Neelsen method[7] at x1000 magnification. Identification of oocysts was based on the size, morphology and staining characteristics. Confirmation of identity was performed at the Scottish Meningococcus and Pneumococcus Reference Laboratory, Glasgow, UK using a modified Ziehl-Neelsen method and autofluorescence.[7,8]

C. cayetanensis oocysts were present in three samples (2.2%) – a lettuce sample from Idi-Araba, a water leaf sample from Yaba market, and a well-water sample taken from a farm on which the water was used for crop irrigation; co-incidentally, also in Idi-Araba.

Vegetables and water remain potential vehicles for the transmission of intestinal parasitic agents worldwide. This study revealed the presence of *C. cayetanensis* oocysts in two commonly eaten vegetables (lettuce and water leaf) in Lagos, Nigeria, and also in a well-water sample used for the irrigation of vegetables on farms. Such wells are about a metre deep and over a metre wide, and are never covered. They are dug on the farm and therefore contamination could be expected, due to surface run-off or it being located in an area of fractured bed-rock. The actual reasons for contamination in this case, however, are not known.

Of the two vegetable types implicated, lettuce is more likely to be a potential vehicle for the transmission of this pathogen because it is eaten raw in salad meals, although it may be washed before consumption. However, washing may not remove all oocysts from a contaminated sample.

The results presented here re-emphasise the need for improved hygiene, especially during the preparation of food, although more studies are necessary to determine the extent of vegetables involvement in the spread of *C. cayetanensis* oocysts.

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Sequence analysis of partial regions of the 5.8S rRNA internal transcribed region 2 and 28S rRNA of *Isospora belli*

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Isospora belli is an important gastrointestinal protozoal pathogen in several mammalian host species and is particularly important in the aetiology of diarrhoeal disease in immunocompromised humans in tropical and subtropical regions, including tropical Africa, Brazil and South East Asia.[1-3] The prevalence of this parasite is particularly high in HIV infection and HIV-AIDS, and recent studies have shown infection rates of 2.5%, 11% and 17% in such patients in northern India, eastern India and Guinea-Bissau, respectively.[4-7]

Historically, laboratory diagnosis of this disease has been difficult, especially during the early years of human isosporiasis (c. 1915), due to morphological confusion with related genera including *Toxoplasma, Eimeria* and *Sarcocystis*.[8] Presently, several morphological criteria are used to identify *I. belli*, including the presence of ellipsoidal oocysts that range in length (20-30 µm) and width (10-19 µm), where sporocysts are rarely seen broken out of oocysts.[9]

The usual diagnostic stage in faeces is the presence of immature oocysts (containing two sporocysts, each with four sporozoites), as visualised by a spherical mass of protoplasm.[9] However, a recent review has highlighted the importance of molecular techniques in overcoming current diagnostic limitations.[10] Previously, Muller *et al*.[11] designed a specific polymerase chain reaction (PCR) assay to target the small-subunit (18S) ribosomal RNA (rRNA) sequence of *I. belli*, and Franzen *et al*.[8] have examined the taxonomical position of this protozoan, based on 18S rRNA sequence analysis.

To date, there have been no published sequence data available

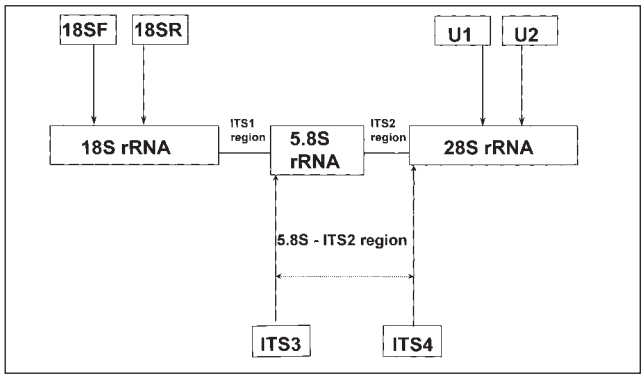

Fig. 1. Arrangement of ribosomal RNA gene loci of *Isospora belli* and location of oligonucleotide primer pairs used in the study.

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Table 1. Details of PCR amplification conditions employed

Primer	Sequence 5'.....3'	Target gene	Region amplified	Approximate amplicon size (bp)	PCR amplification conditions	No. of bases submitted	GenBank accession number of submitted sequence
U1*	GTG AAA TTG TTG AAA GGG AA	28S rRNA	28S rRNA	300	Ref. 11	274	AY063483
U2	GAC TCC TTG GTC CGT GTT	28S rRNA					
18SF	ATT GGA GGG CAA GTC TGG TG	18S rRNA	18S rRNA	490	Ref. 11	430	AF441289
18SR*	CCG ATC CCT AGT CGG CAT AG	18S rRNA					
ITS3*	GCA TCG ATG AAG AAC GCA GC	5.8S rRNA	5.8S/ITS2/28S	600	Ref. 11	288	AF443614
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rRNA					

* sequencing primer

Table 2. Phylogenetic relatedness of *Isospora belli* to closest neighbours by 18S, 28S and 5.8S rRNA/internal transcribed spacer (ITS) region 2 analyses

Region amplified	% homology	No.homologous bases	Position on homologous sequence	Identity	GenBank accession number
18S rRNA	100	430/430	564-993	*Isospora belli*	U94787
	99.3	427/430	564-993	*I. ohioensis*	AF029303
	98.6	426/435	563-994	*I. suis*	U97523
	96.0	413/430	581-1009	*Besnoitia jellisoni*	AF291426
	95.6	411/430	564-990	*Neospora caninum*	U16159
	95.3	410/430	565-991	*Toxoplasma gondii*	M97703
	95.3	410/430	545-971	*Hammondia hammondi*	AF096498
	94.9	408/430	515-943	*Hyaloklassia lieberkuehni*	AF298623
	92.7	404/436	510-943	*Frenkelia glareoai*	AF009245
	92.6	400/432	568-997	*Goussia jenae*	AY043206
	92.4	402/435	569-1002	*Sarcocystis neurona*	U070812
	89.0	387/435	513-942	*Eimeria tropidura*	AF324217
28S rRNA	91.3	253/274	227-303	*Isospora suis*	AF093428
	87.1	236/271	450-718	*I. felis*	U85705
	71.1	194/273	41-306	*Besnoitia besnoiti*	AF076869
	69.2	159/229	56-278	*Neospora caninum*	AF249972
	67.1	188/280	1168-1437	*Toxoplasma gondii*	L25635
	66.9	186/278	35-302	*Hammondia heydorni*	AF0965022
	62.2	168/270	48-311	*Eimeria tenella*	AF076862
	61.7	166/269	470-734	*Sarcocystis singaporensis*	AF237617
5.8S rRNA/ITS2	67.6	192/284	527-797	*Neospora caninum*	L49389
	66.6	190/287	495-770	*Toxoplasma gondii*	L49390
	71.5	128/179	68-242	*Sarcocystis neurona*	BQ784940
	67.4	118/175	2937-3103	*Eimeria tenella*	AF026388
	68.5	111/162	323-379	*Cyclospora cayetanensis*	AF303964

for the 5.8S rRNA/ITS2 and 28S rRNA regions for *I. belli*. Hence, this study aims to examine the sequences of these regions of the rRNA operons and use them to compare the taxonomical position of *I. belli* with its closest phylogenetic neighbours

All molecular procedures were carried out in accordance with the good molecular diagnostics protocol detailed in the guidelines of Millar *et al*.[12] Genomic DNA from a well characterised isolate of *I. belli* was amplified using three sets of PCR primers (Table 1) that targeted the rRNA operons and ITS regions (Figure 1). *Toxoplama gondii* and molecular grade water (Biowhitaker. Maryland, USA) were used throughout as positive and negative PCR controls, respectively.

Amplicons (rDNA) for sequencing were purified using a QIAquick PCR purification kit (Qiagen, UK) eluted in Tris-

HCl (10 mmol/L, pH 8.5) prior to sequencing to remove, in particular, dNTPS, polymerases, salts and primers. Appropriate primers (Table 1) were used for automated sequencing with the ABI PRISM dye terminator cycle sequencing reaction, with AmpliTaq DNA Polymerase and FS (PE Biosystems, Foster City, CA, USA), and a cycling sequence of 96˚C for 1 min followed by 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 4 min and a hold at 4˚C. Products were ethanol-precipitated and analysed on an ABI 373 automatic sequencer (PE Biosystems).

Sequences obtained were compared with those stored in the EMBL database using WU-BLAST2 alignment software (http://www.ebi.ac.uk), in conjunction with the Apicomplexa protozoan database. For identification to species or genus level, criteria previously published by Goldenberger *et al*.[13] were employed.

PCR amplification of *I. belli* target DNA and the positive control (*T. gondii*) with the universal rRNA primer sets (detailed in Table 1) produced amplicons of varying sizes when visualised on ethidium bromide-stained gels. All negative controls containing reaction mixtures (molecular grade water) without DNA were negative. Resulting *I. belli* amplicons were sequenced and deposited in GenBank (Table 1). For the small rRNA subunit (18S rRNA), the sequence (430 bp) obtained in this study showed 100% identity with *I. belli* (accession number U94787), confirming the identity of the DNA extract.

Table 2 shows the homology between the 18S rRNA sequence obtained and the closest phylogenetic neighbours. The sequence data obtained for both the 28S rRNA and the 5.8S rRNA/ITS2 region showed limited homology matches with any of the closely related coccidia, due to the relative lack of available sequence data for these parasites over these regions.

For the 28S rRNA sequence, the closest neighbour was *I. suis* (253/274 bp; 91.3% identity). As with the 18S rRNA sequence, the closest genus was *Besnoitia* (Table 2). For the partial sequence (288 bp) that spanned the 5.8S rRNA /ITS2 region, the closest homology was with *Neospora caninum* (accession number L49389; 192/284 bp 67% identity) (Table 2).

Presently, the genus *Isospora* consists of eight species – *I. belli, I. felis, I. gryphoni, I. insularius, I. ohioensis, I. peromysis, I. robini and I suis* – for which only limited rRNA sequence data is available for phylogenetic analyses. To date, the phylogenetic relationships within this genus and with closely related genera have been based on 18S rRNA alignment comparisons, and the large (28S) rRNA subunit has not been employed in such analyses.

Consequently, the aim of the present study was to sequence partial regions of the large rRNA subunit and ITS2 regions and compare the phylogenetic relationships of these to that of the 18S rRNA. Based on the 18S and 28S rRNA analyses present here, *I. belli* is related most closely to the genus *Besnoitia*, followed by *N. caninum* and *T. gondii*. With respect to the 5.8S/ITS2 region, a similar relationship was observed for *N. caninum* and *T. gondii*. Owing to the limited availability of sequence data for this region of Apicomplexa, however, the phylogeny associated with the genus *Besnoitia* is uncertain.

The findings presented here are in broad agreement with those of previous studies based solely on 18S rRNA phylogenetic analyses. Carreno *et al*.[14] demonstrated that the genus *Isospora* was most closely related to the species *N. caninum* and *T. gondii*, and Franzen *et al*.[8] also demonstrated a similar relationship, although these workers did not report on the relatedness of *Besnoitia* to *I. belli*.

In conclusion, as well as reporting new ribosomal RNA sequence data for the 28S and 5.8S rRNA/ITS2 regions of *I. belli*, examination of these regions confirm that *I. belli* is most closely related to the genus *Besnoitia*, followed by *N. caninum* and *T. gondii*. □

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