Annals of Tropical Medicine & Parasitology, Vol. 105, No. 5, 339–349 (2011)

Cases of cryptosporidiosis co-infections in AIDS patients: a correlation between clinical presentation and GP60 subgenotype lineages from aged formalin-fixed stool samples

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Received 2 March 2011, Revised 6 April 2011, Accepted 13 June 2011

Nine cases of cryptosporidiosis co-infections in AIDS patients were clinically categorised into severe (patients 1, 3, 8 and 9), moderate (patients 4 and 5) and mild (patients 2, 6 and 7). Formalin-fixed faecal specimens from these patients were treated to obtain high quality DNA competent for amplification and sequencing of the 60-kDa glycoprotein (*GP60*) gene. Sequence analysis revealed that one patient was infected with *Cryptosporidium hominis* whereas the remaining eight patients were infected with *C. parvum*. Interestingly, the patients showing severe cryptosporidiosis harboured two subtypes within the *C. parvum* allelic family IIc (IIcA5G3 and IIcA5G3R2), whereas patients with moderate or mild infections showed various subtypes of the *C. parvum* allelic family IIa (IIaA14G2R1, IIaA15G2R1, IIaA17G3R1 and IIaA18G3R1).

DNA extraction and genotyping of *Cryptosporidium* spp. is a challenging task on formalin-fixed stool samples, whose diagnostic outcome is age-dependent. The method herein reported represents a step forward routine diagnosis and improves epidemiology of HIV-related clinical cases. Due to the need to elucidate genetic richness of *Cryptosporidium* human isolates, this approach represents a useful tool to correlate individual differences in symptoms to subgenotyping lineages.

INTRODUCTION

Cryptosporidium spp. are oocysts-forming Apicomplexan protozoa that cause a widespread enteric infection (cryptosporidiosis) in humans and animals, a disease which results in sickness and severe diarrhoea. In the immunocompetent host, the symptoms, when present, last for about 2 weeks before the infection is cleared (Riggs, 2002). Conversely, in high-risk host groups, particularly neonates (Huang *et al.*, 2004; Keating, 2005) or people with suppressed or deficient immune systems (i.e. HIV/AIDS patients), the infection can be severe, long-lasting and

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DOI: 10.1179/1364859411Y.0000000025

even fatal (Amadi *et al.*, 2002; Certad *et al.*, 2005). The vast majority of human cases of cryptosporidiosis worldwide are caused by two species, *Cryptosporidium parvum* and *C. hominis* (Morgan *et al.*, 1999). However, other species, including *C. felis*, *C. meleagridis*, *C. canis*, *C. suis*, *C. muris* (Xiao and Ryan, 2004) and, more rarely, *C. cuniculus* (Robinson *et al.*, 2010) and *C. ubiquitum* (Fayer *et al.*, 2010) can infect humans too, especially children under the age of 5 years and immunocompromised individuals (Abubakar *et al.*, 2007).

The identification and characterization of *Cryptosporidium* species and population variants (genotypes and subgenotypes) is fundamental to study the epidemiology of cryptosporidiosis, being a valid support for prevention and control strategies (Putignani and Menichella, 2010). Oocyst morphology, host specificity or preferences in infection sites do not provide sufficient information for the identification of *Cryptosporidium* species, genotypes or subgenotypes (Fall *et al.*, 2003; Jex and Gasser, 2010). Advances in molecular technologies have led to significant improvements in the characterization of the genetic variability among and within *Cryptosporidium* species (Jex and Gasser, 2008). In particular, the amplification and sequencing of one or more genetic loci (markers) have been used for the categorization of *Cryptosporidium* species, genotypes or subgenotypes (Xiao *et al.*, 2004; Chalmers *et al.*, 2005; Plutzer and Karanis, 2009; Bouzid *et al.*, 2010). Mini- and micro-satellites, or simple sequence repeats, constitute a rich source of polymorphism and have been extensively used for high-resolution genotyping and mapping (Feng *et al.*, 2000). In particular, the *GP60* gene is useful for such studies as it contains multiple regions with high mutation rates, including a 'hyper-variable' microsatellite region (Strong *et al.*, 2000).

Herein we describe a successful retrospective subgenotyping of *C. parvum* and *C. hominis* isolates, performed on aged formalin-fixed stool samples from nine HIV-infected patients, based on a combination of optimized DNA extraction and sequence analysis of a fragment of the GP60 gene. Subgenotype lineages were correlated to clinical manifestations for each patient. The understanding of this relationship may represent the starting point for further extended studies on the correlation between isolate type and symptom severity in HIV-infected patients.

MATERIALS AND METHODS

Collection of Faecal Specimens and Parasite Identification

Formalin-fixed stool samples were collected from nine HIV-infected patients admitted to the National Institute for Infectious Diseases 'Lazzaro Spallanzani' (Rome, Italy) between 2005 and 2006. The research protocol was approved by the Institutional Review Board of the Hospital. Participants had given written informed consent before enrollment into the study. Clinical data were provided for each patient (Table 1).

Faecal samples were collected, fixed and filtrated by using a 10% formalin disposable system (Meridian Biosciences, Cincinnati, OH, USA). *Cryptosporidium* oocysts were detected by a modified Ziehl–Neelsen staining (Becton Dickinson, Franklin Lakes, NJ, USA). After microscopic examination, the residual stool aliquot was stored at 4°C in formalin before DNA extraction.

DNA Extraction from Formalin-fixed Stools

DNA extraction was performed by the following modification of the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). Briefly, 200 µl of formalin-fixed stool samples were washed three times with phosphate-buffered saline (pH 7.4) and centrifuged to remove traces of formalin before DNA extraction. Samples were submitted to 15 freeze–thaw cycles (freezing in liquid nitrogen for 1 minute and heating at 65°C for 1 minute) and 1.4 ml of ASL buffer (QIAamp DNA stool mini kit; Qiagen) was added. The suspension was mixed, incubated for 5 minutes at 95°C and

TABLE 1. *Clinical features of the nine HIV-infected patients recruited in this study*

Patient	Gender/ age (year)	Time of diagnosis	CDC stage/ CD4	Risk factor	PN	Fecal occult blood	Electrolyte disturbances	HAART	Albumin	Comorbidities	Specimen	Viral load (cp/ml)	Clinical presentation	Outcome
1	F/47	September	C3 37	IDU	Yes	Present +	Hypokalemia	Yes	Low	Wasting syndrome Chronic hepatitis C Anaemia Major depression Nephrolithiasis Oxyuriasis Oral candidiasis	Duodenal biopsy, stool	>500 000	Watery diarrhoea Weight loss (~15 kg) Fever Abdominal pain Vomiting Productive cough Headache Arthromyalgias	Discharged
2	F/52	March	C3 6	Sexual blend	No	Present +	Hyponatremia	No	Low	Chronic renal failure Chronic hepatitis C Urinary tract infection Syphilis	Stool	3977	Watery/mucoid diarrhoea Weight loss (~15 kg) Vomiting Productive cough	Discharged
3	M/40	January	C3 30	Homosexual	Yes	Present +	Hyponatremia Hypocalcemia Hypomagnesemia	On failure	Low	Mucocutaneous KS Wasting syndrome Esophageal candidiasis COPD Anaemia	Stool	97 102	Watery diarrhoea Weight loss Fever Abdominal pain Dysphagia Muscle cramps	Died*
4	M/35	February	C3 14	Unknown	Yes	Present +++	Hypokalemia Hypomagnesemia	No	Low	Pneumonia Anaemia	BAL, stool	257 603	Watery diarrhoea Weight loss Fever Vomiting Abdominal pain Cough	Discharged
5	F/44	January	C3 124	Sexual blend	No	Present +++	Hypokalemia Hypocalcemia Hypophosphatema	No	Normal	Urinary tract infection Oral candidiasis Anaemia	Stool	118 316	Watery diarrhoea Weight loss Fever Abdominal pain	Discharged
6	M/35	February	C3 14	IDU	No	Absent	Hypokalemia	No	Low	Drug abuse Alcoholic hepatitis Thrombocytopenia Oral candidiasis	Stool	1938	Watery diarrhoea Abdominal pain Dehydratation	Discharged
7	M/54	January	C3 25	Sexual blend	No	Absent	None	No	Normal	Intestinal ascariasis Oral candidiasis Syphilis	Stool	294 830	Watery diarrhoea Weight loss (~9 kg) Fever Vomiting	Discharged
8	F/44	December	C3 1	Unknown	Yes	Absent	None	No	Normal	Atypical mycobacteriosis Oral candidiasis Chronic hepatitis C Anaemia Wasting syndrome	Stool	69 767	Watery diarrhoea Weight loss (~15 kg) Fever Abdominal pain Vomiting Productive cough	Discharged

Table 1. *Continued*

Patient	Gender/ age (year)	Time of diagnosis	CDC stage/ CD4	Risk factor	PN	Fecal occult blood	Electrolyte disturbances	HAART	Albumin	Comorbidities	Specimen	Viral load (cp/ml)	Clinical presentation	Outcome
9	M/38	March	C3 10	Unknown	Yes	Present ++ ++++	Hypokalemia Hypomagnesemia Hypophosphatemia Hypocalcemia	On failure	Low	Pancytopenia Wasting syndrome Pancreatitis Hepatorenal syndrome Ascites Esophageal candidiasis Epididimitis, CMV infection Urinary tract infection	Sputum, stool	10 224	Diarrhoea Weight loss Abdominal pain Vomiting Dysphagia Jaundice	Died

Patients 1, 3, 8 and 9 were referred as affected by severe cryptosporidiosis, patients 4 and 5 with moderate cryptosporidiosis; patients 2, 6 and 7 were characterized by mild symptoms.
COPD: chronic obstructive pulmonary disease; KS: Kaposi's sarcoma; PN: parenteral nutrition; IDU: intravenous drug user; BAL: bronchoalveolar lavage.
*Cause of final exitus.

processed according to the manufacturer's instructions (QIAamp DNA stool mini kit; Qiagen). DNA was eluted in 50 µl of H_2O, previously heated at 65°C for 15 minutes. As positive control, DNA was extracted with the same procedure from 10^7 Percoll purified oocysts from the *C. parvum* isolate ISSC162, subgenotype IIaA15G2, kindly provided by the Department of Infectious, Parasitic and Immunomediated Diseases (Istituto Superiore di Sanità, Rome, Italy).

GP60-based Subgenotyping

A fragment of the *GP60* gene was amplified using a previously described nested-PCR protocol (Sulaiman *et al.*, 2005). Both PCR reactions, set up to a 25 µl volume, were performed as previously described (Glaberman *et al.*, 2002), except for the annealing temperature (50 and 51°C, for first and second rounds, respectively). An aliquot of 5 µl from the primary PCR reaction was used as template in the secondary PCR reaction. Between primary and secondary PCRs, a purification step, performed by using a PCR clean-up system (Wizard; Promega, Fitchburg, WI, USA), was included to remove residual formalin affecting the secondary PCR. The resulting DNA fragments, loaded on 2.2% agarose gels (Sigma Aldrich, Milan, Italy), were visualized by Gel Doc XR System and analysed using the software Quantity One 4.6.3 (Bio-Rad, Hercules, CA, USA). The size of the amplified PCR products was estimated by comparison with the Flash Gel 50 bp–1.5 kb DNA ladder (Lonza, Rockland, ME, USA). Sequencing reactions were performed by using 1.6 pmol of inner GP60 primers (Sulaiman *et al.*, 2005) and 1.5 µl of amplified DNA in a final volume of 10 µl, hence cycled according to the manufacturer (version 3.1; Applied Biosystems, Foster City, CA, USA). Consensus sequences corrected by base calling were probed against non-redundant GenBank databases using BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). The highest score matching was considered the most likely correct identification, and only an identity

score of more than 98% was used as a threshold to be considered significant. GP60 subgenotypes were classified according to the nomenclature proposed by Sulaiman (Sulaiman *et al.*, 2005). Nucleotide sequences were deposited into GenBank under accession nos. HM-371366 to HM371374.

RESULTS

Clinical Manifestations of Cryptosporidiosis

Cryptosporidium was identified in stools from all nine patients, but in bronchoalveolar lavage (BAL), sputum and duodenal biopsy from three different patients. All patients but one had CD4 counts less than 50 cell/μl and a CDC C3, and were therefore susceptible to severe cryptosporidiosis. None of the subjects had suppressed HIV viraemia, with viral load ranging from 1.938 to more than 500 000 cp/ml, mainly (8/9 patients) due to poor compliance or failure of previous HAART regimens. Eight out of the nine *Cryptosporidium* cases were diagnosed between the moist months December and March, and one in September. Diarrhoea was complained of by all patients, and was watery for 8 of 9. Weight loss was reported by eight patients, with three of them experiencing a strong weight loss ($\sim$15 kg in 1 month) and four patients suffering from wasting syndrome. Other common symptoms were: abdominal pain (7/9 patients), fever and vomiting (6/9 patients), and cough, often productive (4/9 patients); however, *Cryptosporidium* was isolated from sputum and BAL from only two patients. One patient had frank pneumonia and another had diffuse interstitial infiltrates. Nutritional biomarkers were also altered. Plasmatic albumin was low in 6/9 patients, suggesting strong and lasting malabsorption. As a consequence, the majority of patients (5/9 patients) required the administration of parenteral nutrition (PN) to contrast clinical condition worsening. Chronic diarrhoea and malabsorption led to electrolyte disturbances, especially hypokalaemia, except for two patients. The fecal occult blood resulted positive in six patients, but no patient clearly showed haematic stools. For patient 1, the histological examination of duodenal biopsy revealed sphere shapes suggestive of duodenal cryptosporidiosis. Also the patient 9 had symptoms compatible with biliary involvement, confirmed also by imagining evidences, but neither endoscopic retrograde cholecystopancreatography nor biopsies of biliary ducts were performed to certainly diagnose biliary cryptosporidiosis. Candidiasis was a recurrent co-infection, observed in 7/9 patients, mark of mucosal changes or altered intestinal immunity. We employed symptom features to categorize patients into three different groups: severe, moderate and mild cryptosporidiosis.

In the first set, patients 1, 3, 8 and 9 were grouped (Table 1). Patient 1 was a hepatitis C virus co-infected subject who presented with-wasting syndrome, abdominal pain and vomiting. During the hospitalization, an esophagogastroduodenoscopy with biopsy was performed and the histological examination revealed a duodenal cryptosporidiosis (data not shown). Stool examination revealed the presence of *Cryptosporidium* oocysts (Table 2). Treatment with metronidazole and paromomycin failed with progressive worsening of clinical conditions. However, after a few days, the patient refused additional therapies and was discharged against medical advice (Table 1). Patient 3 presented with mucocutaneous Kaposi's sarcoma and wasting syndrome. The patient was on HAART failure. He presented with fever, diarrhoea, dysphagia and weight loss. After hospitalization PN, glucose, amino acids and electrolyte solutions were started, but clinical conditions progressively deteriorated and after 32 days of hospital stay the patient died (Table 1). Patient 8 was a hepatitis C virus co-infected subject presenting with wasting syndrome and atypical mycobacteriosis who began both HAART and antimycobacterial therapy, with further improvement and discharge (Table 1). Patient 9 presented with wasting

syndrome, esophageal candidiasis and systemic CMV infection with recurrent symptoms of diarrhoea and vomiting. Multiple HAART regimens were undertaken but failed. During hospitalization, he presented abdominal pain with marked elevations of amylases, lipases and cholestatic indexes. Abdominal ultrasound and magnetic resonance cholangiography showed intra- and extrahepatic dilatation of the biliary tree, edematous pancreas and a gallbladder filled with dense bile (suggesting duodenal cryptosporidiosis), but a duodenal biopsy was not undertaken. A chest X-ray evidenced diffuse interstitial infiltrates and pleural effusion. Sputum (data not shown) and stool examinations revealed *Cryptosporidium* oocysts (Table 2). His clinical conditions progressively worsened and he died.

The second set (moderate symptoms) included patients 4 and 5 (Table 1). Patient 4 complained lack of appetite and had diarrhoea for about 2 months. Due to diarrhoea persistence, patient underwent a rectosigmoidoscopy suggesting ulcerative colitis; hence, a therapy with mesalazine and metronidazole was begun. Despite the therapy, the diarrhoea persisted and the patient began to additionally present productive cough and fever. A chest X-ray showed an interstitial reticulonodular pattern with a basal parenchymal consolidation (Table 1). Pulmonary tuberculosis was suspected but the BAL revealed the presence of *Cryptosporidium* oocysts (data not shown), also detected in large amounts in the stools (Table 2). Patient received PN, antibiotic therapy (paromomycin/azithromycin) and HAART with progressive improvement and further discharge. Patient 5 was admitted presenting fever, abdominal pain, diarrhoea and ingravescent loss of weight. Therefore, HAART was begun. Stool examination showed numerous *Cryptosporidium* oocysts (Table 2) and a therapy with paromomycin/azithromycin was hence administrated. Afterwards, the clinical manifestations ameliorated and the patient was discharged.

The third set (mild symptoms) included patients 2, 6 and 7. Patient 2 complained watery diarrhoea, abdominal pain and loss of weight for 2 months and productive cough for 1 month. Stool examinations revealed *Cryptosporidium* oocysts (Table 2). HAART was not started; however, amelioration of clinical conditions was observed leading to his discharge. Patient 6 complained fever, watery diarrhoea, abdominal pain and dysphagia. Stool examinations revealed *Cryptosporidium* oocysts (Table 2). After a few days, diarrhoea diminished and the patient was discharged against medical advice. For patient 7, the seropositivity status was unknown on admission. He presented diarrhoea, dyspepsia and progressive loss of weight. Rifaximin was administered without any improvement; hence, the patient was hospitalized and therefore tested HIV-seropositive. Stool examination was positive for both *Ascaris lumbricoides* and *Cryptosporidium* (Table 2). Diarrhoea persisted despite the use of albendazole, but was reduced after paromomycin/azithromycin administration, leading to patient discharge.

DNA Extraction and Amplification from Formalin-fixed Stools

The optimization of the DNA extraction step from aged formalin-fixed stools was based on repeated phosphate-buffered saline washings for formalin excess removal followed by a thermal stress-induced rupture of oocysts for DNA release, by repeated freeze/thaw cycles and heating of the samples at 95°C. Mechanical oocyst disruption was not associated to an improved DNA yield and competence for amplification (data not shown). DNA competence was improved by choosing a small size DNA target (358 bp), thus minimizing the effect of formalin-induced DNA fragmentation.

GP60-based Subgenotyping

Nested-PCR at the *GP60* locus yielded single products of the expected size for all samples. All products were successfully sequenced. BLAST analysis revealed that four isolates

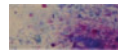
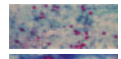
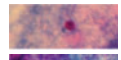
(samples 1, 3, 8 and 9) belonged to the *C. parvum* allelic family IIc: precisely, three samples (1, 3 and 8) were classified as subgenotype IIcA5G3 and one sample (9) as IIcA5G3R2 (Table 2). Four isolates, belonging to the allelic family IIa, were classified as IIaA18G3R1 (sample 2), IIaA15G2R1 (sample 4), IIaA17G3R1 (sample 5) and IIaA14G2R1a (sample 7) (Table 2). A single isolate (sample 6) belonged to the *C. hominis* subgenotype IaA12G1 (Table 2).

DISCUSSION

Based on symptom features, we categorized patients into three different groups: severe, moderate and mild cryptosporidiosis (Table 1). Consistently, the GP60 subgenotyping was discussed for the three patient sets. GP60 subgenotyping, for the first group of patients, identified only the IIcA5G3 lineage, while for the other two groups, a wider variety of lineages was reported. The *C. parvum* allelic family IIc has been frequently recorded (Jex and Gasser, 2010) and described from at least 12 countries: Australia (Jex *et al.*, 2007; Jex *et al.*, 2008; O'Brien *et al.*, 2008), Guatemala (Peng *et al.*, 2001), Japan (Abe *et al.*, 2006), Kuwait (Sulaiman *et al.*, 2005), Malawi (Peng *et al.*, 2003), Perù (Cama *et al.*, 2008), Portugal (Peng *et al.*, 2001; Alves *et al.*, 2006), South Africa (Leav *et al.*, 2002), Spain (Jex and Gasser, 2008), The Netherlands (Wielinga *et al.*, 2008), Slovenia (Soba and Logar, 2008) and Uganda (Akiyoshi *et al.*, 2006). However, to date, this allelic family has been recorded almost exclusively in humans (Xiao and Feng, 2008; Bouzid *et al.*, 2010) and only recently in hedgehogs (Dyachenko *et al.*, 2010). Based on the clinical evidence, wasting syndrome appears strongly linked to infection with parasite of the family IIc (4/4 patients), whereas no wasting syndrome was observed in patients infected with Ia and IIa families (Table 1). Remarkably, for 2/4 IIcA5G3 patients (1 and 9), esophagogastroduodenoscopy and magnetic resonance cholangiography exams suggested an extended and atypical colonization (Lumadue *et al.*, 1998) of the subtype IIcA5G3 to other sites (bile-duct/duodenum tract), different from sigmoid colon and rectum (Velásquez *et al.*, 2010). The infection of the biliary tree represents a reservoir from which intestinal cryptosporidiosis may relapse and allows the organism to avoid luminal antiparasitic agents such as paromomycin (Chalmers and Davies, 2010). In these cases, drugs like nitaxozanide with biliary excretion should be used (Baishanbo *et al.*, 2006). Indeed, the administration of paromomycin, even if integrated by metronidazole, failed as specific treatment of cryptosporidiosis (Palmieri *et al.*, 2005; Pozio and Morales, 2005) for patient 1. For the second patient set (4 and 5), subtypes IIaA15G2R1 and IIaA17G3R1 were observed. The IIa allelic family comprises some 50 subgenotypes, has been detected in 26 countries worldwide, including Italy, with a prevalence of 25.5% in humans and of 57.8% in all other hosts (Jex and Gasser, 2010), and has been described as the only or prevalent family in some extended geographical areas (Plutzer and Karanis, 2007). The IIaA15G2R1 subtype, prevalently reported from farm animals, has zoonotic potential (Jex and Gasser, 2010). However, IIaA15G2R1 has been detected in human samples from Australia (O'Brien *et al.*, 2008) and from sporadic human cases in Portugal (Alves *et al.*, 2003), Belgium (Geurden *et al.*, 2009), Ireland (Zintl *et al.*, 2009), Kuwait (Sulaiman *et al.*, 2005), Canada (Trotz-Williams *et al.*, 2006), Japan (Amer *et al.*, 2010) and the USA (Peng *et al.*, 2003). Remarkably, this subtype was also linked to an outbreak associated with an open farm in Wales (UK) (Chalmers *et al.*, 2005), supporting a zoonotic transmission. The other subtype IIaA17G3R1 was described both in calves and humans in Northern Ireland (Glaberman *et al.*, 2002; Thompson *et al.*, 2007), and in humans in Australia (Waldron *et al.*, 2009).

Interestingly, an extra-intestinal cryptosporidiosis was noted for patients 4, similarly

TABLE 2. *Conventional diagnosis and molecular typing of Cryptosporidium* spp. *isolates from stool samples*

	Microscopy-based diagnosis			Subgenotyping	
Patient/sample code	No. of oocysts/field	Image (×1000)	Species	GP60	GenBank Accession
1	5		*C. parvum*	IIcA5G3	HM371366
2	3		*C. parvum*	IIaA18G3R1	HM371367
3	1		*C. parvum*	IIcA5G3	HM371368
4	120		*C. parvum*	IIaA15G2R1	HM371369
5	10		*C. parvum*	IIaA17G3R1	HM371370
6	1		*C. hominis*	IaA12G1	HM371371
7	1		*C. parvum*	IIaA14G2R1a	HM371372
8	5		*C. parvum*	IIcA5G3	HM371373
9	1		*C. parvum*	IIcA5G3R2	HM371374

Dark and pale grey apply to sets of patients referred as affected by severe and moderate cryptosporidiosis, respectively.

to patient 9. However, for patient 4, after receiving PN, the paromomycin/azithromycin combined therapy gradually improved the clinical conditions leading to patient discharge. The same symptoms were also seen in patient 5, despite the absence of pulmonary involvement. Only stool examination showed numerous *Cryptosporidium* oocysts; the same antibiotic therapy, used for patient 4, induced amelioration of her clinical manifestations.

For the third set of patients (2, 6 and 7), subtypes IIaA18G3R1, IIaA14G2R1a and IaA12G1 were observed. The first subtype has been very commonly reported in humans and cattle in Ireland (Zintl *et al.*, 2009) and Australia (Waldron *et al.*, 2009), and in patient 2, it self-resolved upon patient's discharge. To the best of our knowledge, the IIaA14G2R1a subtype (Table 2), characterized in Germany for the first time by Broglia *et al.* (2008) in cattle, is herein reported as a novel human subtype (patient 7). An important co-infection with *A. lumbricoides* was observed for this patient, but only a specific anti-*Cryptosporidium* treatment resolved diarrhoea. Interestingly, only for patient 6, a *C. hominis* subtype (IaA12G1) was reported. The allelic family Ia has a worldwide distribution and is significantly diverse at the subtype level (25 subtypes reported; Jex and Gasser, 2010). There may be slight differences in the clinical manifestations depending on *C. hominis* subtypes, as previously described (Cama *et al.*, 2007). Apparently, the *C. hominis* infection for patient 6 was associated with the absence of fecal occult blood and required PN; there was no wasting syndrome and his weight loss was not remarkable when compared to the other patients.

Fixatives are essential for preservation of stool specimens. Buffered formalin is a traditional stool fixative, still considered the 'gold standard' in parasitology by virtue of its excellent long term preservative activity on intestinal parasites. However, the extraction of high-quality genomic DNA and its amplification is usually hampered by the high nucleic acid fragmentation chemically induced and by the presence of remnants of formalin that inhibit the amplification reaction. Because of

these difficulties, we have developed a new protocol that yields DNA suitable for amplification of small size targets, which are less affected by sporadic DNA breakages. *Cryptosporidium* DNA extraction was performed directly from stool samples, without prior oocyst purification or concentration steps. The method relies on a chemical lysis sustained by a heating effect acting at two protocol stages before lysis and spin-column steps. However, no mechanical oocyst disruption, based on additional required equipments, was introduced to enhance the DNA yield, as previously reported (Zhu *et al.*, 1998). The heating disruption appeared a resolutive determinant in the breakage of formalinized oocysts/sporozoites and in the release of amplifiable DNA. The protocol was introduced as a convenient bench-side procedure for stool specimen analysis and it was employed to select appropriate subgenotyping markers and to link subgenotyping lineages to individual manifestations of cryptosporidiosis.

Cryptosporidium subgenotyping is an important tool for the understanding its population structure. Given the need to investigate the genetic diversity of *Cryptosporidium* clinical isolates, still underestimated for technical difficulties to handle formalin-fixed stools, the method herein described may offer advantages for routine diagnosis and clinical epidemiological studies, despite referring to a small-scale patient dataset. Therefore, further evidence from larger sample size appears necessary to prove a statistically significant correlation between symptom severity and genotype. The interactionship may therefore provide insights on subgenotype lineages and HIV-associated cryptosporidiosis to unveil, on large patient cohorts, determinants of clinical diversity of cryptosporidiosis associated with genetic complexity of the parasite.

ACKNOWLEDGEMENTS. We would like to thank as source of support a grant from Ricerca Corrente (RC 2010) Children's Hospital and Research Institute 'Bambino Gesù' to Lorenza Putignani.

We wish to thank Dr Fabio Tosini (Istituto Superiore di Sanità, Rome, Italy) for providing purified *C. parvum* oocysts, and Dr Andreina Santoro for her careful revision of the manuscript.

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