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Genetic characterization of selected parasites from people with histories of gastrointestinal disorders using a mutation scanning-coupled approach

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Abbreviations: **BI**, Bayesian inference; **gp60**, 60-kDa glycoprotein; **ITS-2**, second internal transcribed spacer of nuclear ribosomal DNA; **L3s**, third-stage larvae; **Nst**, number of substitutions; **pgp60**, part of the 60-kDa glycoprotein gene; **pp**, posterior probability; **ptpi**, part of the triose phosphate isomerase gene; **REF**, restriction endonuclease fingerprinting; **STH**, soil-transmitted helminth; **tpi**, triose phosphate isomerase gene; **WHO**, World Health Organization.

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[Abstract]

A single-strand conformation polymorphism (SSCP) analysis and targeted sequencing approach was used for the genetic characterization of some major pathogens from a cohort of 227 people with histories of gastrointestinal disorders. Genomic DNAs from faecal samples were subjected to PCR-amplification of regions in the glycoprotein (*gp60*) or triose phosphate isomerase (*tpi*) gene, or the second internal transcribed spacer of nuclear ribosomal DNA (ITS-2). *Cryptosporidium*, *Giardia* and strongylid nematodes were detected in 94, 132 and 12 samples. *Cryptosporidium hominis* subgenotypes IbA10G2, IdA15G1, IgA17, IgA18 and IfA13G1 were identified in 74.6, 16.9, 5.6, 1.4 and 1.4% of 71 samples, respectively. For *C. parvum*, subgenotypes IIaA17G2R1 (47.6%) and IIaA18G3R1 (23.8%) were identified in 23 samples. *Giardia duodenalis* assemblage B (78%) was more common than assemblage A (22%). In addition, DNA of the nematodes *Ancylostoma ceylanicum* (n = 2), *Ancylostoma duodenale* (4), *Necator americanus* (5) and *Haemonchus contortus* (1) was specifically detected. This is the first report of *A. ceylanicum* in two persons in Australia and, we provide molecular evidence of *H. contortus* in a child. This SSCP-based approach should provide a useful diagnostic and analytical tool for a wide range of pathogens.

Keywords:

Cryptosporidium / *Giardia* / hookworm / pathogen / single-strand conformation polymorphism (SSCP) analysis.

1 Introduction

Gastrointestinal parasites of humans cause destructive diseases of major socio-economic importance worldwide. For instance, in the developed world, waterborne protists, such as *Cryptosporidium* and *Giardia*, can be responsible for up to 20-30% of cases of diarrhoea in some countries [1]. Diarrhoea has been recognized by the World Health Organization (WHO) as the second leading cause of death in children, and the leading cause of malnutrition in children of less than five years of age [2]. In 2004, *Giardia* and *Cryptosporidium* were added to the WHO's 'Neglected Diseases Initiative' [3] in an effort to boost the global profile and improve the detection and control of these pathogens. Parasitic worms (i.e., trematodes, cestodes and nematodes) have a massive, long-term impact on human health and cause substantial suffering (e.g., [4-9]). WHO estimates that 2.9 billion people are infected with nematodes [10] and that morbidity from worms surpasses diabetes and lung cancer in disability adjusted life years (DALYs) [11]. Soil-transmitted helminths (STHs), such as the hookworms *Necator americanus* and *Ancylostoma duodenale*, are of major importance, infecting ~740 million people and causing anaemia and malnutrition worldwide [12].

The diagnosis of parasitic infections and diseases is central to investigating their epidemiology, treatment and control. Methods routinely used for parasite diagnosis mostly

rely on the microscopic detection of parasite stages (e.g., [1, 13-16]). These approaches can be time-consuming to perform and are quite often unreliable [17, 18]. For instance, using coproscopic methods, it is usually not possible to identify or distinguish different parasite species based on the morphology of the structures detected (e.g., oocysts, cysts or eggs), because of an absence or lack of distinguishing morphological features. In addition, immunological assays, such as commercial coproantigen detection methods, are often not entirely specific (e.g., [19-24]). The limited specificity and/or sensitivity of some of these phenetic methods represent a major constraint for the routine diagnosis of parasitic infections.

The use of DNA-based methods as complementary tools can circumvent this limitation. Particularly polymerase chain reaction (PCR)-coupled methods are useful for the genetic identification and characterization of pathogens from tiny amounts (pg to fg) of parasite DNA [25, 26]. Although a wide range of PCR-coupled methods is available, mutation scanning [27] provides a powerful tool for both diagnosis and genetic analysis, provided suitable molecular markers are employed. In particular, single-strand conformation polymorphism (SSCP) has found applicability because a standard protocol can be applied to a wide range of genetic markers and organisms. For instance, PCR-based SSCP analysis of internal transcribed spacer sequences of nuclear ribosomal DNA allows the specific identification of key parasitic nematodes of major importance [27] and nuclear genes, such as 60-kDa glycoprotein (*gp60*) and triose phosphate isomerase (*tpi*), are commonly employed for the specific detection or characterization of protozoa, such as *Cryptosporidium* [21, 28-34] and *Giardia* [35, 36]. The method has been applied to DNA isolated directly from parasitic protozoans and from biological matrices, including faeces.

Although SSCP has been used for systematic, epidemiological and population genetic investigations of pathogens [27, 37], its dual diagnostic/analytical capacity has not been fully exploited in a pathology, microbiology or parasitology laboratory setting to complement routine diagnostic testing (using phenetic, including immunological, methods). In the present study, we employed PCR-coupled SSCP analysis and targeted sequencing to genetically characterize key pathogens in the faeces from patients with histories of gastrointestinal disorders. The main focus here was on the protozoa *Cryptosporidium* and *Giardia* as well as strongylid nematodes, such as species of *Ancylostoma* and *Necator* (hookworms), because of their major socioeconomic impact.

2 Materials and methods

2.1 Samples and conventional diagnostic testing

A total of 227 faecal samples from anonymous humans with histories of gastrointestinal disorders were tested in two diagnostic pathology service laboratories in Australia (between Mar. 2010 and Nov. 2011), and aliquots thereof provided to us for genetic testing. Most samples ($n = 217$) were from localities in Western Australia, five were from the Northern Territory, and five were from unknown localities in Australia. The faecal samples had been tested for the presence of parasitic protozoa and helminths using conventional methods. Cysts and oocysts of *Giardia* and *Cryptosporidium*, respectively, were detected microscopically either by a formalin-ethyl acetate concentration technique [38], immunofluorescence [39], modified acid-fast staining [40] or using a commercial coproantigen immunoassay (CELISA, Cellabs, Australia). Eggs of strongylid nematodes (i.e. of the order Strongylida) were detected microscopically following the formalin-ethyl acetate concentration method [15]. Following conventional testing, the remaining amounts of individual faecal samples were stored at -80°C .

2.2 Testing of DNA isolated from faecal samples by PCR-coupled SSCP analysis and targeted sequencing

2.2.1 Genomic DNA isolation and PCR

Genomic DNA was extracted directly from 0.1 to 0.4 g of individual faecal samples using the PowerSoil DNA Isolation Kit (MoBio, USA), according to the manufacturer's protocol. This kit was used because it is known to be effective at removing components that are inhibitory to PCR [41, 42]. Following DNA isolation, individual genomic DNA samples were subjected to nested or single-step PCR, targeting different regions of nuclear DNA. Each PCR was carried out in a 50 µl volume containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega, USA), 3.0 mM of MgCl₂, 200 µM of each deoxynucleotide triphosphate, 100 pmol of each primer and 1 U of GoTaq (Promega) DNA polymerase.

For human-infective *Cryptosporidium*, the *gp60* gene (~1 kb) was amplified using primers gp15-ATG (forward: 5'-ATGAGATTGTCGCCTCATTATC-3') and gp15-STOP (reverse: 5'-TTACAACACGAATAAGGCTGC-3') [43], followed by the nested amplification of a portion of the *gp60* gene (designated *pgp60*; 250-350 bp) using primers gp15-15A (forward: 5'-GCCGTTCCACTCAGAGGAAC-3') and gp15-15E (reverse: 5'-CCACATTACAAATGAAGTGCCGC-3') [44]. Primary amplification of *gp60* utilized the cycling protocol: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 sec (denaturation), 55 °C for 45 sec (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C/10 min. For the secondary amplification of *pgp60*, we employed 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec, with a final extension at 72 °C for 10 min.

For *Giardia*, the *tpi* gene of *Giardia* was amplified using primers AL3543 (forward: 5'-AAATTATGCCTGCTCGTCG-3') and AL3546 (reverse: 5'-CAAACCTTTTCCGCAAACC-3'), followed by nested amplification of a portion of the *tpi* gene (called *ptpi*; ~530 bp) employing primers AL3544 (forward: 5'-CCCTTCATCGGTGGTAACTT-3') and AL3545 (reverse: 5'-GTGGCCACCACTCCCGTGCC-3') [45]. For the primary amplification, the cycling protocol was 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 sec (denaturation), 50 °C for 45 sec (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min. Secondary amplification of *ptpi* was achieved employing 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 sec, 55 °C for 30 sec, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

For strongylid nematodes (including human hookworms), the second internal transcribed spacer (ITS-2; 310-420 bp) was amplified using primers NC1 (forward: 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') [27, 46]. The cycling protocol was: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 sec (denaturation), 55 °C for 30 sec (annealing) and 72 °C for 30 sec (extension), with a final extension of 72 °C for 5 min.

2.2.2 Agarose gel electrophoretic analysis of amplicons

The quality, intensity and size of individual amplicons were examined on ethidium bromide-stained 1.5% agarose gels, using TBE (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad, USA) as the buffer and ΦX174-*Hae*III (Promega, USA) as a size marker. Amplicons were then subjected to non-isotopic SSCP or restriction endonuclease fingerprinting (REF) analysis (see section 2.2.3).

2.2.3 SSCP and targeted sequencing

Direct SSCP (protocol B; [27]) was used for the analysis of *pgp60* (*Cryptosporidium*) and ITS-2 (strongylid nematodes) amplicons. In brief, 1 µl of each secondary amplicon was mixed with 5 µl sequencing-stop solution (Promega) and, heat-denatured at 94 °C/30 min, snap-cooled on a freeze-block (-20 °C) and then subjected to electrophoresis at 74 V at 7.4 °C (constant) for 16-18 h in a GMA Wide Mini S-2x25 gel in a SEA 2000 rig (Elchrom Scientific AG) using TAE buffer (40 mM Tris base, 20 mM acetic acid, 1.0 mM EDTA, Bio-Rad). Control samples (representing known species or genotypes) were included on each gel to ensure the reproducibility of profiles representing this sample among gels.

REF [35, 47] was used for the analysis of *ptpi* amplicons, because their size (~530 bp) exceeded that usually employed for direct SSCP analysis. In brief, an aliquot (10 µl) of each amplicon was cleaved with 10 IU of the restriction endonuclease *Hae*III (Promega, USA) in 20 µl, incubated at 37 °C for 2 h, and the enzyme inactivated at 70 °C for 5 min. One µl of this digest was then subjected to analysis by SSCP (protocol B; [27]) using the same conditions as for amplicons of ≤ 450 bp.

Following these SSCP-based analyses, selected *ptpi* (n = 52), *pgp60* (n = 33) and ITS-2 (n = 5) amplicons representing each distinct electrophoretic profile were treated with ExoSAP-IT® (Affymetrix, USA), according to the manufacturer's instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) using the same primers employed in the prior PCR. Sequence quality was verified by comparison with corresponding electro-pherograms using Geneious v.6.0.4 (Biomatters, New Zealand).

2.2.4 Phylogenetic analysis of sequence data

Representative sequences were aligned with 12 (*C. parvum* *pgp60*), 6 (*C. hominis* *pgp60*), 13 (strongylid ITS-2) or 13 (*Giardia* *ptpi*) reference sequences (Supplementary Tables S1-S4). Sequences were aligned using the program ClustalW [48], and alignments adjusted manually using Mesquite v.2.75 [49]. Phylogenetic analysis of sequence data was conducted by Bayesian inference (BI) using Monte Carlo Markov Chain analysis in the program MrBayes v.3.1.2 [50]. The likelihood parameters set for the BI analysis of sequence data were based on the Akaike Information Criteria test in jModeltest v.2.1 [51]. For the separate analyses of *pgp60* (*C. parvum*), *ptpi* (*Giardia*) and ITS-2 (strongylid nematodes) sequence data, the number of substitutions (Nst) was set at 6, with a gamma distribution. For the *pgp60* sequence data of *C. hominis*, Nst was also set at 6, using a proportion of invariable sites. Posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was < 0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Analyses were run three times to ensure convergence and insensitivity to priors. Outgroups used in the analyses were *C. hominis* for *C. parvum* (*pgp60*), *C. parvum* for *C. hominis* (*pgp60*), *Giardia ardeae* and *G. muris* for *G. duodenalis* (*ptpi*) and *Oesphagostomum dentatum* for strongylid nematodes (ITS-2).

3 Results and discussion

Using three distinct PCR assays, amplicons were produced from 227 genomic DNAs from individual faecal samples from humans with histories of gastrointestinal disorders (including diarrhoea, abdominal pain, vomiting and/or anaemia) and known to be passing *Cryptosporidium* oocysts, *Giardia* cysts and/or strongylid nematode eggs, based on conventional copro-diagnostic testing. In total, 88 samples were PCR test-positive for *Cryptosporidium*, 122 for *Giardia*, 13 for both *Cryptosporidium* and *Giardia*, 11 for one or more strongylid nematodes, and one sample for both *Giardia* and at least a strongylid nematode. All of the amplicons were subjected to SSCP (see Fig. 1) or REF analysis. All SSCP profiles were recorded, and multiple amplicons representing each individual profile selected for subsequent sequencing.

3.1 *Cryptosporidium*

Amplicons (*pgp60*) were amplified by PCR from 94 DNA samples. Following SSCP analysis and selective sequencing, 71 (75.5%), 21 (22.3%) and two (2.1%) of the 94 DNA samples represented *C. hominis*, *C. parvum* and *C. meleagridis*, respectively. Phylogenetic analysis of the nucleotide sequence data showed that *C. hominis* was represented by genotypes Ib, Id, Ig and If, with subgenotype IbA10G2 being most prevalent ($n = 53$; 74.6%) (Table 1; Fig. 2). *C. parvum* was represented by genotype IIa, with IIaA18G3R1 being the commonest subgenotype (Table 1; Fig. 3). *C. meleagridis* was represented by a previously published reference sequence (GenBank accession number JX548300). There was no significant difference between the number of male and female patients excreting *C. hominis* or *C. parvum* (goodness-of-fit test: $P = 0.561$ and $P = 0.664$, respectively) (Table 2). *Cryptosporidium* was found predominantly in patients of ≤ 9 years of age (Table 2).

The numbers of samples PCR-test positive for *C. hominis* (75.5%) and *C. parvum* (22.3%) were comparable with those reported previously in Australia [21, 52-55] and other countries (reviewed by Xiao *et al.* [56]), and are consistent with the finding that the majority of human cryptosporidiosis cases in industrialized countries relate to *C. hominis* [56]. To date, IbA10G2 is the commonest subgenotype recorded in humans worldwide [57]. A previous study of samples from humans from Western Australia showed that IdA15G1 was the main subgenotype [53], whereas, here, *C. hominis* IbA10G2 dominated. This discrepancy might relate to a significant regional outbreak of subgenotype IdA15G1 in 2010 in Western Australia [53]. For *C. parvum*, the two most dominant subgenotypes recorded here were IIaA17G2R1 and IIaA18G3R1, which is in accordance with previous findings in Australia [53].

The two patients with *C. meleagridis* were both one-year-old females presenting with diarrhoea, but there was no evidence of other, concurrent parasitic infections. There are numerous published reports of *C. meleagridis* in humans around the world [58]. Mostly, this species of *Cryptosporidium* is detected in children and immuno-compromised or -suppressed persons who have had direct or indirect contact with infected birds, such as turkeys or chickens [59]. Although *C. meleagridis* has a specific affiliation with birds, it is directly transmissible to humans and can be transmitted among humans, particularly those suffering from immunological disorders [58].

3.2 *Giardia*

Amplicons (*ptpi*) were produced by PCR from 136 DNA samples. Following REF analysis and selective sequencing, 103 amplicons represented assemblage B (78%), and 29 belonged

to assemblage A (22%). A subset of 25 amplicons representing unique profiles was subjected to sequencing, and all of the (distinct) sequence types subjected to phylogenetic analysis. The tree showed that all samples belonged to either assemblage A or B (Fig. 4), and a pairwise comparison of all sequences revealed that assemblage A was represented by two distinct sequence types, and assemblage B by 23 distinct types. Of these 25 *ptpi* sequences, eight were present in the GenBank database and, interestingly, the remaining 17 are new sequence types differing by 1-4 bp from previously published sequences. The number of male patients with *G. duodenalis* was significantly greater than that of females (binomial goodness-of-fit test: $P = 0.037$) (Table 2). *G. duodenalis* was detected predominantly in patients of ≤ 9 years of age (Table 2).

G. duodenalis assemblage B was far more frequently detected than assemblage A (78% and 22%, respectively). The predominance of assemblage B is concordant with the findings from studies of *Giardia*/giardiasis of humans in Australia ([60]: assemblage B = 75%; assemblage A = 25%; $n = 124$; [61]: assemblage B = 70%; assemblage A = 30%; $n = 23$). Worldwide, based on current evidence, assemblage B appears to be only slightly more prevalent than assemblage A in both developed and developing countries [62]. Although a higher (apparent) prevalence of this assemblage might relate to higher reproductive potential and faecal cyst counts compared with assemblage A [63], there is presently no clear evidence to indicate that assemblage A is more pathogenic than assemblage B [62]. This statement is supported by the study of Lebbad *et al.* [64] who did not find an association between particular assemblages and clinical symptoms in 214 patients with giardiasis in Sweden.

3.3 Nematodes

Amplicons (ITS-2) were produced from 12 of the 227 genomic DNA samples. SSCP analysis revealed four distinct profiles. Following sequencing, the hookworms *Ancylostoma ceylanicum* ($n = 2$), *Ancylostoma duodenale* (4) and *Necator americanus* (5) were detected, and the barber's pole worm, *Haemonchus contortus* (1), which only usually infects small ruminants, such as sheep and goats (Fig. 5).

Both *A. duodenale* and *N. americanus* have been reported in Australia, and were first recorded during the Australian Hookworm Campaign (1919-1924) [65]. Originally, the dominant species in Queensland and New South Wales was *N. americanus*, but in more recent times only *A. duodenale* has been documented in indigenous communities of Western Australia and the Northern Territory [65, 66]. In the present study, all five people inferred to harbour *N. americanus* likely imported this parasite into Australia from overseas, because all patients were recent immigrants from Sierra Leone or refugees from Sudan where *N. americanus* is endemic [67, 68].

In addition, the first two cases of *A. ceylanicum* in humans were recorded in Western Australia. These people were both in their mid twenties, one from Perth the other from the Pilbara region. *Ancylostoma ceylanicum* is the only hookworm of dogs and cats that can be transmitted to humans and cause patent intestinal infection [69, 70]. Recently, *A. ceylanicum* has been found in dogs from Western Australia, Queensland and the Northern Territory [71]. Although it possible that these people might have acquired this hookworm outside of Australia, there was no documented evidence of travel; therefore, it is more likely that they became infected in Australia by *A. ceylanicum* derived from dogs or cats.

Interestingly, *H. contortus* DNA was amplified from a faecal sample from a one-year-old female child, which is the first case of *H. contortus* detected by molecular means in a human. This particular patient presented with diarrhoea, a cough and fever and might have been immuno-compromised, as she was shown to harbour mixed infections of *Blastocystis hominis*, *Chilomastix mesnili*, *Endolimax nana* and *Entamoeba coli* by coproscopy, although

no helminth eggs were detected upon the microscopic examination. Globally, there are seven documented cases of *H. contortus* infection in humans, following the detection of either eggs or worms in faecal samples [72, 73]. Most of these cases are believed to relate to humans accidentally ingesting infective third-stage larvae (L3s) from environments stocked with large numbers of small ruminants (including sheep and goats) infected with *H. contortus* [73]. This child may have become infected or been exposed to *H. contortus* L3s or eggs through coprophagia (ingestion of faecal matter), or geophagia (ingestion of soil), recognized as a significant risk factor for children (between 1-3 years of age) acquiring diseases [74]. The present case might also be the result of pseudo-parasitism, whereby PCR detected *H. contortus* DNA originating from L3s or eggs accidentally ingested and passing through the gastrointestinal tract of the non-permissive human host, but not relating to an actual infection. Interestingly, three of all seven previously published cases of *H. contortus* infection in humans worldwide relate to indigenous persons from Western Australia [73].

4 Concluding remarks

PCR-based SSCP analysis and targeted sequencing allowed the genetic characterization of major pathogens from a cohort of 227 people with histories of gastrointestinal disorders. *Cryptosporidium* and *Giardia* were characterized to the genotypic/assemblage and subgenotypic levels by comparison with homologous reference sequences from previous national and global studies, accessible from the GenBank database. Although no novel genotypes or assemblages of *Cryptosporidium* and *Giardia* were discovered, two interesting cases of *C. meleagridis* (typically found in birds) were identified in faecal samples from children. We detected, for the first time, *A. ceylanicum* in two persons from Australia that most likely originated from infected dogs or cats. In addition, we provided molecular evidence of *H. contortus* exposure or infection in a young child, probably *via* a small ruminant source.

Although the SSCP-based analyses have been used routinely in our laboratory for the detection and characterization of species and genotypes/assemblages of *Cryptosporidium* or *Giardia* [21, 29, 31-36, 75, 76], it had not been employed previously for the analysis of ITS-2 products PCR-amplified directly from human faecal DNA samples (rather than parasite DNA). The detection of single ITS-2 bands on agarose gels, discrete profiles by SSCP and unambiguous sequences resulting from selective sequencing of amplicons has inferred exquisite specificity of the PCR (determined by primer NC1). Therefore, this SSCP-based approach should find significant utility as an epidemiology tool for the simultaneous diagnosis and genetic analysis of hookworms and other strongylid nematodes of humans.

Nucleotide sequences reported in this paper are available from the GenBank database under accession nos. KC632529-KC632571.

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Table 1. Summary of the species and/or subgenotypes of *Cryptosporidium* identified by PCR-based SSCP analysis and targeted sequencing in the present study.

Species	Subgenotype	Number	Percentage
<i>Cryptosporidium hominis</i>			
	IbA10G2	53	74.6
	IdA15G1	12	16.9
	IgA17	4	5.6
	IgA18	1	1.4
	IfA13G1	1	1.4
<i>Cryptosporidium parvum</i>			
	IIaA18G3R1	10	47.6
	IIaA17G2R1	5	23.8
	IIaA15G2R1	3	14.3
	IIaA19G4R1	2	9.6
	IIaA20G3R1	1	4.8
<i>Cryptosporidium meleagridis</i>			
		2	100
		Total: 94	

Table 2. Age and gender of the present cohort of 227 people with histories of gastrointestinal disorders, and evidence of infections with *Cryptosporidium*, *Giardia* and/or nematodes based on testing using conventional copro-diagnostic techniques

Pathogen	Gender	Age range (years)								Unknown	Total
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+		
<i>Cryptosporidium hominis</i>	Female	20	2	5	11	2	0	0	0	0	40
	Male	20	1	2	4	4	0	0	0	0	31
<i>Cryptosporidium meleagridis</i>	Female	2	0	0	0	0	0	0	0	0	2
	Male	0	0	0	0	0	0	0	0	0	0
<i>Cryptosporidium parvum</i>	Female	4	1	2	2	0	0	0	0	0	9
	Male	4	4	2	0	1	0	1	0	0	12
<i>Giardia duodenalis</i> (A)	Female	4	0	1	2	0	0	0	0	0	7
	Male	7	2	0	4	5	2	0	0	0	20
	Unknown	1	0	0	0	1	0	0	0	0	2
<i>Giardia duodenalis</i> (B)	Female	19	2	6	4	0	4	8	1	1	45
	Male	30	2	4	9	3	5	2	1	1	57
	Unknown	1	0	0	0	0	0	0	0	0	1
<i>Ancylostoma ceylanicum</i>	Female	0	0	1	0	0	0	0	0	0	1
	Male	0	0	1	0	0	0	0	0	0	1
<i>Ancylostoma duodenale</i>	Female	0	0	1	1	0	0	0	0	0	2
	Male	1	0	0	0	1	0	0	0	0	2
<i>Necator americanus</i>	Female	0	3	0	0	0	0	0	0	0	3
	Male	0	0	0	1	0	0	0	0	0	1
<i>Haemonchus contortus</i>	Female	1	0	0	0	0	0	0	0	0	1
	Male	0	0	0	0	0	0	0	0	0	0

Figure 1. Representative examples of single-strand conformation polymorphism (SSCP) analyses of amplicons. Panel A: ITS-2 amplicons representing *Ancylostoma ceylanicum* (lanes 1 and 3-5), *Necator americanus* (lane 2). Panel B: *pgp60* amplicons representing *Cryptosporidium parvum* IbA10G2, *C. hominis* IIaA18G3R1, *C. hominis* IIaA20G3R1, *C. hominis* IIaA15G2R1 (lanes 1-4) and *C. hominis* IbA10G2 (lanes 5-8). Subgenotypic classification was made following the sequencing of amplicons.

Figure 2. Phylogenetic relationships of *Cryptosporidium hominis* from humans in the present study (bold-type, followed by sample code) based on analysis of *pgp60* sequence data by Bayesian inference; the tree is rooted to *C. parvum*. Posterior probabilities are indicated adjacent to nodes. The number of samples with a particular sequence is in round parentheses.

Figure 3. Phylogenetic relationships of *Cryptosporidium parvum* from humans in the present study (bold-type followed by sample code) based on analysis of *pgp60* sequence data by Bayesian inference; the tree is rooted to *C. hominis*. Posterior probabilities are indicated adjacent to nodes. The number of samples with a particular sequence is in round parentheses.

Figure 4. Phylogenetic relationships of *Giardia duodenalis* from humans in the present study (bold-type, followed by sample code) based on analysis of *ptpi* sequence data by Bayesian inference; the tree is rooted to *G. muris* and *G. ardeae*. Posterior probabilities are indicated adjacent to nodes. The number of samples with a particular sequence is in round parentheses.

Figure 5. Phylogenetic relationships of strongylid nematodes from humans in the present study (bold-type, followed by sample code) based on analysis of ITS-2 sequence data by Bayesian inference; the tree is rooted to *Oesophagostomum dentatum*. Posterior probabilities are indicated adjacent to nodes. The number of samples with a particular sequence is in round parentheses.