Cyclospora cayetanensis travels in tap water on Italian trains

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ABSTRACT

Tap water samples from the toilets of an Italian national railway train were collected over a period of 10 months and tested for the presence of *Cyclospora cayetanensis* (*C. cayetanensis*) using EvaGreen® real-time polymerase chain reaction (RT-PCR) assay coupled with high resolution melting (HRM) analysis for protozoan detection and oocyst quantification. *C. cayetanensis* positive samples were detected in March, April, and May 2013, with the number of oocysts of 4, 5, and 11 per liter, respectively. This is the first finding of *C. cayetanensis* in water samples in Italy. The findings call for an improvement of hygiene and water safety by the Italian national railway company.

Key words | *Cyclospora cayetanensis*, HRM, Italy, real-time PCR, tap water, train

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INTRODUCTION

Cyclospora cayetanensis (*C. cayetanensis*) (Apicomplexa, Eimeriidae) is an important emerging human intestinal parasite (Ortega *et al.* 1993, 1994) responsible for anorexia, nausea, abdominal cramping, prolonged self-limiting, and relapsing diarrhea, low-grade fever, and weight loss in both children and adults (Zhou *et al.* 2011).

Cyclosporiasis is endemic in several tropical and subtropical developing countries; in industrialized countries, cyclosporiasis has been observed mainly in travelers but also in people with no history of foreign travel and/or immunocompetent people (Ortega & Sanchez 2010; Chacin-Bonilla 2010).

Contaminated water and food are the primary means by which *C. cayetanensis* infections spread (Ortega & Sanchez 2010; Hall *et al.* 2011), but it also spreads via soil, particularly in impoverished communities in endemic areas and in some areas of developed countries (Chacin-Bonilla 2010).

Oocysts may survive for long periods in external environmental conditions: 2 months at 4 °C and 7 days at 37 °C (Smith *et al.* 1996; Ortega *et al.* 1998). They may also be resistant to routine chemical disinfectants or sanitizing water-processing methods.

These, and their low specific gravity, facilitate their spread. The infectious dose has not been determined, but based on outbreak investigations and extrapolations from other coccidians is thought to be relatively low (Sterling & Ortega 1999; Dixon *et al.* 2005), possibly as low as between 10 and 100 oocysts (Dixon *et al.* 2005).

C. cayetanensis oocysts have been found in drinking water, wastewater, and recreational water in several countries – not always undeveloped countries – and are responsible for waterborne outbreaks worldwide (Ortega & Sanchez 2010; Chacin-Bonilla 2010). In endemic regions, *Cyclospora* prevalence shows a marked seasonality in both clinical and environmental samples. However, this seasonal trend does not appear to be univocal, since differences have been shown between geographical areas and between periods of maximal rainfall to periods with hot and dry weather (reviewed by Chacin-Bonilla (2010), Ortega & Sanchez (2010), and Galván *et al.* (2013)).

As a protozoan with waterborne transmission potential, *Cyclospora* has been included in the US Environmental Protection Agency (EPA) (http://www.water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm).

figu is not, however, included in the European legislation list of fecal indicators (Directive 98/83/ EEC) and its detection is left to national/local sanitary authorities. The Italian reference standards for the quality of water for

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human consumption are Legislative Decrees Nos. 31/2001 and 27/2002, implemented by Directive 98/83/EEC, which do not provide for detection of *Cyclospora*.

Although cases of cyclosporiasis have been recorded in Italy (Scaglia *et al.* 1994; Caramello *et al.* 1995; Maggi *et al.* 1995; Drenaggi *et al.* 1998; Masucci *et al.* 2008) and an outbreak has also been registered which was possibly due to water contamination (Doller *et al.* 2002) there are, as yet, no data available on the presence of *Cyclospora* in any water samples.

In order to carry out a preliminary investigation into the presence of *C. cayetanensis* in water, we used a recently developed fast EvaGreen® real-time polymerase chain reaction (RT-PCR) protocol coupled with high resolution melting (HRM) analysis to test samples of tap water from the toilets on trains.

METHODS

From October 2012 to July 2013, 1 liter tap water samples were collected once a month from the toilets of 10 different coaches of a busy passenger train, traveling the 820-km Adriatic route daily from south to north and vice versa.

DNA extraction

Genomic DNA was isolated from individual water samples using the Nucleospin Soil kit (Macherey-Nagel, The Netherlands), according to the manufacturer's instructions. The extracted DNA was eluted in 50 μL of DEPC water and all the samples were stored at −20 °C until the molecular analyses were performed.

RT-PCR and HRM assay

Plasmid control

The genomic DNA from *C. cayetanensis* was selected as reference to design a plasmid control. A sequence of about 116 bp from *C. cayetanensis* ITS-2 gene (AF301382; Olivier *et al.* 2001) was cloned into a pEX-A vector (Eurofins, MWG/Operon, Ebersberg, Germany). In order to assess the dynamic range of linearity in the RT-PCR assay, 10-fold serial dilutions of the *C. cayetanensis* ITS-2 plasmid with a range from 16.5×10^8 to 0.165 copies/μL, respectively, was subjected in duplicate to the RT-PCR assay, and the threshold cycle (C_t) mean value was plotted against the logarithm of their copies/μL. Each standard curve was generated by a linear regression of the plotted points. PCR efficiency (E) was calculated according to the equation: $E = 10 - 1/\text{slope} - 1$ (Rasmussen 2001). E between 90 and 130% and a correlation $R^2 < 1$ values were considered optimal for validation of the RT-PCR assay.

RT-PCR and HRM assay set up

Amplification and melting analysis were performed in a CFX-96 Real Time Instrument (BioRad, Italy). *C. cayetanensis* ITS-2 gene primers were CCITS2-F (5′-GCAGTCACAGGAGGCATATATCC-3′) and CCITS2-R (5′-ATGAGAGACCTCACAGCCAAAC-3′) (Lalonde & Gajadhar 2008). A PCR mixture of 20 μL reaction contained 5 μL of DNA samples (or plasmid control), 5× EvaGreen® Reagent (BioRad, Italy) and 10 μM concentration of each primer. The RT-PCR protocol was as follows: initial denaturation at 98 °C for 2 min, followed by amplification for 45 cycles at 98 °C for 5 s and at 59 °C for 30 s, then elongation at 95 °C for 60 s and at 70 °C for 60 s. Fluorescence data were collected at the end of each cycle as a single acquisition. The melting program was performed at the end of each reaction, and consisted of temperature increases from 70 to 95 °C at intervals (ramps) of 0.2 °C/10 s. Each sample was analyzed in duplicate, and the C_t and melting temperature (T_m) mean values were recorded. The criteria used to define a positive sample were (1) detectable amplification curves, (2) T_m value of ±0.5 °C vs T_m value of plasmid control, and (3) dF/dT fluorescence value above 2.

Sensitivity and specificity of the RT-PCR assay

All DNA samples were controlled for the presence of PCR inhibitors: 1.5 μL of the DNA sample was mixed with 0.5 μL plasmid control and analyzed by RT-PCR assay. Samples with a C_t value greater than the C_t value of the plasmid control alone (diluted two-fold) were subjected to RT-PCR assay after a 10-fold dilution. The sensitivity and specificity of the RT-PCR assay were calculated for all the

samples. Sensitivity was determined as the number of true positives divided by the sum of true positives and false negatives. Specificity was determined as the number of true negatives divided by the sum of true negatives and false positives.

DNA sequencing

C. cayetanensis positive samples were purified with Exonuclease I (EXO I) and Thermosensitive Alkaline Phosphatase (FAST AP) (Fermentas) enzymes, and directly sequenced in both directions using the ABI PRIMS BygDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) with the same primers as the respective PCRs, according to the manufacturer's instructions. Sequences were determined on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), electropherograms were inspected by eye and consensus sequences were determined. BLASTn software was used to compare each sequence to nucleotide sequences of *C. cayetanensis IT-2* gene in publicly available databases (Altschul *et al*. 1997).

Quantitative analysis

Absolute quantification was performed for the positive samples; the amount of DNA (copies/μL) was calculated by relating the C_t mean value of each sample to a standard curve obtained from a plasmid control. Moreover, the number of oocysts was calculated for *C. cayetanensis* according to Varma *et al.* (2003).

RESULTS

Set-up and validation of RT-PCR assay

A good range of linearity was observed for *C. cayetanensis* ITS-2 plasmid and for seven of 10 tested serial dilutions; *E* was 128.2% with a slope of −2,791 and a high fitting value ($R2 = 0.985$) (Figures 1(a) and 1(b)). The lower limit of detection was 5 copies/μL of DNA pathogens in samples. Amplified DNA fragments were 116 bp with T_m mean values of 84.5 °C as shown in Figures 1(c) and 1(d).

Positive samples and quantification

Of the 10 water samples collected, the three (30%) taken in March, April, and May tested positive to *C. cayetanensis*. These positive samples contained copies of DNAs corresponding to 4, 5, and 11 oocysts, respectively.

HRM analysis and sequencing

Melting curve shapes and T_m points showed the same curves as those shown by *C. cayetanensis* ITS-2 plasmid control (Figure 2). No other curves attributable to other species were detected. Sequencing analysis confirmed that all samples belonged to *C. cayetanensis*.

DISCUSSION

Trains are supplied with water each day at the departure station from a tank carrying potable water; water is stored in 1,500–1,800 liter (steel or resin) tanks on the roof of the train coaches; each tank serves one coach, and the water is delivered to the toilets via a hydraulic system of pipes and pumps. The railway company should program a routine twice-yearly sanitation program.

Advanced molecular tools have improved detection of parasites. EvaGreen® combined with HRM analysis can be used for rapid and selective detection of *C. cayetanensis* (Aksoy *et al*. 2014) due to its high specificity and ability to quantify an extremely low number of oocysts.

Cases of cyclosporiasis have been reported in Spain, Greece, Germany, the UK, Switzerland, France, Sweden, and the Netherlands, and in some of these countries outbreaks have also been described (Ortega & Sanchez 2010). In Italy, *Cyclospora* has been detected in both immunocompromised (Scaglia *et al*. 1994; Maggi *et al*. 1995) and immunocompetent natives (Masucci *et al*. 2008) and in travelers (Caramello *et al.* 1995; Drenaggi *et al.* 1998). An outbreak involving 34 people at four independent sites in Germany has been associated with a salad, which may have been irrigated with contaminated water; some of the vegetables involved came from southern Italy (Apulia and Campania regions) (Doller *et al.* 2002). Workers working in contact with soil or water are also considered as a

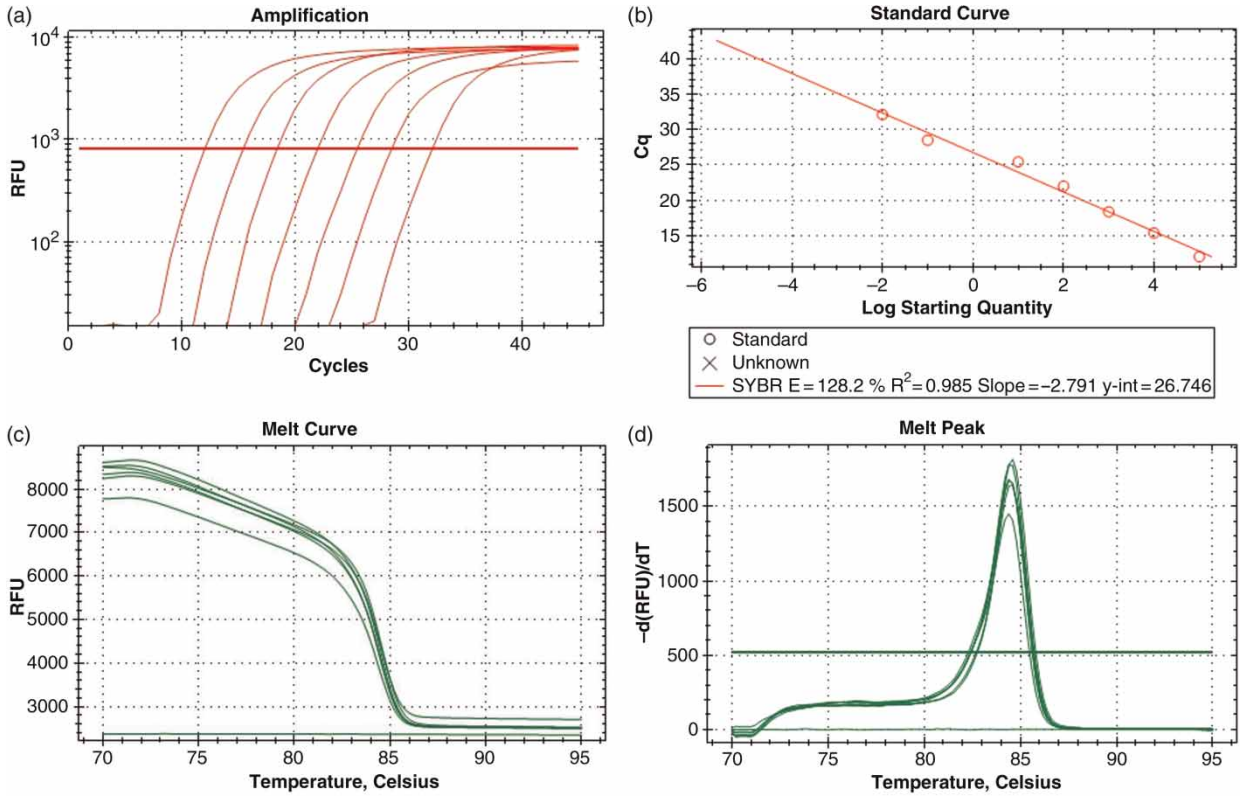

Figure 1 | Amplification and standard curves (a) and (b) of seven-fold serial dilutions and melting curves and melt peaks (c) and (d) of *C. cayetanensis* ITS-2 plasmid, respectively.

possible cause of contamination (Chacin-Bonilla *et al.* 2007; Chacin-Bonilla 2008).

In this study, *Cyclospora* was detected in 30% of 10 water samples, and this is the first reported finding of *C. cayetanensis* oocysts in water samples in Italy.

Thus far, humans have been recognized as the definitive hosts of *C. cayetanensis* infections, and humans eliminate the protozoans by the fecal route. Assuming that the train tanks where the water is stored are – or should be – sealed, it is important to understand how the water became contaminated. Drinking water supplied to trains in the spring could be contaminated at its origin or by the tanks used to transport it to the trains, or stagnant water in the storage tanks could have contaminated the water supply after a pump failure. In fact, it is also possible that coprophagic animals (i.e., cockroaches, beetles, millipedes, perhaps also rats or birds) act as reservoirs/paratenic hosts; if these then enter water pipes and tanks, they may play a role in the dissemination of oocysts. However, their suitability as reservoirs or paratenic hosts of *C. cayetanensis* is unknown (Ortega & Sanchez 2010).

At present, we do not know if the *C. cayetanensis* DNA oocysts detected in this study were alive. However, the high viability of *Cyclospora* oocysts in water (Smith *et al.* 1996; Ortega *et al.* 1998) and the still unknown but possible low infectious dose (Dixon *et al.* 2005) mean that the detection of 4–11 *Cyclospora* oocysts in 1 liter of water is a cause for concern. Although tap water in the train is labeled as 'not drinking water', it might accidentally be drunk by children, and is usually used for washing hands, cleaning fruit, brushing teeth, etc.

Different studies have confirmed worldwide the seasonality of *Cyclospora* in both clinical and environmental samples; however, as reviewed by Chacin-Bonilla (2010), it is not similar among different countries/areas and lacks easy explanation. In relation to environmental samples, only a few studies have detected *Cyclospora* and evaluated its seasonal behavior. Some studies have reported the

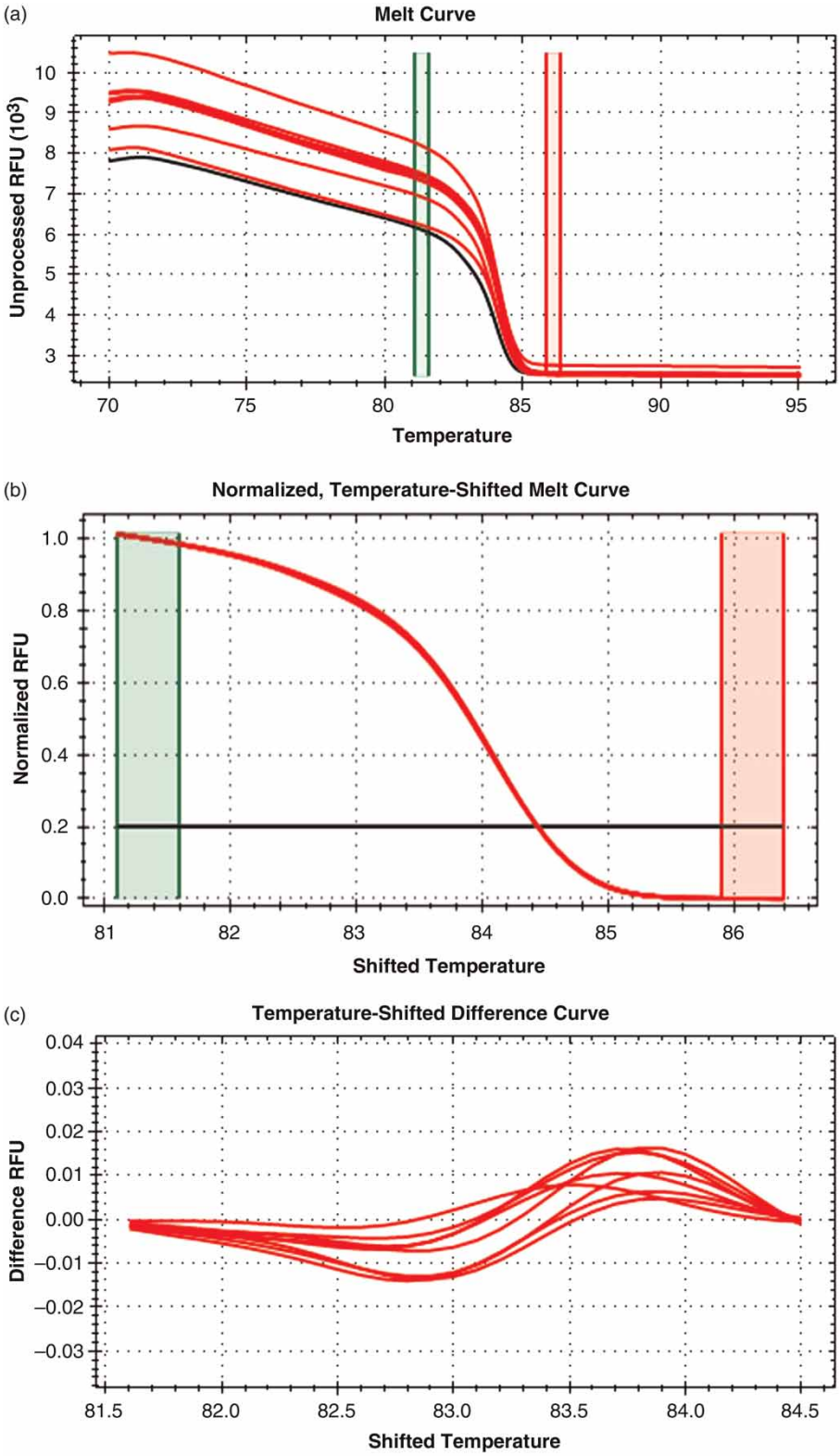

Figure 2 | Raw data (a), normalized (b), and temperature shifted melting curves (c) of *C. cayetanensis* ITS-2 plasmid and positive samples analyzed using HRM.

presence of this protozoan throughout the year in treated potable water from tanks (el-Karamany *et al*. 2005) and treated piped water (Elshazly *et al*. 2007), but in this study, the detection of *Cyclospora* in train drinking water tanks in March, April, and May, i.e., in spring, overlaps the very recent results obtained by Galván *et al.* (2013) in Spain, where a high prevalence of positive samples from drinking water treatment plants were found in spring months, even if significant difference prevalence rates between the seasons could not be found (Galván *et al*. 2013).

In Italy, few cases of cyclosporiasis have been reported in the native population (Scaglia *et al*. 1994; Maggi *et al*. 1995; Masucci *et al*. 2008, 2011); it is possible that cyclosporiasis infections in Italy remain undiagnosed because this protozoan is not routinely investigated. Consequently, it is possible that the prevalence of *C. cayetanensis* infected people may be higher than believed.

The present study was carried out over the course of approximately one year, but the results obtained cannot be considered conclusive, due to the complete lack of longitudinal studies/surveys on *Cyclospora* prevalence in environmental and human samples (in both symptomatic and asymptomatic subjects) in Italy and to the consequent impossibility of correlating the infection pattern with *C. cayetanensis* and the seasonal pattern reported in this paper.

In-depth and broad longitudinal epidemiological studies are required in Italy to define the seasonal trend of *Cyclospora* in the environment, in humans and also in animals, since the latter could act as possible reservoirs. This would show whether the spring months coincide with human infection in Italy and represent a risk for health in this country.

CONCLUSIONS

This study supports the presence of *Cyclospora* in tap water in Italy. A large-scale study is needed to investigate whether this contamination is an isolated episode, or if the problem is more widespread, and involves many Italian trains. In the meantime, these preliminary results highlight the need for improved hygiene by the Italian national railway company, which should be alerted to the problem in order to guarantee hygienic traveling conditions.

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