Molecular characterization of *Cystoisospora belli* and unizoite tissue cyst in patients with Acquired Immunodeficiency Syndrome

JORGE NÉSTOR VELÁSQUEZ[1], GERMÁN ASTUDILLO OSVALDO[2], CECILIA DI RISIO[3], CRISTINA ETCHART[3], AGUSTÍN VÍCTOR CHERTCOFF[2], GLADYS ELISABET PERISSÉ[3] *and* SILVANA CARNEVALE[2,4]*

[1] *Hospital Municipal de Infecciosas 'Dr. Francisco Javier Muñiz', Uspallata 2272, (CP 1282) Ciudad de Buenos Aires, Argentina*
[2] *Instituto Nacional de Enfermedades Infecciosas - ANLIS 'Dr. Carlos G. Malbrán', Avenida Vélez Sarsfield 563, (CP 1281) Ciudad de Buenos Aires, Argentina*
[3] *Hospital Municipal General de Agudos 'Dr. José María Penna', Pedro Chutro 3380, (CP 1437) Ciudad de Buenos Aires, Argentina*
[4] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Avenida Rivadavia 1917, (CP 1033), Ciudad de Buenos Aires, Argentina*

(*Received 17 June 2010; revised 8 July 2010; accepted 22 July 2010; first published online 9 September 2010*)

SUMMARY

Cystoisospora belli is a coccidian protozoan that can cause chronic diarrhoea, acalculous cholecystitis and cholangiopathy in AIDS patients. We applied molecular methods to identify *Cystoisospora* at species level in AIDS patients presenting with and without the presence of unizoites in lamina propria. Coprological and histological analyses were performed in stool and/or biopsy samples from 8 *Cystoisospora*-infected patients. DNA from the same samples was used to amplify 2 fragments of the SSU-rRNA gene and the ITS-1 region. Sequencing of the resulting amplicons identified *C. belli* infections in all cases, independent of the presence or absence of unizoite tissue cysts. Further work should be considered in order to find molecular targets related to strain variations in *C. belli*.

Key words: *Cystoisospora belli*, AIDS, PCR, unizoite tissue cyst.

INTRODUCTION

Cystoisospora belli is an obligatory intracellular protozoan responsible for human cystoisosporosis (Lindsay *et al*. 1997*a*). In patients with the acquired immunodeficiency syndrome (AIDS) it has been described as another opportunistic agent that can cause chronic diarrhoea, acalculous cholecystitis and cholangiopathy (Benator *et al*. 1994; Zenta and Topazian, 2009). Reports of disseminated cystoisosporosis with unizoite tissue cysts in the lamina propria of the intestines, lymph nodes, liver and spleen in patients with AIDS have been published (Comin and Santucci, 1994; Restrepo *et al*. 1987; Michiels *et al*. 1994; Velasquez *et al*. 2001; Frenkel *et al*. 2003). The *Cystoisospora felis* species infects cats and several hosts (Dubey and Frenkel, 1972; Costa and Lopes, 1998; Melo *et al*. 2003). *C. felis* has an extraintestinal cycle with unizoite tissue cysts in cats as definitive hosts and in other hosts including mice, rats, hamsters, birds, rabbit and swine (Dubey and Frenkel, 1972; Costa and Lopes, 1998; Melo *et al*. 2003). Another species *Cystoisospora ohioensis* has an extraintestinal cycle with unizoite tissue cysts in dogs as definitive hosts and in other hosts such as mice and broiler chicken (Dubey and Frenkel, 1972; Dubey and Mehlhorn, 1978; Massad *et al*. 2003). The systemic distribution of unizoite tissue cysts in the viscera of the hosts showed a tropism by lamina propria of the intestines, lymph nodes, liver and spleen for both species (Dubey and Frenkel, 1972; Melo *et al*. 2003; Massad *et al*. 2003). Histological and ultrastructural electron microscopy findings of unizoite tissue cysts in humans and animals revealed similar structures, and this explains the difficulty in distinguishing zoites of *C. belli, C. ohioensis* complex and *C. felis* on the basis of their morphology (Dubey and Mehlhorn, 1978; Lindsay *et al*. 1997*a*). The ultrastructure of *Cystoisospora canis* monozoic cysts produced *in vitro* is similar to that of *C. belli* unizoite tissue cysts in immunocompromised patients (Mitchell *et al*. 2009). Immunohistological studies using antisera against *Cystoisospora suis* and other Apicomplexans showed variable reactions with unizoite tissue cysts in a patient with AIDS and *C. belli* (Lindsay *et al*. 1997*b*), but this procedure was not carried out on *C. felis* and *C. ohioensis*. Reports of molecular tools to differentiate genus and species level of *Cystoisospora* have been published (Müller

* Corresponding author: Av. Vélez Sarsfield 563, (CP 1281) Ciudad de Buenos Aires, Argentina. Tel: +54 11 4301 7437. E-mail: jorgeysilvana@speedy.com.ar

doi:10.1017/S0031182010001253

Table 1. Microbiological and molecular results for the eight *Cystoisospora*-infected patients

Case	Sample	Coprological analysis	Histopathology		18S rRNA gene		ITS-1 of the rRNA genes		
			Asexual and/or sexual stages of *Isospora* in epithelium	Unizoites in lamina propria	Nested PCR (396 bp product)	Sequencing	Nested PCR (440 bp product)	*Alu* I digestion (fragments length in base pairs)	Sequencing
1	Stool	*Cystoisospora* oocysts	ND	ND	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
2	Stool and biopsy	*Cystoisospora* oocysts	Yes	Yes	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
3	Stool and biopsy	*Cystoisospora* oocysts	Yes	No	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
4	Stool and biopsy	*Cystoisospora* oocysts	Yes	No	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
5	Biopsy	ND	Yes	Yes	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
6	Biopsy	ND	Yes	No	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
7	Biopsy	ND	Yes	No	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*
8	Biopsy	ND	Yes	No	+	*Cystoisospora belli*	+	200, 118, 101, 21	*Cystoisospora belli*

et al. 2000; Samarasinghe *et al*. 2008). These molecular tools could be used for distinguishing zoites of *C. belli*, *C. ohioensis* complex and *C. felis* species.

The present study was undertaken to identify *Cystoisospora* isolates, with and without the presence of zoites, with molecular tools in order to differentiate *C. belli* from other species of *Cystoisospora* in AIDS patients.

MATERIALS AND METHODS

Studied population

Eight adult patients with AIDS who were evaluated for chronic diarrhoea and diagnosis of cystoisosporosis were included in the present study. These individuals had an age range between 51 and 23 years, 6 were male and 2 were female, and all of them were living in Buenos Aires, Argentina.

Cystoisospora *isolates*

Fecal samples were collected from patients daily for 1 week. Stool specimens were fixed in 5% formalin and screened for the presence of *Cystoisospora* oocysts using formalin-ether concentration followed by microscopy.

Five biopsy specimens from patients were obtained from the distal duodenum by flexible fiberglass endoscopy. Two samples were fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin-eosin and Giemsa. Two specimens were fixed in 2·5% buffered glutaraldehyde and routinely processed for transmission electron microscopy (TEM). Tissue samples embedded for TEM were also stained with Azur II and examined by light microscopy. The fifth sample was stored at –20 °C in saline solution.

DNA extraction

DNA purification from frozen duodenal biopsy samples was carried out by phenol-chloroform extraction. Each sample was centrifuged for 5 min at 15 000 ***g***. The pellet was resuspended in 200 µl of phosphate-buffered saline pH 8, with 20 μl of 5% trypsin and incubated overnight at 37 °C with orbital shaking. Then, 200 μl of lysis buffer 2× (200 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1% SDS, 300 mM NaCl) and 4 μl of proteinase K stock solution (200 mg/ml) were added. Samples were incubated for 3 h at 58 °C and overnight at 37 °C.

After lysis, a standard phenol-chloroform extraction was carried out (Maniatis *et al*. 1989), and DNA was precipitated in absolute ethanol, dissolved in 10 µl of double-distilled water and kept at –20 °C until use.

When stool samples were employed, 2 ml of feces in 5% formaldehyde were gauze-filtered, treated with ether and centrifuged for 3 min at 15 000 ***g***. The pellet

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

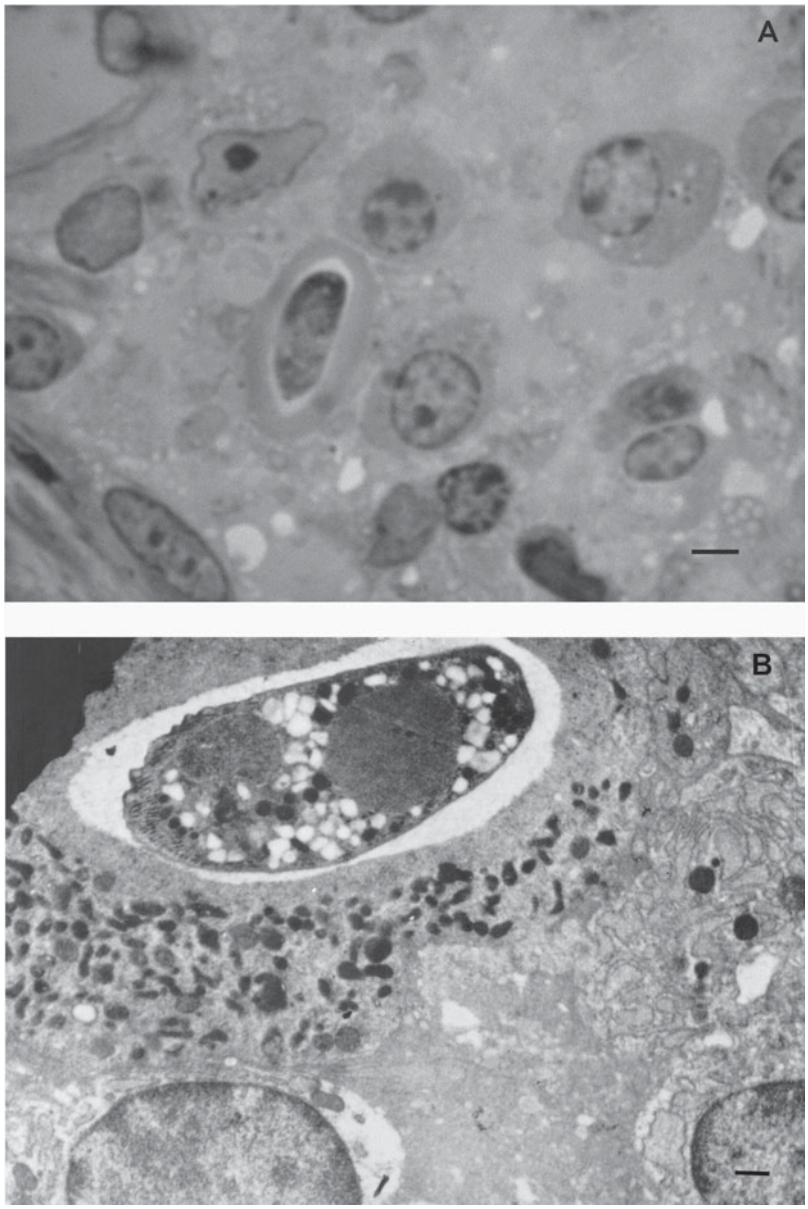

Fig. 1. Unizoite tissue cyst in lamina propria of the duodenum. (A) Light microscopy of an Azur II-stained biopsy specimen. (B) TEM. Scale bars: (A) 5 μm; (B) 1 μm.

was resuspended in 1 ml of 70% ethanol and kept overnight at –20 °C. Then samples were centrifuged for 5 min at 15 000 ***g*** and washed twice with 1 ml of double-distilled water by centrifugation at 15 000 ***g*** for 3 min each wash. Final pellets were evaporated at 37 °C until they appeared dry and then resuspended in 200 μl of lysis buffer 2× and 4 μl of proteinase K stock solution. The following steps were the same as those employed for biopsy specimens.

Nested PCR amplification for the 18S rRNA gene

Nested PCR to amplify a fragment of the 18S ribosomal RNA gene from *Cystoisospora* sp. was carried out essentially as described by Müller *et al*. (2000). For the first round the outer primer pair IsoFO (5′-GTGCCTCTTCCTCTGGAAGG-3′), corresponding to nucleotides 174 to 193 of the small subunit ribosomal RNA (SSU-rRNA) sequence of *Cystoisospora belli* (GenBank Accession nos. AF106935 and U94787), and IsoRO (5′-GCACTCCACCCAGTTAAGTGC-3′), corresponding to nucleotides 712 to 732 was employed in order to amplify a 559 bp DNA fragment. For the second round the inner primer pair IsoFI (5′-CGATGGATCATTCAAGTTTC-3′), corresponding to position 286 to 305, and IsoRI (5′-ACCACGTACACACCCCTAAG-3′), corresponding to position 662 to 681 was used to amplify a 396 bp DNA fragment.

For the first round, the sample was 1 μl of genomic DNA. Amplifications were performed in 50 μl reaction mixtures containing 0·5 μM each primer, 200 μM each deoxinucleotide triphosphate (dNTP), 2·5 U *Taq* DNA polymerase (Fermentas International Inc.), 20 mM $(NH4)_2SO_4$, 75 mM Tris-HCl, 0·01%

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

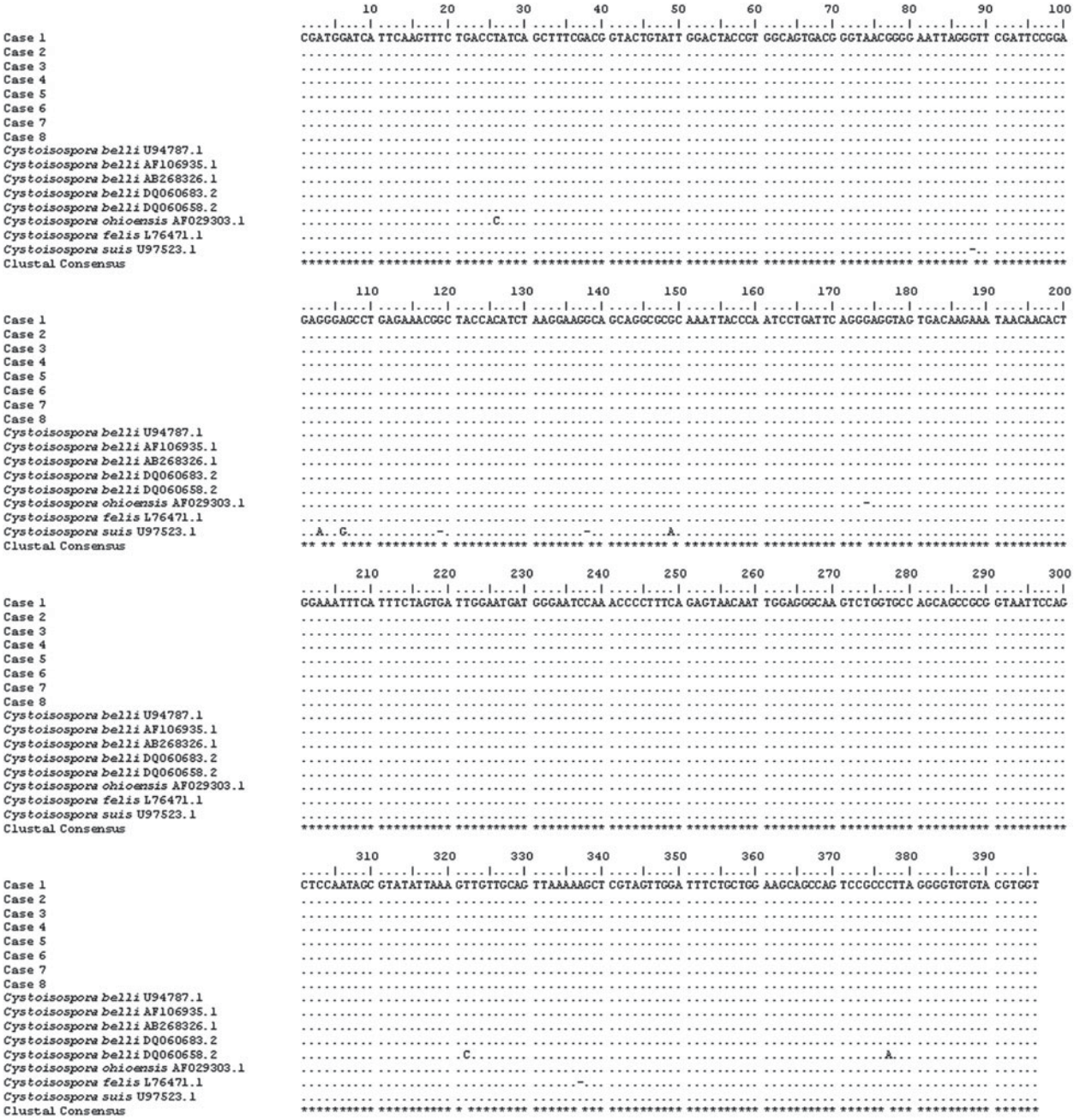

Fig. 2. Alignment of the sequences from the 8 amplicons generated by the 18S rDNA nested PCR with those of different *Cystoisospora* species. Dots and dashes represent identical residues and deletions, respectively. GenBank Accession numbers and species are detailed in Table 2.

Tween 20, 1·5 mM $MgCl_2$, 400 ng/μl bovine serum albumin (BSA), template.

After the first round 10 μl of amplification products were employed for the second reaction in a total volume of 50 μl using the same concentrations of reagents.

A P × 2 Thermal Cycler (Thermo Electron Corporation) was employed. After initial denaturation at 94 °C for 3 min, 35 cycles were run at 94 °C for 1 min, 61 °C (56 °C for the second round) for 2 min, and 72 °C for 3 min, with a 10 min final extension at 72 °C.

Twenty μl of amplicons from the second round were run on ethidium bromide-stained 2% agarose gels and visualized under UV illumination.

Nested PCR amplification for the internal transcribed spacer 1 (ITS-1) of the rRNA genes and RFLP analysis

The procedure was performed employing the primer pairs designed by Samarasinghe *et al*. (2008). The outer primer pair corresponded to ITSF (5′-CCGTTGCTCCTACCGATTGAGTG-3′) located in the 3′ end of the 18S rRNA gene, and EMR7 (5′-GCATTTCGCTGCGTCCTTCATCG-3′) located at the 5′ end of the 5·8S gene. For the first round reactions were carried out in 25 μl containing 0·5 μM each primer, 200 μM each dNTP, 1·25 U *Taq* DNA polymerase (Fermentas International Inc.), 20 mM

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

Table 2. Identity of the SSU-rRNA amplified fragment with different *Cystoisospora* sequences available at the GenBank database

Organism	GenBank Accession number	Identity (%)	Compared fragment (Nucleotide positions)	Reference
Cystoisospora belli	U94787.1	100	286–681	Pieniazek, (1997) (unpublished)
Cystoisospora belli	AF106935.1	100	286–681	Franzen *et al*. (2000)
Cystoisospora belli	AB268326.1	100	267–662	Abe and Kimata (2006) (unpublished)
Cystoisospora belli	DQ060683.2	100	280–675	Jongwutiwes *et al*. (2007)
Cystoisospora belli	DQ060658.2	99·4	280–675	Jongwutiwes *et al*. (2007)
Cystoisospora ohioensis	AF029303.1	99·4	287–681	Carreno *et al*. (1998)
Cystoisospora felis	L76471.1	99·4	269–663	Carreno *et al*. (1998)
Cystoisospora suis	U97523.1	98·4	288–680	Carreno *et al*. (1998)

Table 3. Identity of the ITS-1 amplified fragment with different *Cystoisospora* sequences available at the GenBank database

Organism	GenBank Accession number	Identity (%)	Compared fragment (Nucleotide positions)	Reference
Cystoisospora belli	DQ060683.2	99·5	1783–2221	Jongwutiwes *et al*. (2007)
Cystoisospora belli	EU124687.1	99·7	1–394	Samarasinghe *et al*. (2008)
Cystoisospora ohioensis	EU124688.1	81·5	1–398	Samarasinghe *et al*. (2008)
Cystoisospora rivolta	EU124686.1	81·2	1–400	Samarasinghe *et al*. (2008)
Cystoisospora felis	EU124689.1	58·8	1–389	Samarasinghe *et al*. (2008)
Cystoisospora suis	EU124685.1	81·9	1–404	Samarasinghe *et al*. (2008)

$(NH4)_2SO_4$, 75 mM Tris-HCl, 0·01% Tween 20, 1·5 mM $MgCl_2$, 400 ng/μl BSA, template. Cycling conditions consisted of a pre-cycle (94 °C for 2 min, 62 °C for 1 min, 72 °C for 2 min), 45 cycles (94 °C for 30 sec, 62 °C for 20 sec, 72 °C for 35 sec) and a final extension step (72 °C for 7 min). The inner primer pair ITSGF (5′-GATCATTCACACGTGGCC-CTTG-3′) and ITSR2 (5′-GACGACGTCCAAA-TCCACAGAGC-3′) were used to amplify an approximately 450-bp portion of the ITS-1 rDNA locus of *Cystoisospora* sp. For the second round, reactions were carried out in 50 μl containing 0·5 μM each primer, 200 μM each dNTP, 2·5 U *Taq* DNA polymerase (Fermentas International Inc.), 20 mM $(NH4)_2SO_4$, 75 mM Tris-HCl, 0·01% Tween 20, 1·5 mM $MgCl_2$, 400 ng/μl BSA, template. Cycling conditions were as for the first round except that the annealing temperature was raised to 68 °C. Both rounds were performed in a P×2 Thermal Cycler (Thermo Electron Corporation). Fifteen microlitres of amplification products from the second round were analysed by 2% agarose gel electrophoresis, stained with ethidium bromide and UV visualized.

Restriction fragment length polymorphism (RFLP) analysis was carried out according to the method described by Samarasinghe *et al*. (2008). Ten microlitres of amplification products from the second round of the nested PCR were digested in a final volume of 30 μl with 10 U of *Alu* I restriction enzyme (Fermentas International Inc.) by incubation overnight at 37 °C. Digestion products were run on ethidium bromide-stained 3% agarose gels.

DNA sequencing

To confirm the sequence of the amplification fragments of the 18S and ITS-1 rDNA locus, amplicons of the right size were purified from 1·2% agarose gels with the centrifugal filter device Ultrafree®-DA (Millipore). DNA sequencing of the PCR products was performed using a Hitachi 3130XL Genetic Analyzer (Applied Biosystems) with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence similarity was analysed using the Blast program of the National Center for Biotechnology Information, and multiple alignments employing the ClustalW 2.0.12 free software. A sequence identity matrix was performed using the BioEdit Sequence Alignment Editor version 7.0.9.0.

RESULTS

General results for samples from the 8 *Cystoisospora*-infected patients are summarized in Table 1. Cases 2 and 5 had unizoites in duodenal samples (Fig. 1). Nested PCR amplification of a fragment of the 18S

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

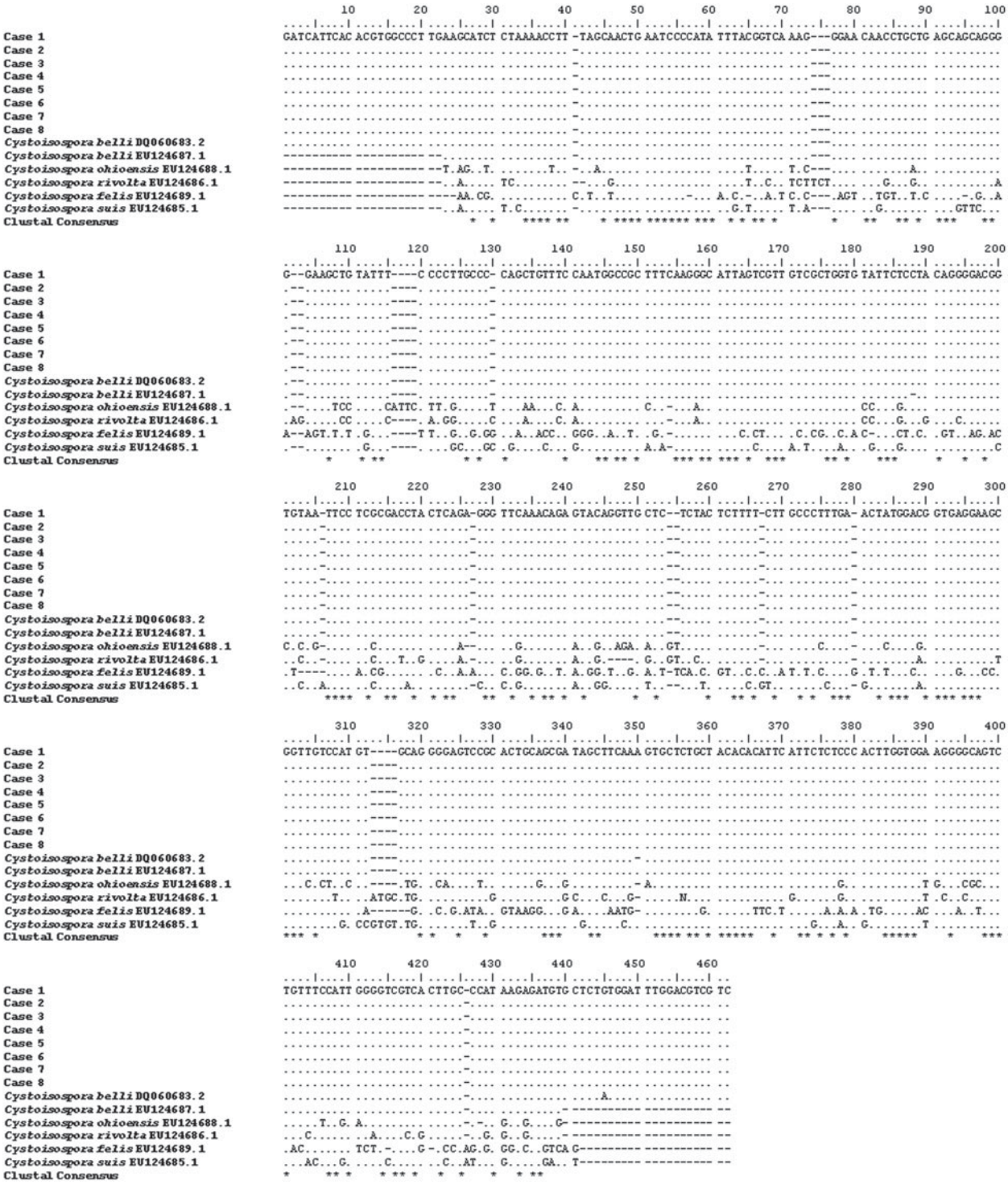

Fig. 3. Alignment of the sequences from the 8 amplicons generated by the ITS-1 rDNA nested PCR with those of different *Cystoisospora* species. Dots and dashes represent identical residues and deletions, respectively. GenBank Accession numbers and species are detailed in Table 3.

rRNA gene produced the expected 396 bp amplicon in all samples and sequences of the 8 cases were identical to those previously reported for *C. belli*. No differences were observed between cases, especially when compared with samples containing unizoites (Fig. 2). Identity was 100% for previously reported sequences for *C. belli,* with the exception of 1 case described by Jongwutiwes *et al.* (2007) of an immunocompetent patient with multiple relapses. When compared with other *Cystoisospora* species, this value reduces to 98·4% (Table 2).

The DNA fragment generated from the nested PCR for the ITS-1 contained 440 bp. Sequences were generated for the 8 cases and are available from GenBank under the Accession nos. HM630352-HM630359. All isolates yielded RFLP fragments of

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

identical size when digested with *Alu* I. Sequences of the 8 isolates examined were identical between them, but differed from the isolates reported by Jongwutiwes *et al*. (2007) and Samarasinghe *et al*. (2008) (GenBank Accession nos. DQ060683.2 and EU124687.1, respectively). They differed from DQ060683.2 at an insertion and a base substitution and from EU124687.1 at an insertion (Fig. 3). Identity was 99·7% and 99·5% for previously reported *C. belli* ITS-1 sequences and ranged from 81·9% to 58·8% for other *Cystoisospora* species (Table 3).

Identity was 99·7% and 99·5% for previously reported *C. belli* ITS-1 sequences because they showed a low variation from the isolates reported by Jongwutiwes *et al*. (2007) and Samarasinghe *et al*. (2008). When our isolates were compared with the sequences of the other *Cystoisospora* species that have unizoite tissue cysts in their life cycles, identity values varied from 81·9% to 58·8%.

In conclusion, it is clear that we still need another molecular tool that could be used to identify species strain variation in *C. belli* with and without the presence of unizoites.

DISCUSSION

Several authors have used the SSU-rRNA sequence for detection and investigation of species/strain variation of the genus *Cystoisospora* in oocysts, bile and duodenal biopsy samples (Müller *et al*. 2000; Franzen *et al*. 2000).

The detection of *C. belli* in duodenal biopsy and bile samples by PCR using primers based on the SSU-rRNA sequence was described for 2 AIDS-patients (Müller *et al*. 2000). Two reports determined the sequences of the SSU-rRNA gene of *C. belli* (Jongwutiwes *et al*. 2007; Franzen *et al*. 2000). One study used only the oocyst stage in fecal samples from HIV-infected patients and immunocompetent individuals in Thailand (Jongwutiwes *et al*. 2007). Bile juice sample from 1 patient was used in another report (Franzen *et al*. 2000). The presence or absence of unizoites in tissues was not studied in these reports (Müller *et al*. 2000; Jongwutiwes *et al*. 2007; Franzen *et al*. 2000).

In our study we compared samples with and without the presence of unizoites in lamina propria and we did not detect differences by sequencing a 396-bp fragment of the SSU-rRNA gene. This fragment showed also high identity values (over 99%) with the sequences of other *Cystoisospora* species in which the presence of unizoites in their life cycles is common, namely *C. ohioensis* and *C. felis*.

The ITS-1 region has been shown to be useful for the distinction of *Cystoisospora* species (Samarasinghe *et al*. 2008). This report used fecal samples collected from cats, dogs, pigs and humans and developed a PCR-RFLP assay that detected and differentiated the *Cystoisospora* species *C. suis*, *C. rivolta*, *C. felis*, *C. ohiohensis*-like and *C. belli* (Samarasinghe *et al*. 2008). The identification of unizoites in tissue was not studied.

In this report, we analysed the DNA fragments generated from the nested PCR for the ITS-1 and all isolates yielded RFLP fragments of the sizes expected for *C. belli* species. Sequences of the 8 isolates examined were identical between them and it was not possible to identify species or strain variation in isolates of *C. belli* with or without unizoite tissue cysts.

REFERENCES

Benator, D. A., French, A. L., Beauder, L. M., Levy, C. S. and Orenstein, J. M. (1994). *Isospora belli* infection associated with acalculous cholecystitis in a patient with AIDS. *Annals of Internal Medicine* **121**, 663–664.

Carreno, R. A., Schnitzler, B. E., Jeffries, A. C., Tenter, A. M., Johnson, A. M. and Barta, J. R. (1998). Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora*. *Journal of Eukaryotic Microbiology* **45**, 184–188.

Comin, C. E. and Santucci, M. (1994). Submicroscopic profile of *Isospora belli* enteritis in a patient with acquired immune deficiency syndrome. *Ultrastructural Pathology* **18**, 473–482.

Costa, P. S. and Lopes, C. W. G. (1998). Avaliacação do parasitismo por *Cystoisospora felis* (Wenyon, 1923) Frenkel, 1977. (Apicomplexa: Cystoisosporinae) em coelhos do tipo carne. *Revista Brasileira de Parasitologia Veterinária* **7**, 15–19.

Dubey, J. P. and Frenkel, J. K. (1972). Extra-intestinal stages of *Isospora felis* and *Isospora rivolta* (protozoa: eimeriidae) in cats. *Journal of Protozoology* **19**, 89–92.

Dubey, J. P. and Mehlhorn, H. (1978). Extraintestinal stages of *Isospora ohioensis* from dogs in mice. *Journal of Parasitology* **64**, 689–695.

Franzen, C., Müller, A., Bialek, R., Diehl, V., Salzberger, B. and Fätkenheuer, G. (2000). Taxonomic position of the human intestinal protozoan parasite *Isospora belli* as based on ribosomal RNA sequences. *Parasitological Research* **86**, 669–676.

Frenkel, J. K., Oliveira Silva, M. B., Saldanha, J. C., De Silva Vergara, M. L., Correia, D., Barata, C. H., Lages Silva, E., Ramirez, L. E. and Prata, A. (2003). Presença extra-intestinal de cistos unizoicos de *Isospora belli* em paciente com SIDA. *Revista da Sociedade Brasileira de Medicina Tropical* **36**, 409–412.

Jongwutiwes, S., Putaporntip, C., Charoenkorn, M., Iwasaki, T. and Endo, T. (2007). Morphologic and molecular characterization of *Isospora belli* oocysts from patients in Thailand. *American Journal of Tropical Medicine and Hygiene* **77**, 107–112.

Lindsay, D. S., Dubey, J. P. and Blagburn, B. L. (1997*a*). Biology of *Isospora* spp. from humans, nonhuman primates, and domestic animals. *Clinical Microbiology Reviews* **10**, 19–34.

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press

Lindsay, D. S., Dubey, J. P., Toivio-Kinnucan, M. A., Michiels, J. F. and Blagburn, B. L. (1997*b*). Examination of extraintestinal tissue cysts of *Isospora belli*. *Journal of Parasitology* **83**, 620–625.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Massad, F. V., Oliveira, F. C. R., Albuquerque, G. R. and Lopes, C. W. G. (2003). Hypnozoítas de *Cystoisospora ohioensis* (Dubey, 1975) Frenkel, 1977 (Apicomplexa: Cystoisosporinae) em frangos. *Revista Brasileira de Ciência Veterinária* **10**, 57–58.

Melo, P., De Carvalho Filho, P. R., Lopes, C. W. G., Flausino, W. and De Oliveira, F. C. R. (2003). Hypnozoítas de *Cystoisospora felis* (Wenyon, 1923) Frenkel, 1977 (Apicomplexa: Cystoisosporinae) isolated from piglets experimentally infected. *Revista Brasileira de Ciência Veterinária* **12**, 82–84.

Michiels, J. F., Hofman, P., Bernard, E., Saint Paul, M. C., Boissy, C., Mondain, V., LeFichoux, Y. and Loubiere, R. (1994). Intestinal and extraintestinal *Isospora belli* infection in an AIDS patient. *Pathology, Research and Practice* **190**, 1089–1093.

Mitchell, S. M., Zajac, A. M. and Lindsay, D. S. (2009). Development and ultrastructure of *Cystoisospora canis* Nemeséri, 1959 (syn. *Isospora canis*) monozoic cysts in two cell lines. *Journal of Parasitology* **95**, 793–798.

Müller, A., Bialek, R., Fätkenheuer, G., Salzberger, B., Diehl, V. and Franzen, C. (2000). Detection of *Isospora belli* by polymerase chain reaction using primers based on small-subunit ribosomal RNA. *European Journal of Clinical Microbiology & Infectious Diseases* **19**, 631–634.

Restrepo, C., Macher, A. M. and Radany, E. H. (1987). Disseminated extraintestinal isosporiasis in a patient with acquired immune deficiency syndrome. *American Journal of Clinical Pathology* **87**, 536–542.

Samarasinghe, B., Johnson, J. and Ryan, U. (2008). Phylogenetic analysis of *Cystoisospora* species at the rRNA ITS1 locus and development of a PCR-RFLP assay. *Experimental Parasitology* **118**, 592–595.

Velasquez, J. N., Carnevale, S., Mariano, M., Kuo, L. H., Caballero, A., Chertcoff, A., Ibañez, C. and Bozzini, J. P. (2001). Isosporosis and unizoite tissue cyst in patient with acquired immunodeficiency síndrome. *Human Pathology* **32**, 500–505.

Zenta, W. and Topazian, M. D. (2009). *Isospora belli* cholangiopathy: case study with histologic characterization and molecular confirmation. *Human Pathology* **40**, 1342–1346.

https://doi.org/10.1017/S0031182010001253 Published online by Cambridge University Press