

# First Italian case of Cyclosporiasis in an immunocompetent woman: local acquired infection

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## SUMMARY

*Cyclospora cayetanensis* is a coccidian agent of chronic diarrhea in humans with a worldwide distribution. We report the first documented case of acquired Cyclosporiasis in Italy. The patient was an immunocompetent woman with no recent history of travel outside the country. Microscopy detected *Cyclospora* oocysts in a feces sample. PCR detected the pathogen in a second sample, which had tested negative by microscopy. The patient was investigated to detect other microorganisms in feces, such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., and enteroviruses: all were negative. All symptoms disappeared 72h after the beginning of therapy.

**KEY WORDS:** *Cyclospora cayetanensis*, Laboratory diagnosis, PCR, Chronic diarrhea

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## INTRODUCTION

*Cyclospora cayetanensis* is a coccidian protozoa, Phylum Apicomplexa, implicated as pathogen agent of gastrointestinal infections in humans, prolonged watery diarrhea, fatigue and anorexia. In some people infection could be asymptomatic. Several years ago, *Cyclospora cayetanensis* was linked to travellers and immunocompromised patients, but then cases of immunocompetent hosts were reported in literature (Doller *et al.*, 2002; Koru *et al.*, 2006; Ooi *et al.*, 1995; Sancak *et al.*, 2006; Yazar *et al.*, 2004). This organism has since been identified in patients with a worldwide geo-

graphic distribution. Infection is acquired by the fecal-oral route through ingestion of infective oocysts in contaminated water or on contaminated fruits and vegetables. Person to person transmission is unlikely, because *Cyclospora cayetanensis* oocysts are not infectious at the time passing via stool and require a developmental period in the environment. Incubation period for infection usually appears to be approximately from a few days to ten, but typically about one week and two weeks in immunocompetent patients.

Laboratory diagnosis presents several problems given the morphologic similarities of *Cyclospora cayetanensis* oocysts to those of *Cryptosporidium* as well as the clinical similarities between the two diseases.

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## MATERIALS AND METHODS

### Patient

In March 2007, a 63-year-old woman presented with a history of chronic secretory diarrhea. She

was having 8-10 discharges of diarrhea every 24 hours, during the night as well as the day. The discharges were preceded by abdominal pain and flatulence, without rectal bleeding. The symptoms had begun two months before with one stool a day. After about two weeks of symptoms, she had taken bacitracin and neomycin at home for one week and the symptoms had worsened. At the time of examination, the patient had lost about 8Kg (16-lb) in weight. Abdominal ultrasonography showed hepatomegaly and meteorism. Abdominal CT showed multiple enlarged mesenteric, hepatic and aortic lymph nodes. Enteric CT showed a normal bowel wall. Laboratory tests showed increased values for acute phase reactants.

The patient, who was retired, lived in a large Italian city and had not traveled to tropical or developing countries during the previous 12 months. She was neither immunosuppressed nor immunocompromised. For many years she had suffered from diabetes and hypertension, taking metformine, gliclazide, irbesartan and doxazosin as chronic therapy. She had chronic mild renal failure (Cr clearance 40 ml/min) and had smoked 15 cigarettes a day for about 40 years. Two months before the symptoms began, she had suffered a painful cyst in her right knee and had been treated with NSAIDs and bed rest.

#### *Microscopy and sampling*

For parasite analysis, we performed routine direct microscopy examinations for ova and parasites, using three unpreserved stool samples collected over a seven day period. Samples were stored in fresh normal saline smear. We then applied the formalin ethyl acetate concentration technique using a Midi PARASEP (DiaSys Europe Ltd - Wokingham, UK) with Lugol's iodine. Both sets of smears were examined using a 40X phase contrast objective. To create permanent smears we applied the acid-fast modified Ziehl-Neelsen procedure, working directly with unpreserved stool. We examined the samples using a 100X oil immersion objective.

#### *DNA extraction and PCR qualitative assay*

To perform the molecular tests, we prepared DNA templates from unpreserved stool frozen at -20°C, using the DNASTool miniKit (Qiagen, Valencia, Calif.) according to the manufacturer's instruc-

tions. For PCR detection of *Cyclospora* spp., we applied the procedure described by (Yoder *et al.*, 1996) using the nested primer pairs F1E/R2B (primary amplification) and F3E/R4B (secondary amplification) (MWG-Biotech AG). For primary amplification, we used HotStarTaq Master Mix (Qiagen, Valencia, Calif.) in a 50- $\mu$ l reaction volume, containing 200  $\mu$ M concentrations of dATP, dCTP, dGTP, and dTTP. The reaction mixture had an adjusted final concentration of 2 mM MgCl<sub>2</sub> and 0.2  $\mu$ M of primers F1E (5'-TACCCAAT-GAAAACAGTTT-3') and R2B (5'-CAGGAGAAGC-CAAGGTAGG-3'). The amplification profile consisted of initial denaturation at 95° for 15 minutes followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 90 s. A final extension cycle at 72°C for 10 min was followed by soaking at 4°C. The expected size of the primary amplicon was 636 bp. The second, nested PCR was conducted with 5  $\mu$ l of the primary amplicon as a template in a reaction volume of 50  $\mu$ l using the primer pair F3E (5'-CCTTCCGCGCTTCGCTGCGT-3') and R4B (5'-CGTCTTCAAACCCCCTACTG-3'). The cycling parameters were identical to those used in the first round of amplification with the exception of the annealing temperature, which was raised to 60°C. Secondary amplification yielded a 294-bp amplicon. Amplicons were detected by electrophoresis: 10  $\mu$ l of amplified product were run in a 2% agarose gel (Eurobio, Les Ulis, France) and visualized using ethidium bromide staining with the Fluor-S<sup>TM</sup> multimaginer (Bio-Rad) UV light transilluminator. The molecular size of amplicons were determined by comparison with a commercial DNA molecular size marker (molecular weight marker VIII, Roche Diagnostics S.p.A, Monza, MI).

## RESULTS

Microscopy clearly detected oocysts in the second sample of three after a seven day period. In the wet mounts the oocysts appeared as nonrefractile spheres with a diameter of 8-10  $\mu$ m. Each sphere contained a cluster of refractile globules surrounded by a thick membrane. In iodine-stained wet mounts, the internal globules stained brown (Figure 1). Permanent procedure stained the oocysts from pink to red or deep purple and

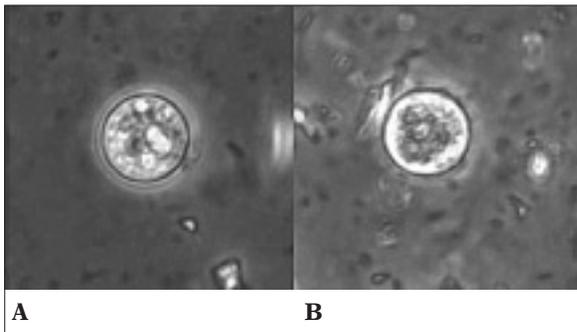


FIGURE 1 - *Cyclospora* oocysts in stool smear preparations. A: *Cyclospora* oocysts in native preparation. B: iodine-stained wet mount (original magnification 400X).

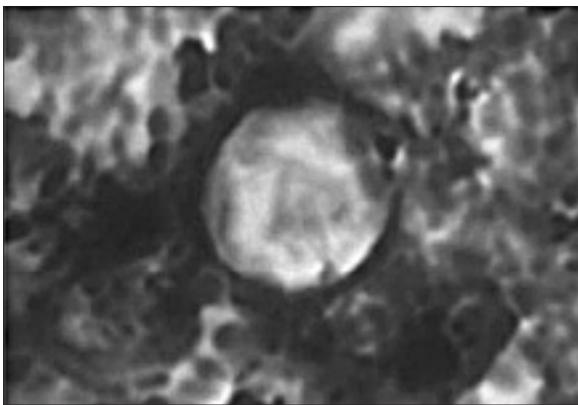


FIGURE 2 - *Cyclospora* oocysts in stool smear preparation stained with the acid-fast modified Ziehl-Neelsen procedure (original magnification 1000X).

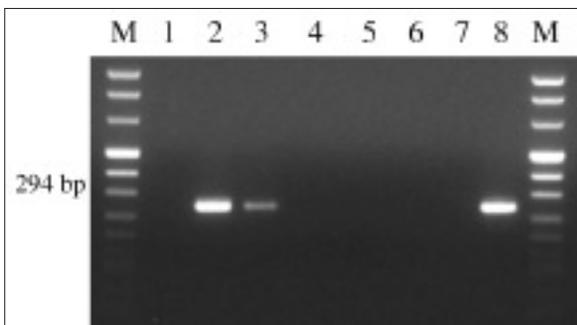


FIGURE 3 - Detection of PCR-amplified *Cyclospora* DNA in agarose gel. M: DNA Molecular Markers; lane 1: negative sample; lane 2: positive sample; lane 3: positive sample; lane 4: negative sample; lane 5: negative sample; lane 6: negative sample; lane 7: negative control; lane 8: positive control (From lanes 1 to 3 before therapy; from lanes 4 to 6 after therapy).

many did not stain at all but appeared bubbly, clear, glassy and refractile spheres (Figure 2).

Subsequently we applied molecular methods to the samples which had been tested by microscopy. In the molecular assay, the third sample also tested positive (Figure 3).

We evaluated the patient for the presence of other pathogen microorganisms such as *Salmonella* spp., *Shigella* spp, *Campylobacter* spp., *Yersinia* spp, and enteroviruses. The results were negative in all cases. The patient refused an enteroscopy to search for parasites in small bowel biopsy samples.

We treated the patient with IV fluids and thrimethoprim-sulphamethoxazole 160/800 mg twice daily for seven days, as suggested for cases of *Cyclospora* infection. The diarrhea disappeared after 72h, together with the patient's abdominal pain, fatigue and anorexia. Values for acute phase reactants returned to normal. On the seventh day of therapy the patient developed urticaria. Thirty days later, we used microscopy and PCR to test three samples of stool collected over a seven day period. All tests were negative for *Cyclospora* (Figure 3).

## DISCUSSION

*Cyclospora cayetanensis* is a coccidian protozoa belonging to the Apicomplexa. It was first recognized as a human pathogen in 1977 (Ashford, 1979). It appears that humans are the only host. Gastrointestinal infection causes prolonged watery diarrhea, fatigue and anorexia. Early reports associated the pathogen with travelers and immunocompromised patients. However, more recently there have been reports of Cyclosporiasis and associated diarrhea in immunocompetent patients (Doller *et al.*, 2002; Koru *et al.*, 2006; Ooi *et al.*, 1995; Sancak *et al.*, 2006; Yazar *et al.*, 2004). In Italy there have been few reports of infection, mainly in immunocompromised patients and travelers (Drenaggi *et al.*, 1998; Maggi *et al.*, 1995). Cyclosporiasis appears to be distributed throughout the world. It seems that patients of all ages are at risk of infection. As with other parasitic protozoa such as *Cryptosporidium* and *Giardia* (Dawson, 2005), infection is acquired by the fecal-oral route through ingestion of infective oocysts in contaminated water or on contaminated fruits

and vegetables. *Cyclospora cayetanensis* oocysts passed in stool only become infectious after a period of development in the environment. Person to person transmission is thus unlikely. In immunocompromised patients, the incubation period ranges from a few days up to 10 days, in immunocompetent patients typical values are between 1 and 2 weeks.

Laboratory diagnosis of *Cyclospora cayetanensis* is difficult and requires close collaboration with clinicians to establish a correct choice of techniques, including permanent stain, and/or the use of molecular methods. During tests, it is particularly important to distinguish between infections with *Cyclospora cayetanensis* and with *Cryptosporidium*. This is particularly hard because of the morphological similarities between oocysts from the two organisms and the clinical similarities between the two diseases. Both organisms require the same permanent stain, however oocysts from *Cyclospora cayetanensis* have a diameter of 8-10 µm; oocysts from *Cryptosporidium* are smaller (Garcia and Bruckner, 1997).

The case reported here shows the value of molecular methods. One of our samples that tested negative with microscopy resulted positive with PCR. If this sample had been tested only with microscopy, and if it had been the only sample sent for analysis, it would not have been possible to diagnose the patient's condition. This suggests that the prevalence of *Cyclospora cayetanensis* could be underestimated in the epidemiological literature. More generally, it is evidence of the value of molecular methods in the diagnosis of parasitosis – an area where direct microscopy is laborious and time-consuming and may not be entirely reliable.

#### ACKNOWLEDGMENTS

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