclospora detected in the present study and the actual prevalence and spread of Cyclospora in/among humans in Italy using a cohort-based survey. Pending more information, in our opinion, public health authorities should: (a) implement effective sanitary controls on water used in agriculture, i.e. irrigation of vegetables and fruit trees; (b) carry out frequent routine monitoring of wastewater treatment plant performance; and (c) promote the use of advanced inactivation technology in wastewater treatment plants. In the mean-time, it seems prudent for diagnostic service laboratories to establish sensitive molecular tools for the specific and quantitative detection of Cyclospora, and the viability and infectivity testing of oocysts. In addition, the EEC Directive 98/83 (Europe) should include this protist in the list of water contaminants of potential human health concern.

The following are the supplementary data related to this article.

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Supplementary Table 1. Distribution and percentage of Cyclospora cayetanensis in patients (n = 40) tested in the present study, according to gender, age group, nationality, and presence or absence of diarrhoea.

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