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Detection of selected intestinal helminths and protozoa at Hospital Universiti Sains Malaysia using multiplex real-time PCR

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Abstract. Intestinal parasites are the causative agents of a number of important human infections in developing countries. The objective of this study was to determine the prevalence of selected helminths and protozoan infections among patients admitted with gastrointestinal disorders at Hospital Universiti Sains Malaysia, Kelantan, Malaysia using multiplex real-time PCR. In addition microscopic examination was also performed following direct smear, zinc sulphate concentration and Kato-Katz thick smear techniques; and the presence of protozoan parasites was confirmed using trichrome and acid-fast stains. Of the 225 faecal samples analysed, 26.2% were positive for intestinal parasites by the multiplex real-time PCR, while 5.3% were positive by microscopy. As compared to microscopy, the multiplex real-time PCR detected 5.8 and 4.5 times more positives for the selected helminth and protozoan infections respectively. Among the selected helminths detected in this study, hookworm was the most prevalent by real-time PCR, while *Ascaris lumbricoides* was detected the most by microscopy. Meanwhile, among the selected protozoa detected in this study, *Entamoeba histolytica* was the most prevalent by real-time PCR, however microscopy detected equal number of cases with *E. histolytica* and *Giardia lamblia*. This study showed that real-time PCR can be used to obtain a more accurate prevalence data on intestinal helminths and protozoa.

INTRODUCTION

Intestinal parasitic infections are endemic worldwide and remain as major public health problems in many tropical and subtropical countries (Mehraj *et al.*, 2008; Ngui *et al.*, 2011). It is estimated that more than three billion people are infected with intestinal helminths and protozoa (Balcioglu *et al.*, 2007; Kurt *et al.*, 2007).

Several factors affect the distribution and the prevalence of intestinal parasites. Personal hygiene, dietary habits, education level of the community, socio-economic status and climate are among the common factors that influence the prevalence of intestinal parasitic infections (Balcioglu *et al.*, 2007; Mahsol *et al.*, 2008).

Common intestinal protozoa such as *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum;* and intestinal helminth such as roundworms (*Ascaris lumbricoides*) and whipworms (*Trichuris trichiura*) are transmitted by ingestion of contaminated food or water or when contaminated hands come into contact with the mouth. Blood feeding hookworms (*Necator americanus* and *Ancylostoma duodenale*) and threadworm (*Strongyloides stercoralis*) are transmitted by skin penetration of the larva when in direct contact with contaminated soil.

The effects of intestinal parasitic infections depend on the species of the parasites, the affected organ and the host immunological status. In an immuno-

competent person, early infection of intestinal helminths or protozoa may cause mild diarrhoea and other gastrointestinal discomfort such as vomiting and bloating; and prolonged infection may lead to more serious conditions such as anemia, malabsorption and mental retardation especially among children (Varkey *et al.*, 2007; Amuta *et al.*, 2009).

In Malaysia, improvements in socio-economic status had significantly reduced the prevalence of intestinal parasitic infections in general, however many rural areas remain endemic. About 80.0% of studies in rural areas carried out after 1978 still reported high prevalence rates of intestinal parasites. In particular, among Orang Asli communities, the prevalence rates were more than 50.0%, and soil-transmitted helminths are the most common infections (Lim *et al*., 2009).

The infection prevalence largely depends on the method(s) employed and the number of examinations performed on the sample (Esparar *et al.*, 2004). In the routine diagnostic laboratory, the presence of faecal helminths and protozoa are usually diagnosed by microscopic examination. Microscopy can be labour intensive, especially when a large number of samples need to be screened in a relatively short time such as in epidemiological studies. Additionally, some of the parasites cannot be differentiated microscopically because they are morphologically similar. Therefore this may result in misdiagnosis leading to delayed or inadequate treatment or overdiagnosis that results in unnecessary treatment to the patient (Rayan, 2005; Leiva *et al*., 2006).

Molecular diagnosis is a very useful method to be used in epidemiological studies of intestinal parasitic infections, as well as in routine diagnosis of patients. In particular, real-time PCR can detect and quantify a relatively small amount of PCR product from a low concentration of parasite DNA. It is also able to detect PCR amplicons in a shorter time and allow the elimination of post-PCR steps which reduce the risk of contamination. Multiplex assay that simultaneously detects several parasite species is important to be used since multiple infections by intestinal helminths or protozoa are often seen, especially in children. The high incidence of multiple infections is exemplified by the results of a study performed in 1996 to 2001 using routine ova and parasite examinations of multiple stool samples from 10,358 new refugees in Minnesota, USA. The result showed that 19.0% were infected with pathogenic parasites, 12.3% of them were infected with more than one species of parasites and 52.0% were in the age of 6-18 years old (Varkey *et al.*, 2007).

The objective of this study was to determine the prevalence of selected intestinal helminths and protozoa infections among patients admitted at Hospital Universiti Sains Malaysia (HUSM) by multiplex real-time PCR assays, and the results were compared with those obtained using microscopic examination.

MATERIALS AND METHODS

Sample collection

The study was conducted from October 2008 to October 2010. As part of the routine diagnostic service, a total of 225 faecal samples were obtained from patients admitted at HUSM with general gastro-intestinal disorders. The minimum sample size was calculated according to Carley *et al*. (2005), based on 60.0% prevalence for both helminths and protozoan infections, 5% confidence interval, 0.05 desired precision on specificity and sensitivity, the availability of the samples in HUSM and the collection time. Samples were processed within four hours after collection for microscopic examination and one aliquot of each sample was stored at -20°C for subsequent DNA extraction. Ethical clearance for the use of the faecal samples was obtained from the USM Human Research Ethics Committee.

DNA extraction

DNA was extracted using the QIAamp Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Briefly, about 100 mg of sample was suspended in 200 µL phosphate buffered saline (pH 7.2) containing 2%

polyvinylpolypyrolidone (PVPP; Sigma, Steinheim, Germany). After heating for 10 minutes at 100°C, the suspension was treated with ATL buffer containing proteinase K for 3 hours at 55°C before performing the DNA extraction. Phocine herpesvirus 1 (PhHV-1) at 10^{-3} PFU/mL was added to the AL lysis buffer. The PhHV-1 is a universal and non-human virus control which is usually used in PCR assays for clinical samples (Harder *et al.*, 1996). The viral DNA serves as the internal control for the efficiency of the DNA extraction and PCR inhibition. This provides the evidence that negative results were real negatives and not caused by inhibition. The PhHV-1 was a kind contribution from Dr. Martin Schutten, Erasmus MC, Department of Virology, Rotterdam, The Netherlands.

Real-time PCR amplification and detection

The real-time PCR was performed with Corbett Rotor Gene 6000 real-time analyzer. It has a centrifugal rotary design that keeps each tube spinning in a chamber of moving air. This keeps all the samples at a precisely same temperature during rapid thermal cycling, thus ensuring well-to-well variation is insignificant. Samples were tested by established primers and probes which have been shown to be 100% when tested against DNA controls derived from a wide range of intestinal microorganisms (Verweij *et al.*, 2004, 2007, 2009; Wiria *et al.*, 2010). The pentaplex assay for the detection of four helminth species and internal control was performed according to our previous published report (Basuni *et al.*, 2011). Each sample in this study was tested by two multiplex assays: (i) detection of intestinal helminths i.e *A. duodenale*, *A. lumbricoides*, *S. stercoralis*, *N. americanus;* and PhHV-1 and (ii) detection of intestinal protozoa i.e. *E. histolytica*, *G. lamblia*, *C. parvum;* and PhHV-1. Each amplification reaction mixture (25 µL) consisted of PCR buffer (HotStar Taq master mix; Qiagen), 5 mM $MgCl_2$ (MBI Fermentas Inc., Amherst, NY), 0.1 mg/mL bovine serum albumin (Sigma Aldrich Corp.), 5 µL of template DNA, optimized concentrations of each species specific primers and probes.

For detection of intestinal helminths, the concentrations of each reverse and forward primers were 40 nM for *A. duodenale* and *A. lumbricoides*, 60 nM for *S. stercoralis* and PhHV-1, 80 nM for *N. americanus* and 250 nM of each probe. For detection of intestinal protozoa, the primers concentrations were 40 nM for *G. lamblia*, 60 nM for *E. histolytica* and PhHV-1, 200 nM for *C. parvum* and 250 nM of each probe. Included in each PCR run were negative control which consists of PCR mixture without DNA template, five positive controls comprising DNA of each helminth, and PhHV-1 viral DNA. The amplification program consisted of 15 minutes at 95°C, followed by 50 cycles of 9 seconds at 95°C and 60 seconds at 60°C, performed with the Rotor Gene 6000 real-time analyzer (Rotorgene-Q, Hilden, Germany). Amplification reactions were repeated for samples with Ct values above 35. Real-time PCR assay was considered as negative when the Ct value was more than 40 or no amplification curve was obtained. In addition, samples with a Ct value above 37 from the PhHV-1 internal control amplification are considered to be hampered by faecal inhibitory factors. In order to prevent contamination, preparation of master mix, extraction of DNA, and handling of PCR products were all performed in separate rooms using dedicated pipettes and equipments.

Microscopic examination

Samples were examined using direct faecal smear, zinc sulphate concentration, Kato-Katz thick smear, trichrome stain and modified acid-fast stain. These methods are part of the routine parasitological examination at the Medical Microbiology and Parasitology Laboratory, School of Medical Sciences, which routinely processes all samples for parasitological investigations from HUSM. For each sample, three slides for direct faecal smears, three slides from zinc sulphate concentration, one Kato-Katz preparation, one trichrome stain smear and one modified acid-fast stain smear were examined. Samples were considered positive if the organism was detected by any one of these methods.

Direct faecal smear

With an applicator stick, approximately 2 mg of faeces was mixed with a drop of normal saline at the left half of a slide to form a suspension. This step was repeated for iodine solution at the right half of the slide. Each drop was covered with a coverslip and examined.

Zinc sulphate concentration

Approximately 1 g faeces was emulsified in 10 ml of zinc sulphate solution (SG 1.18). The suspension was strained on two layers of gauze into a 15 ml centrifuge tube and centrifuged at 2 000 x g for 2 minutes. The entire surface samples obtained were placed on a slide several times, until the volume was equivalent of a large drop on the slide. Following that, iodine solution was added to the sample and the slide was examined. The above steps were also repeated for the sediment of the sample.

Trichrome stain

The faeces was smeared on a slide and allowed to air dry. The slide was placed in two changes of 70% ethanol for (i) 5 minutes (ii) 3 minutes and stained in trichrome stain for 10 minutes. The smear was destained in 90% acid ethanol for 1 to 3 seconds and dipped in 100% ethanol to rinse several times. The slide was placed in two changes of 100% ethanol for 3 minutes each and two changes of xylene for 10 minutes. A coverslip was placed on the smear by using mounting medium. About 200 oil immersion fields were examined on a slide using the 100X objective.

Modified acid-fast stain

The faeces was smeared on a slide and allowed to air dry. The smear was fixed in methanol for 2 to 3 minutes and stained with cold carbol-fuchsin for 5 to 10 minutes. The smear was differentiated in 1% HCl-ethanol until the colour stopped to flow out of the smear and then rinsed in tap water. The smear was counterstained with 3% malachite green for 30 seconds and then rinsed again in tap water and drained. A coverslip was placed on the smear by using mounting medium. About 200 oil immersion fields were examined on a slide using the 100X objective.

Kato-Katz thick smear

A small amount of faecal material was placed on a piece of newspaper. A small nylon screen was pressed on top of the faecal material. The faeces accumulated on top of the screen and the sieved faeces was collected. A template was placed on the centre of a microscope slide and the sieved faeces was added until the hole in the template was completely filled. The template was removed and the faecal material was covered with cellophane strip pre-soaked in glycerol-malachite green solution. The microscope slide was inverted and the faecal material was firmly pressed against the cellophane strip on microscope slide. The faecal material was spread evenly between the microscope slide and the cellophane strip. The second slide was carefully removed to avoid lifting the cellophane strip off. The slide was placed on the bench with the cellophane upwards. The slide was examined after 15 minutes under the microscope for hookworm ova and examined after one hour for *Ascaris* and *Trichuris* ova.

Statistical analysis

Statistical analysis was performed by using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL). The difference between the detection rates of microscopy and multiplex real-time PCR assay was analyzed using the chi-square test (χ^2). A P-value <0.05 was considered significant.

RESULTS

Table 1 shows the detection rates by multiplex real-time PCR and microscopy. As compared to microscopy, there was a 6.6 times increase in helminth detection and 4.5 increase in protozoa detection using multiplex real-time PCR ($P < 0.001$). There were 46 samples positive for helminths by real-time PCR, compared to 7 positive samples by microscopy. Meanwhile, for the three selected intestinal protozoa, 18 samples were positive by real-time PCR whereas 4 samples were positive by microscopy.

Of the total 225 faecal samples analyzed, 64 cases of intestinal helminths and protozoa

Table 1. Statistical comparison of positive samples detected by microscopy and real-time PCR method

Organism	Method	Positive samples detected	χ^2 statistic (df)	P-value
Helminths (N=225)	Microscopy	8	28.114 (1)	<0.001
	Real time PCR	46		
Protozoa (N=225)	Microscopy	4	46.833 (1)	<0.001
	Real time PCR	18		

Table 2. Details of each multiple infection of intestinal helminths and protozoa

Organisms detected in multiple infections	No. of samples
By microscopy	(N=2)
A. lumbricoides and *T. trichiura*	2
By multiplex real-time PCR	(N=9)
A. lumbricoides, *A. duodenale* and *N. americanus*	1
A. duodenale and *N. americanus*	1
A. duodenale and *S. stercoralis*	1
N. americanus and *S. stercoralis*	1
N. americanus and *G. lamblia*	4
A. lumbricoides and *E. histolytica*	1

infections were detected by the multiplex real-time PCR assays. Five cases had co-infections of intestinal helminths and protozoa, thus the number of positive samples by real-time PCR was 59/225. Samples with multiple helminth infections were 4/225, single protozoan infections were 13/225 (*E. histolytica* 3/13; *G. lamblia* 10/13; *C. parvum* 0/13) and 37/225 were samples with single infection of helminth species (*N. americanus* 13/37; *A. lumbricoides* 10/37; *A. duodenale* 11/37; *S. stercoralis* 3/37). The details of the multiple infections are tabulated in Table 2.

In comparison, out of the 225 samples, 12 samples were found to harbour one of the intestinal parasites using one or more of the conventional parasitological methods. Single infection with *A. lumbricoides* was found in most of the positive samples (5/12), followed by multiple infections with *A. lumbricoides* and *T. trichiura* (2/12), single infection by *G. lamblia* (2/12), single infection by *E. histolytica* (2/12), and single infection by *Trichuris trichiura* (1/12).

Table 3 shows the comparison of detection of the selected species of helminths and protozoa by the two methods. *Necator americanus* 20/225 (8.9%) was the most frequent parasite detected by multiplex real-time PCR, followed by *A. duodenale* 14/225 (6.2%), *G. lamblia* 14/225 (6.2%), *A. lumbrocoides* 12/225 (5.3%), *S. stercoralis* 5/225 (2.2%) and *E. histolytica* 4/225 (1.8%). On the other hand, by microscopy, the most common organism seen among the patients was *A. lumbricoides* 7/225 (3.1%). This was followed by *E. histolytica* 2/225 (0.9%), *G. lamblia* 2/225 (0.9%) and no positive case was recorded for *S. stercoralis*, hookworm and *C. parvum*. Other parasite seen by microscopic examination was *T. trichiura* 3/225 (1.3%). Similar with real-time PCR, no positive case was recorded for *C. parvum* by microscopy.

Overall, among the admitted patients at HUSM, multiplex real-time PCR recorded a prevalence of 26.2% for intestinal parasitic infection, while microscopic examination detected a prevalence of 5.3%. Three samples which were positive for *A. lumbricoides* by microscopy were not detected by real-time PCR. Instead two of them were detected as negative and one sample was detected as *N. americanus* by real-time PCR. In addition there were two samples detected as *E.*

Table 3. Detection of each species of helminths and protozoa by multiplex PCR and microscopy (n=225)

No.	Species of parasite	No of positives detected by multiplex real-time PCR (%)	No of positives detected by microscopy (%)
1.	*A. lumbricoides*	12 (5.3%)	7 (3.1%)
2.	*A. duodenale*	14 (6.2%)	0*
3.	*N. americanus*	20 (8.9%)	0*
4.	*S. stercoralis*	5 (2.2%)	0
5.	*T. trichiura*	Not available	3 (1.3%)
6.	*E. histolytica*	4 (1.8%)	2 (0.9%)
7.	*G. lamblia*	14 (6.2%)	2 (0.9%)
8.	*C. parvum*	0	0

* Microscopy is unable to differentiate hookworm ova of *A. duodenale* and *N. americanus*, zero is stated for both since no hookworm ovum was detected by microscopy

histolytica-positive by microscopy, but were negative by real-time PCR assay.

DISCUSSION

Patients infected with intestinal parasites often experience abdominal discomfort, dysentery or other general symptoms such as bloating, diarrhoea and fever. In heavy infection, serious health conditions and problems such as malnutrition and mental retardation especially in children may occur (Amuta *et al.*, 2009). Immunocompromised individuals are more vulnerable to acquire serious infections which may lead to death (Graczyk & Fried, 2007).

There are many studies on prevalence of intestinal helminths and protozoa in Malaysia. In one study, out of 716 sample collected from a rural area in West Malaysia, the prevalence of soil-transmitted helminth (STH) infections (73.2%) was found to be significantly more common compared to protozoan infections (21.4%), P<0.001 (Ngui *et al.*, 2011). Similarly, studies on children in Kelantan showed a higher prevalence of intestinal helminths compared to protozoa (Rahmah *et al.*, 1997; Menon *et al.*, 1999, 2001). Furthermore, a study on 246 stool samples collected from University Malaya Medical Center, Kuala Lumpur showed an overall infection rate of 6.9% (17/246), in which *T. trichiura* was the most common parasite (4.5%), followed by *A. lumbricoides* (0.8%), *Clonorchis sinensis* (0.8%), hookworm (0.4%), and *E. histolytica* (0.4%) (Jamaiah & Rohela, 2005). In a review of the epidemiology of STH in Malaysia from the 1970s to 2009, high prevalence rates persist among the rural aborigines, estate workers, urban slums and squatter areas (Al Mekhlafi *et al.*, 2007; Ahmed *et al.*, 2011). It was reported that prior to 1978, hookworm infection was the most predominant soil-transmitted helminth but trichuriasis is currently the most common STH infection (Lim *et al.*, 2009).

Intestinal protozoa are found in all communities in Malaysia and among all ethnic groups (Lai, 1992). According to a study by Nor Aza *et al.* (2003), intestinal protozoan infection rates in Malaysia varied between 18.8% and 91.4%. Norhayati *et al.* (2003) reported on the prevalence of amebiasis (1-14%) and giardiasis (2-19%); while Nor Aza *et al.* (2003) reported a high prevalence of *E. histolytica* (21.0%) and *G. lamblia* (8.6%). Noor Azian *et al.* (2007) showed the prevalence of intestinal protozoa among the aborigines in Pos Senderut, Pahang was 72.3% (94/130) in which *Blastocystis hominis* (52.3%), *G. lamblia* (29.2%), *Entamoeba coli* (26.2%), *E. histolytica* (18.5%) and 3-18% of mixed infections were demonstrated. A study among 346 HIV-infected individuals in Malaysia showed a high prevalence of intestinal protozoa infections (18.8%) compared to helminths (7.5%). The most common protozoa observed were *E. histolytica/dispar* (16.8%), *C. parvum*

(12.4%), *Isospora belli* (10.1%), *Cyclospora cayetanensis* (4.9%) and *G. duodenalis* (3.2%) (Asma *et al*., 2011).

According to Brown *et al*. (2003), performing a range of techniques on a single sample may enhance the detection of parasites since different techniques vary in their sensitivity for different parasite species. Therefore, in this study, more than one parasitological methods were used to diagnose intestinal parasitic infection to increase the diagnostic yield. The prevalence of intestinal parasitic infection was found to be 26.2% (59/225) by multiplex real-time PCR and 5.3% (12/225) by microscopic examination among the patients at HUSM. This shows that the prevalence by real-time PCR was 4.9 times higher than by microscopy. This study also showed that intestinal helminth infections were more common than intestinal protozoa infections by both parasitological and DNA detection methods i.e. 1.8 times and 2.6 times respectively. This is also in concordance with the results of the previous studies in Kelantan by Rahmah *et al*. (1997) and Menon *et al*. (1999) in which the intestinal helminths infections were about 3.6 to 4.0 times greater than protozoan infections.

With regard to the helminth species detected by microscopy in Kelantan, *A. lumbricoides* (7/225 or 3.1%) remains the most common intestinal parasitic pathogen since the last reports by Rahmah *et al*. (1997) and Menon *et al*. (1999). Meanwhile, the most common organism detected by real-time PCR among the selected helminths was *N. americanus* (20/225 or 8.9%). Thus this study showed that the use of molecular method showed a different epidemiological picture than the use of microscopy, not just in terms of prevalence rates but also in terms of the dominant species.

There were three samples detected positive for *A. lumbricoides* by microscopy, however by real-time PCR, two samples were found to be negative while the other sample was positive for *N. americanus*. The negative samples may be caused by PCR failure caused by poor quality of DNA template, most probably due to DNA degradation from improper storage and transportation. On the hand, the apparent misidentification of *N. americanus* as *A. lumbricoides* in the other sample may be due to visual fatigue of the microscopist. In addition, two microscopy-positive *E. histolytica* were also detected as negative by real-time PCR. This may be be due to the fact that microscopic examination cannot differentiate between pathogenic *E. histolytica* and non-pathogenic *E. dispar*.

In this study, real-time PCR assay was found to be simpler and more rapid than the panel of parasitological techniques that was performed for microscopic examination of the samples. In terms of cost, each multiplex real-time PCR assay was approximately US$2.61; while the cost for three wet smears, and ZnSO4 concentration technique followed by examination of six slides (three from surface sample, and three from sediment samples) cost approximately US$2.60 per sample. In addition, the parasitological traditional technique requires an experienced microscopist, and is less sensitive than the DNA detection method.

Therefore multiplex real-time PCR is very useful for the detection of intestinal parasitic infections, either for patient diagnosis, epidemiological studies or monitoring of the prevalence and intensity of intestinal parasitic infections during intervention programs. In this study, the multiplex real-time PCR on the selected helminths and protozoa showed a high prevalence of intestinal parasites among patients admitted to HUSM for gastro-intestinal disorders.

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