Multiplex real-time PCR monitoring of intestinal helminths in humans reveals widespread polyparasitism in Northern Samar, the Philippines

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Abstract

The global socioeconomic importance of helminth parasitic disease is underpinned by the considerable clinical impact on millions of people. While helminth polyparasitism is considered common in the Philippines, little has been done to survey its extent in endemic communities. High morphological similarity of eggs between related species complicates conventional microscopic diagnostic methods which are known to lack sensitivity, particularly in low intensity infections. Multiplex quantitative PCR diagnostic methods can provide rapid, simultaneous identification of multiple helminth species from a single stool sample. We describe a multiplex assay for the differentiation of *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale*, *Taenia saginata* and *Taenia solium*, building on our previously published findings for *Schistosoma japonicum*. Of 545 human faecal samples examined, 46.6% were positive for at least three different parasite species. High prevalences of *S. japonicum* (90.64%), *A. lumbricoides* (58.17%), *T. saginata* (42.57%) and *A. duodenale* (48.07%) were recorded. Neither *T. solium* nor *N. americanus* were found to be present. The utility of molecular diagnostic methods for monitoring helminth parasite prevalence provides new information on the extent of polyparasitism in the Philippines municipality of Palapag. These methods and findings have potential global implications for the monitoring of neglected tropical diseases and control measures.

Keywords: Multiplex qPCR, Intestinal helminths, Schistosomiasis, STH infection, Polyparasitism, The Philippines, Taeniasis

1. Introduction

Soil-transmitted helminths (STH) pose a significant public health risk in many of the developing regions of south eastern Asia (SEA), including the Republic of the Philippines. Approximately one-third of global STH cases occur in SEA (Jex et al., 2011). *Ascaris lumbricoides* is the most common STH infecting approximately 1 billion individuals, followed by *Trichuris trichiura* and hookworm spp. which collectively infect approximately 600 - 800 million people (Bethony et al., 2006). The highest number of STH cases within SEA occur in Indonesia, followed by the Philippines (de Silva et al., 2003). STH are considered to be highly endemic in the Philippines due to high prevalences of *A. lumbricoides*, *T. trichiura* and hookworm infection reported (Belizario et al., 2009; Ezeamama et al., 2005; Jex et al., 2011). STH infection in children has been linked to lower cognitive function, malnutrition and health problems such as anaemia (Ezeamama et al., 2005; Fleming et al., 2006; Steinmann et al., 2010). The high prevalence of STH in the Philippines also reflects a high prevalence of co-infections, with some suggestions of synergism, susceptibility to other infections and compounding effects on pathology related to infection with multiple helminths, bacteria and protozoa (Abruzzi and Fried, 2011; Fleming et al., 2006; Papier et al., 2014; Ross et al., 2014).

Morphologically the eggs of the many STH are very similar, causing difficulty in distinguishing between many nematode species and, particularly, the hookworms, *Necator americanus* and *Ancylostoma duodenale*. Microscopic diagnostic techniques such as the Kato-Katz (KK) method fail to provide conclusive identification not only between species, but also between genera. Another limitation with methods such as KK is the fast clearance or lysis of hookworm eggs, if they are not preserved soon after the stool is produced, and if the KK slide is not read within 30 min of preparation (Dacombe et al., 2007; McCarthy et al., 2012).

Taenia is another helminth genus of public health concern in the Philippines, with the three major human species, *Taenia saginata*, *Taenia solium* and *Taenia asiatica* all considered endemic. In terms of health risks, *T. solium* is known to cause cystercercosis and neurocystercercosis (Flisser, 2013; González et al., 2004). *Taenia saginata* and *T. asiatica* infections cause taeniasis which, while generally asymptomatic, may produce symptoms including dizziness, abdominal pain, diarrhoea, headache and nausea (Schmidt and Roberts, 2005; Yamasaki, 2013). Allergic responses to antigen release by the adult tapeworms, or through the rupturing or death of the cysticercus, in the case of *T. solium*, can be fatal (Schmidt and Roberts, 2005). Cysticercal infections in humans occur as a result of ingesting eggs of *T. solium* (and possibly *T. asiatica* (Ale et al., 2014)). The presence of eggs in the faeces is not indicative of cystercercosis but reflects the presence of adult worms in the gastrointestinal tract (GIT). Consuming raw or undercooked meat containing *Taenia* spp. cysts will result in adult tapeworm infections where the adult worms live and reproduce in the GIT. Despite this the prevalence of *T. solium* eggs in a clinical sample is a good means for monitoring the possibility of cystercercosis in a population, particularly as infected individuals can auto-infect themselves, and others, resulting in cystercercosis through the ingestion of the eggs they excrete. Poor hygiene is considered the main driving factor of this aspect of disease transmission.

The eggs of *T. saginata*, *T. asiatica* and *T. solium* are morphologically identical, and visual differentiation of gravid proglottids is also problematic (González et al., 2004). The development of molecular techniques has facilitated the development of sensitive assays which can distinguish between the three species (González et al., 2004; Yamasaki et al., 2004; Yamasaki et al., 2006). Gene sequencing has identified considerable DNA similarity between *T. saginata* and *T. asiatica* (Bowles and McManus, 1994; Jeon et al., 2007). Despite these sequence similarities, *T. saginata* and *T. asiatica* do not share a common animal intermediate host, with *T.*

asiatica, similar to *T. solium*, being primarily found in pigs, while *T. saginata* infects bovines (Schmidt and Roberts, 2005).

A multiplex quantitativePCR (qPCR) diagnostic approach allows for rapid screening for multiple helminth species within a single human stool sample (Gordon et al., 2011). In this study we utilize a multiplex qPCR and show that many Filipino villagers in the municipality of Palapag in northern Samar Province harbor multiple helminth infections.

2. Materials and methods

2.1. Ethics

Informed written consent was received from all human participants in the study and ethical approval for the human work was provided by the Ethics Committee of the Research Institute of Tropical Medicine (RITM), Manila, Philippines and the QIMR Berghofer Medical Research Institute Human Research Ethics Committee, Brisbane, Australia (Approval Number: H0309-058 (P524)).

2.2. Study design

A cross-sectional survey was carried out in 2011 in the municipality of Palapag, northern Samar Province, the Philippines, in order to determine the level of STH infections in humans using a multiplex qPCR method. Samples were initially collected as part of a schistosomiasis intervention trial where subjects positive for schistosome eggs were treated with 60 mg/kg of praziquantel (PZQ) on days 1 and 14 (Ross et al., 2014). Local and government health officials

were informed of subjects who were positive for STH infection so that follow-up treatment could be provided.

2.3. Study area

The study was undertaken in six barangays (villages): Napo, Capacujan, Mabaras, Matambag, Manajao and Magsaysay, in the municipality of Palapag in northern Samar Province, the Philippines (Fig. 1). These barangays have been subjected in the past to annual mass treatment with PZQ as part of the national schistosomiasis control program in the Philippines, although none of the barangays selected had been treated in 2011. Individual human samples were obtained by handing out labeled stool cups which were collected over 1 week for each barangay. Stool samples from 545 humans were randomly selected and stored in 80% (v/v) ethanol for subsequent DNA isolation and molecular analysis.

2.4. Multiplex qPCR

Two multiplex qPCR amplification strategies were designed to identify hookworm, *Taenia* spp. and *A. lumbricoides* eggs in human stool samples. In the first stage, a multiplex qPCR was performed utilising previously published primers and probes (Basuni et al., 2011; Taniuchi et al., 2011; Verweij et al., 2007; Wiria et al., 2010) for two species of hookworm, *N. americanus* and *A. duodenale*, and *A. lumbricoides* (Table 1). Primers and probes were specifically designed that were generic for all three *Taenia* spp. (*T. saginata*, *T. solium* and *T. asiatica*). Only samples positive for generic *Taenia* spp. were subjected to the second multiplex qPCR. The second multiplex qPCR distinguished *T. saginata* from *T. solium* with primers and probes specific for either species (Table 1).

The first multiplex qPCR, in a total volume of 18 µl, contained 10 µl of iTaq supermix (Bio-Rad, Hercules, CA, USA), 1 µl of H₂O, 4 pM of each primer and 2 pM of each probe for *A. lumbricoides*, *N. americanus* and generic *Taenia*; and 8 pM of each primer and 2 pM of probe for *A. duodenale*. The second multiplex reaction of 18 µl contained 10 µl of iTaq supermix (Bio-Rad), 3 µl of H₂O, 1 ng of each primer and 2 pM of each probe for *T. saginata* and *T. solium*.

The qPCR cycling conditions consisted of 3 min at 95°C followed by 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s followed by 5 min at 72°C. A 5-plex Corbett RotorGene 6000 (Qiagen, Hilden, Germany) was used for the qPCR. Normal melt curves and comparative quantification analyses were used to determine the positive status of individual samples.

2.5 Positive and negative controls

Positive controls for generic *Taenia*, *T. saginata*, *T. solium*, *A. duodenale*, *N. americanus* and *A. lumbricoides* were cloned copies of G-block gene fragments purchased from Integrated DNA Technologies (IDT, Coralville, USA) of 300 bp, specific for the gene of interest from each species (Table 2). The positive control for *Schistosoma japonicum* was DNA extracted from eggs isolated from the livers of infected mice (Dalton et al., 1997). The G-blocks are custom, double-stranded, sequence-verified fragments of DNA and can be up to 500 bp. The qPCR amplicon for each positive control was sequenced, thereby confirming the fidelity of the assay. Negative controls of distilled H₂O were run with each qPCR.

2.6. Statistical analyses

Microsoft Excel and SAS software (SAS Institute, Cary, NC, USA) were used for data analyses. Ninety-five percent confidence intervals (95% CI) were calculated using standard formulae based on the binomial distribution (prevalence) and the lognormal distribution (infection intensity). Significance was calculated by using a general estimating equation model to calculate a *P* value. $P \geq 0.001$ was considered significant.

3. Results

The first qPCR strategy employing primers specific for *A. duodenale*, *N. americanus*, *A. lumbricoides* and the generic *Taenia* primer set, returned positive results for *A. duodenale*, *A. lumbricoides* and for generic *Taenia* spp. using genomic DNA isolated from the 545 human faecal samples collected. None of the 545 samples were positive for *N. americanus*, although the positive G-block control resulted in successful amplification. The qPCR assay identified 122 samples positive for *Taenia* spp. and those were then subjected to the second qPCR assay to determine species-specific identification; all were positive for *T. saginata*.

The results from these multiplex qPCR assays were compared with our previously published findings from the same faecal samples that were used to determine the presence of *S. japonicum* infection using a singleplex qPCR assay (Gordon et al., 2015b; Lier et al., 2006). Accordingly, four species of parasitic helminth - *S. japonicum*, *A. lumbricoides*, *A. duodenale* and *T. saginata* - were identified within the study cohort. The majority of samples were positive for more than one parasite species, with only 15.6% of individuals having a single infection and 46.6% infected with three or more different parasites (Table 3, Fig. 2).

The presence of single and multiple helminth infections were stratified by gender, age and barangay (Table 4). There were no statistically significant differences evident between

gender or age groups for any of the helminth infections. Examination of infection levels by barangay indicated the prevalence of dual infections was lowest in Napo, being significantly lower than in Capacujan, Mabaras and Magsaysay ($P = 0.003$) (Table 4). Conversely, co-infection with three helminth species was highest in Napo, being significantly higher than in Capacujan, Matambag, Mabaras and Magsaysay. Co-infection with the four helminth species was lowest in Magsaysay, being significantly lower than in Capacujan and Mabaras ($P = 0.006$) (Table 4).

The most highly prevalent helminth species in the study cohort was *S. japonicum* (90.64%; 95% CI: 88.2 - 93.1), followed by *A. lumbricoides* (58.17%; 95% CI: 54.9 - 61.4), *A. duodenale* (48.07%; 95% CI: 43.9 - 52.3) and *T. saginata* (42.57% 95% CI: 38.4 - 46.7). Infection intensity was estimated based on cycle threshold (Ct) scores (Table 5) and reflected the entire dynamic range of the assay. The cut-off for a low infection intensity bracket was a Ct above 17 but less than 35, for medium infection intensity above 12 and below 17, and for high infection intensity a Ct below 12. These cutoffs were selected after performing a dilution series from the G-block gene fragments for each species. Cts for each dilution step were considered and cut-offs for categories of low, medium and high selected. Dilutions containing more than 0.9 ng/μl of DNA were considered high, 0.10 – 0.9 ng/μl, medium and lower than 0.10 ng/μl, low.

4. Discussion

Extensive polyparasitism was a feature of individuals in the Palapag study villages with approximately 89% of residents infected with two or more different helminth species. Intensity of infection was estimated based on Ct scores and categorised as low, medium or high infection intensity. Unlike our previous reports for *S. japonicum* (Gordon et al., 2015a; Gordon et al., 2015b; Gordon et al., 2012) eggs per gram of faeces (EPG) could not be calculated more

precisely for *A. lumbricoides*, *A. duodenale* and *T. saginata*. Ct cut-offs for the three categories were made based on the results of a dilution series. A direct correlation between Cts and EPG would not be possible with the current experimental set-up. As with our work on *S. japonicum*, where we had access to eggs (Gordon et al., 2015a; Gordon et al., 2015b; Gordon et al., 2012), we would need to do seeding and extraction experiments to estimate more precisely how much DNA is contained per egg and extrapolate that data to EPG for unknown samples. While these intensity of infection calculations are not truly representative of EPG, they give an indication of the infection intensity. Approximately 50% of *A. lumbricoides* and *A. duodenale* infections were in the high intensity infection category, while for *S. japonicum* more than 90% of infections were in the low or medium infection intensity categories. Only 33.62% of *T. saginata* infections were in the high category. The high infection intensities for the STH may correlate with higher levels of morbidity and thus increase the significance of the presence of these helminths in this population. The World Health Organization (WHO) guidelines characterise light infections of *A. lumbricoides* as 1 – 4, 999 EPG, moderate as 5, 000 – 49, 999 EPG, and heavy intensity infection as $\geq 50,000$ (WHO, 2011). For hookworm, light intensity infections are 1 – 1, 999 EPG, moderate are 1, 000 – 9, 999 and heavy are $\geq 10,000$ (WHO, 2011). Future molecular studies will look at quantifying EPG using qPCR.

The results indicated that *N. americanus* was not present in this study cohort despite being found in other areas of the Philippines (Carney et al., 1980; Carney et al., 1981). A recent malnutrition study which included a survey using KK of STH infection in children from the Palapag area showed prevalences of 54.42%, 71.36% and 25.34% for *A. lumbricoides*, *T. trichiura* and hookworm, respectively (Papier et al., 2014).

Infection with one species of STH can cause significant disease in humans and reduce productivity, cognitive function and physical growth, and can aggravate the effects of malnutrition (Bethony et al., 2006; Stephenson et al., 2000). Helminth co-infections not only

increase the burden of parasitaemia in individuals but can also increase disease morbidity. For example, co-infections of *A. lumbricoides* or *S. japonicum* with hookworm spp. can lead to an increase in anaemia (Abruzzi and Fried, 2011; Ezeamama et al., 2008), as well as increased egg production by both species (Abruzzi and Fried, 2011; Fleming et al., 2006). Maternal infection with STH can cause an increase in susceptibility to STH infection in the unborn child (Mehta et al., 2012). In the Palapag villages under study, a very high rate of chronic malnutrition in children was recently reported that was shown to be significantly associated with STH infection (Papier et al., 2014).

Chemotherapy using mebendazole (500 mg) or albendazole (400 mg) is recommended by the WHO for treating STH infections (<http://www.who.int/mediacentre/factsheets/fs366/en/>). Co-infections necessitate combination therapy with benzimidazoles supplemented with ivermectin and/or diethylcarbamazine (Belizario et al., 2003; Keiser and Utzinger, 2010) so it is important to differentiate individuals with polyparasitemia from those with mono infections as this can impact the selection of treatment regimens and subsequently the effectiveness of disease control. Mass treatment of school children for STH infections is regularly carried out by the Departments of Health and Education in the Philippines (Bacon et al., 2012; Belizario et al., 2014) (<http://www.doh.gov.ph/content/soil-transmitted-helminth-control-program.html>). Annual community mass treatment with PZQ is also undertaken for schistosomiasis but, in general, coverage is less than 50% (Tallo et al., 2008) (<http://www.doh.gov.ph/content/schistosomiasis-control-program.html>). Mass treatment for tapeworm infection in the Philippines has not been reported.

The main risk factors promoting STH infection in humans are poor personal hygiene, lack of toilets, occupation and inadequate or contaminated water supply (de Silva et al., 2003; Ohta and Waikagul, 2007). Wearing shoes, toilet use, washing hands after defecation and before eating are also proven evidence-based behavioural risk factors which greatly impact STH

prevalence (Nasr et al., 2013a, b). A recently developed and tested education-based intervention package, targeting rural schools in Hunan province, People's Republic of China (PR China) included an educational cartoon video and focused on increasing students' knowledge about STH; the package led to a change in behaviour and a reduced incidence of infection by 50% within one school year (Bieri et al., 2013). This and other studies have shown that educational intervention, in combination with mass drug administration (MDA), can have a significant impact on STH infection and improve the quality of life for those living in endemic areas (Bieri et al., 2013; Monse et al., 2013; Nasr et al., 2013a, b). A video-based study is currently underway in the Philippines with the aim, as in PR China, of reducing STH infections (McManus et al., 2014), and it is an approach that could be readily extended to include a component to prevent schistosome and tapeworm infections.

Bovines and pigs are present in the Palapag study area, suggesting that the three *Taenia* spp. of medical importance could be endemic there. However, only *T. saginata* was identified. Due to the high sequence similarity between *T. asiatica* and *T. saginata*, and the small size of ideal qPCR amplicons, we were unable to create qPCR primers that are specific for *T. asiatica*. The qPCR results suggest that *T. solium* is not endemic in Palapag but *T. asiatica* may be transmitted there, due to the presence of pigs. High resolution melting (HRM) (Erali et al., 2009) was attempted to distinguish between the two species (data not shown) but it was neither possible to differentiate them nor to determine whether *T. saginata* and *T. asiatica* are co-endemic in this area. Other methods, such as restriction fragment length polymorphism (RFLP) analysis (Bowles and McManus, 1994) have been used in the past to differentiate *T. saginata* from *T. asiatica* but these methods may not be as sensitive as qPCR.

While *T. solium* was not identified in Palapag, the possibility of *T. asiatica* causing cysticercosis is still being explored and may be of importance there (Ale et al., 2014). Sentinel pigs have previously been used to monitor environmental contamination with *T. solium* eggs

(Gonzalez et al., 1994). Thus, while the presence of eggs in human faeces is not itself indicative of cysticercosis, the results of human prevalence for *Taenia* spp. based on eggs may serve a similar role to that of sentinel pigs and should be considered in parasitological surveys.

Trichuris trichiura is known to be highly endemic in the Philippines (Belizario et al., 2009; Ezeamama et al., 2005; Jex et al., 2011) and the recent study on childhood nutrition in Palapag referred to earlier found the prevalence of *T. trichiura* to be 71.36% (Papier et al., 2014). However, this species was not included in the initial multiplex assay undertaken here as the egg shells of *T. trichiura* are known to be resistant to standard copro-extraction methods (Areekul et al., 2010; Demeler et al., 2013; Mejia et al., 2013). As such, these eggs require an additional homogenization step prior to extraction to break open the eggs and effectively extract the DNA (Demeler et al., 2013). The human faecal samples used in this study were originally used to extract DNA for qPCR studies on *S. japonicum* (Gordon et al., 2015a; Gordon et al., 2015b) so the additional step required to extract DNA for *T. trichiura* eggs was not performed. In future, bead homogenization should be included as part of the DNA procedure for helminth identification using faecal samples from this and other STH-endemic areas. It is clear, however, that *T. trichiura* infections contribute substantially to the overall helminth disease burden and polyparasitism in Palapag municipality.

The sensitivity of microscopic techniques can vary depending on the experience and skills of the microscopists. In addition, the eggs of hookworm species cannot be distinguished morphologically and taeniid cestode eggs are identical in morphology. Furthermore, the 'gold standard' KK method for faecal egg detection has been shown to lack sensitivity in low intensity schistosome and STH infections (Glinz et al., 2010; Gordon et al., 2015b; Gordon et al., 2012; Knopp et al., 2009). An additional complication with hookworm eggs is that they are known to lyse rapidly and will not be recorded by the KK procedure if the KK slide is not read soon after preparation (Dacombe et al., 2007; McCarthy et al., 2012).

Multiplex qPCR allows for the rapid diagnosis of single, co- and multiple helminth infections that are commonly found, especially in SEA. The results obtained in this study for STH prevalence are concordant with other studies undertaken in Palapag and other areas of the Philippines, and the approach using molecular data only is consistent with recent publications (Basuni et al., 2011; Taniuchi et al., 2011). However, it should be stressed that multiplex qPCR has some limitations if being considered as a routine diagnostic method; these include, primarily, the high costs of reagents, the requirement for expensive equipment and the necessity for trained personal to undertake the tests and analysis of the results. It is also a procedure that cannot be performed in the field, necessitating transport of samples for analysis in a reasonably well-equipped laboratory.

In conclusion, the high level of polyparasitism we report in the human population of Palapag is likely to be mirrored in many other areas of the Philippines and other parts of SEA. We found no significant difference in gender or age groups for STH or tapeworm infection. Currently, mass treatment with PZQ for schistosomiasis is the only program for helminth control that targets the whole community in the Philippines, although the results have been sporadic (Ross et al., 2014). The high prevalence of STH infection recorded in this study suggests that a similar program of drug administration for the STH should be considered in combination with health education and other evidence-based strategies such as water, sanitation, and hygiene ‘WASH’ (Campbell et al., 2014) and ‘Blue Bay’ (Barreto et al., 2007; Mascarini-Serra et al., 2010), to prevent rapid re-infection. Additionally, combination drug therapy is advisable for multiple helminth infections which, as we report, are likely very common throughout the Philippines. Despite its limitations, the sensitive and specific diagnosis of single, co- and multiple helminth infections by multiplex qPCR can help to establish accurate baseline prevalences and to determine whether control interventions such as drug treatment have been effective at follow-up.

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Figure legends

Fig. 1. Map showing study location sites. (A) Map of the Philippines showing the location of northern Samar province, highlighted in green. (B) Map of the municipality of Palapag showing barangay study locations. Reprinted with permission from Gordon et al. (2015a).

Fig. 2. Venn diagram showing numbers (n) of single, double, triple and quadruple infections with *Schistosoma japonicum* (SJ), *Taenia saginata* (TS), *Ancylostoma duodenale* (AD) and *Ascaris lumbricoides* (AL) in the study sample.