**Clinical helminthic disease diagnosis in border regions of Thailand shows elevated prevalence levels using multiplex qPCR combined with traditional microscopic methods**

Poom Adisakwattana, 1 Tippayarat Yoonuan,1 Orawan Phuphisut,1 Akkarin Poodeepiyasawat, 1 Nirundorn Homsuwan,1 Catherine A. Gordon,2 Donald P. McManus,2 Louise E. Atkinson,3 Angela Mousley,3 and Geoffrey N. Gobert3

1 Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

2 Molecular Parasitology Laboratory, Infectious Diseases Division, QIMR Berghofer Medical Research Institute, Brisbane, 4006, Australia.

3 School of Biological Sciences, Queen's University Belfast, Belfast, BT9 5DL, UK.

**JID-** Major Article: 3500 words, 50 references, 7 inserts (tables and figures, with no more than 4 panels per figure) in print, and no more than 25 MB of online-only supplementary data.

**ABSTRACT (150 words)**

***Background.*** Under-regulated national borders in Southeast Asia represent potential regions for enhanced parasitic helminth transmission and present barriers to helminthiasis disease control.

***Methods.*** Three Thailand border regions close to Myanmar, Laos and Cambodia, were surveyed for clinical parasitic helminth disease. In-field microscopy was performed on stools from 567 individuals. Sub-samples were transported to Bangkok for molecular analysis comprising three multiplex qPCR assays.

***Results.*** The overall helminth infection prevalence was 18.34% as assessed by Kato Katz and 18.87% by qPCR. The combined prevalence of the two methods was 28.75%; the most predominant species detected were *Opisthorchis viverrini* [18.34%], hookworm [6.88%] (*Ancylostoma* spp. and *Necator americanus*), *Ascaris lumbricoides* [2.29%] and *Trichuris trichiura* [1.76%].

***Conclusions.*** These data demonstrate the value of molecular diagnostics for determining more precise prevalence levels of helminthic disease in Southeast Asia. Availability of such accurate prevalence information will help guide future public health initiatives and highlights the need for more rigorous surveillance and timely intervention in these regions.

**Key words:** Helminthiases; Kato Katz; molecular diagnostics; qPCR; Thailand; Thai border regions; Southeast Asia.

**Abstract word count:** 157 (needs to be under 150)

**Main Text word count:** ~3000 (3500 max)

**INTRODUCTION**

Parasitic worm infections (helminthiases) of humans greatly impact low to medium income countries, including Thailand and bordering countries. Worldwide, helminths infect an estimated 1.45 billion people [[1](#_ENREF_1)], with one third of cases occurring in Southeast Asia (SEA) [[2](#_ENREF_2)]. Most affected are young people, particularly pre- and primary school-aged children (0-12yrs), leading to a range of health issues including stunted growth and delayed mental development [[3](#_ENREF_3)]. Since most helminth diseases cause chronic morbidity (95% of health-attributed losses) rather than acute disease, the impact on the health and economic output of endemic areas is frequently reported in terms of DALYS (Disability-Adjusted Life Years); these measure the years lost due to morbidity, illness, and premature death. Helminth infections world-wide in 2010 were responsible for 14 million DALYs [[4](#_ENREF_4)]. Hookworm infection alone, largely due to anaemia, was responsible for over 4 million DALYs, with the total economic loss due to reduced productivity estimated to be between US$7.5 and 138.9 billion [[5](#_ENREF_5)].

Helminth infections are routinely field diagnosed using the “gold standard” Kato Katz (KK) method, a low-sensitivity microscopic technique, which is limited by species range and quantitative accuracy. The KK can underestimate prevalence [[6](#_ENREF_6)] and therefore impact on MDA (mass drug administration) programs and outcomes.

The previous national surveillance on intestinal helminthiases in Thailand, conducted by the Thai Ministry of Public Health in 2009, revealed an overall prevalence among 15,555 Thai people of 18.1% with the highest prevalence in the North-eastern region of Thailand [[7](#_ENREF_7)]. Recently, only limited spatial and temporal surveillance has been conducted, which may be insufficient to adequately guide the national public health initiatives [[8-10](#_ENREF_8)]. Moreover, prevalence surveillance studies carried out on helminth prevalence to date [[7](#_ENREF_7), [9](#_ENREF_9), [11](#_ENREF_11)] have been undertaken primarily using microscopy-based faecal examination (KK) without combining with molecular detection, which may underestimate the actual extent of helminth infection.

In this study we explore the utility of molecular diagnostics procedures for the detection of active helminth disease in Thai populations close to border regions with Myanmar, Laos and Cambodia. Comparisons with the traditional KK microscopy are made and the strengths and weakness of both methods assessed and discussed. The central aim of this work was to provide a more accurate picture of the prevalence of helminthiases in these areas. With this key information public health officials will be able to better plan control measures for the future to reduce helminth disease transmission.

**METHODS**

**Ethics statement**

This study, which included the collection of human stool samples and their examination for helminth infection using microscopy and real time PCR, was approved by the Human Research Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The results from the two diagnostic approaches will be reported to health promoting hospitals in the targeted areas for further action by local medical doctors or heath officers according to treatment guidelines provided by the Department of Disease Control, Ministry of Public Health, Thailand [[12](#_ENREF_12)].

**Study Areas**

Allstudy sites were located near three Thai border regionsVillages within these border regions were surveyed ( **Figure 1**): (i) the Mae Song Sub-District, Song Yang District and Tak Province close to the Thai-Myanmar border; (ii) Kham Khuean Sub-District of Kaeo Sirindhorn District within Ubon Ratchathani Province at the Thai-Lao border; and (iii) the Phran Sub-District of Khun Han District in Sisaket Province at close proximity to the Thai-Cambodia border**.** All sites were located within Thailand but were close (1-10 kilometres) to the bordering countries. The three sites included multiple villages within the provinces of Sisaket, Tak and Ubon Ratchathani (**Figure 1**). Within these provinces at total of 14 villages were surveyed (7,1 and 6, respectively).

**Sample Collection**

The three field sites were visited on one occasion each between December 2017 – February 2018. In the 14 villages surveyed, 567 clinical faecal samples were collected. Across the Sisaket and Ubon Ratchathani sites 200 faecal samples from both provinces were collected (400 in total) and 167 were collected from the Tak site. Ages of participants (311 females; 256 males)

ranged from 1.5 to 88 years; age demographic data were not collected for 43 sampled individuals.

Labelled sample cups were provided to health volunteers for distribution to participants. The samples were collected the next day either by health volunteers or brought to a collection point by participants. Information on age, gender, occupation, and geographical location, was collected at the same time as faecal samples. KK analysis was conducted at the site. Parallel faecal samples were stored in 80% (v/v) ethanol for transport at room temperature to Bangkok for molecular analysis.

KK was performed on all human faecal samples collected as previously reported [[13](#_ENREF_13)]. In brief, individual stool samples were pressed through a stainless sieve (size 40 mesh), and the non-retained material used to fill a standard volume template which equated to 39.2 mg of material. Glycerin-malachite green soaked cellophane was placed on the sample with a glass slide and firmly pressed to spread the stool evenly across the surface. The slide was viewed on a light microscope after 30 minutes. Two slides were prepared from each sample and analysed independently by two trained microscopists. All eggs were identified as observed when possible. All samples were anonymised and blinded.

**Multiplex qPCR**

Due to the breadth of the potential helminth infection profile amongst the participants, this study screened for 9 helminth species. As a result, three separate multiplex PCR assays were developed to identify the presence of helminth DNA in faecal samples. Faecal DNA was isolated using QIAamp® Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany). Positive controls were prepared by isolation of genomic DNA from adult parasites using QIAamp® DNA Mini Kits (Qiagen GmbH). Each assay was designed to detect three different helminth *spp.* Simultaneously as follows: Assay 1: *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Trichuris trichiura*; Assay 2: *Ancylostoma spp*., *Necator americanus*, *Opisthorchis viverrini*; Assay 3: *Taenia solium*, *T. saginata*, *Schistosoma japonicum*/*S. mekongi*. Primers and probes used in each assay are presented in **Supplementary Table 1** [[14-17](#_ENREF_14)]. Multiplex qPCR was performed in duplicate in a final volume of 20 µl by mixing 1 µl of faecal DNA (100 ng/µl) with 10 µl of iQ Multiplex Powermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 200 nM each of forward (Fw) and reverse (Rv) primers in addition to 100 nM of the appropriate TaqMan probe. Amplification was performed using the a CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA) with pre-incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. All positive control samples and a subset of clinical samples were checked for assay specificity by sequencing amplicons to confirm the identity of the helminth species detected.

**Statistical Analyses**

Excel (Microsoft) and SAS software (SAS Institute) were employed for statistical analysis. A sample was considered positive if there was at least one egg on a KK slide, or if a positive cycle threshold (CT) score was generated by qPCR (CT >37 was considered negative, CT <37 was considered positive). For the KK, egg counts were transformed to eggs per gram of faeces (EPG) by multiplying the average egg count from all slides by 24, based on the amount of material retained and examined after processing. Geometric mean EPG (GMEPG) was calculated using log-transformed egg counts. Standard formulae were used to calculate 95% confidence intervals (CI) for prevalence and intensity using biomodal distribution (prevalence) and the lognormal distribution (infection intensity). Relative sensitivity and specificity were calculated in two ways: (i) by combining the results of both KK and qPCR to act as the reference standard, and (ii) by using the qPCR results as the reference standard to calculate sensitivity and specificity of the KK, and the KK as the reference standard to calculate sensitivity and specificity of qPCR. Significance (Chi square, p-value) was calculated using general estimating equations in SAS; P≤ 0.05 was considered significant. The kappa coefficient was calculated to show agreement between the KK and qPCR methods. A coefficient between 0.81-1.00 was considered as almost perfect agreement, 0.61-0.80 high, 0.41-0.60 moderate, and 0.01-0.40 as low agreement.

**RESULTS**

A summary of individual participant demographics within the three field-site cohorts, as well as the KK and qPCR results for each participant are presented in **Supplementary Table 2**.

**KK field-based analyses**

KK analysis of all faecal samples collected from the three field sites indicated an overall prevalence of any helminth infection in all individuals of 18.34% (95%CI: 15.15-21.54%). The most common parasitic worms present were *O. viverrini* (8.82%; 95%CI: 6.48-11.16%), *A. lumbricoides* (2.12%; 95%CI: 0.93-3.30%), *T. trichiura* (1.59%; 95%CI: 0.56-2.62%) and hookworm (*Ancylostoma spp*. and/or *N. americanus*; 5.82%; 95%CI: 3.89-7.75%) ( **Table 1**). Hookworm infection was recorded based on the identification of the egg of either hookworm species since definitive species-level diagnosis is challenging by microscopy. There were no statistical differences in helminth infection prevalence? based on age, gender, or geographical location. No indication of active *S. mekongi* or *S. japonicum* infections were evident by KK analysis in any of the samples collected, across the three field sites. Intensity of infection was calculated by KK as the GMEPG. The highest GMEPG was evident with *A. lumbricoides* (2595.6) followed by *Taenia* spp. (1009.19) ( **Table 1**).

**Molecular diagnostics**

Stool samples were transported to Mahidol University, Bangkok for processing and molecular analysis. A panel of three separate multiplex qPCR assays was applied to all collected (n=567) samples.. When qPCR was used independently, the overall prevalence (for any helminth infections) was similar to that obtained by the KK method (18.87%; 95%CI: 15.64-22.10; **Table 2**). Major helminth species identified were: *O. viverrini* (17.28%; 95%CI: 14.16-20.41%), *A. lumbricoides* (2.12%; 95%CI: 0.93-3.30%), hookworm designated as either *Ancylostoma* spp. (1.59%; 95%CI: 0.56-2.62%) or *N. americanus* (2.29%; 95%CI: 1.06-3.53%) and *T. trichiura* (1.59%; 95%CI: 0.56-2.62%). All qPCRs were negative for *S. mekongi* and *S. japonicum* for all samples collected across the three field sites surveyed.

The range of CT scores for each helminth species detected, compared to the egg burdens determined by KK, is presented in **Supplementary Table 3**. CT cut offs were based on egg intensity (eggs/per gram). For *O. viverrini,* detected by KK in the largest number of samples (n=38), the CT range presented for lower egg numbers (1-5) was 22.63-32.09 and for higher egg numbers (>5) was 21.93-25.19. Other parasite species, despite having smaller positive sample numbers, presented similar differentiation of CT ranges between lower and higher egg burdens ( **Supplementary Table 3)**.

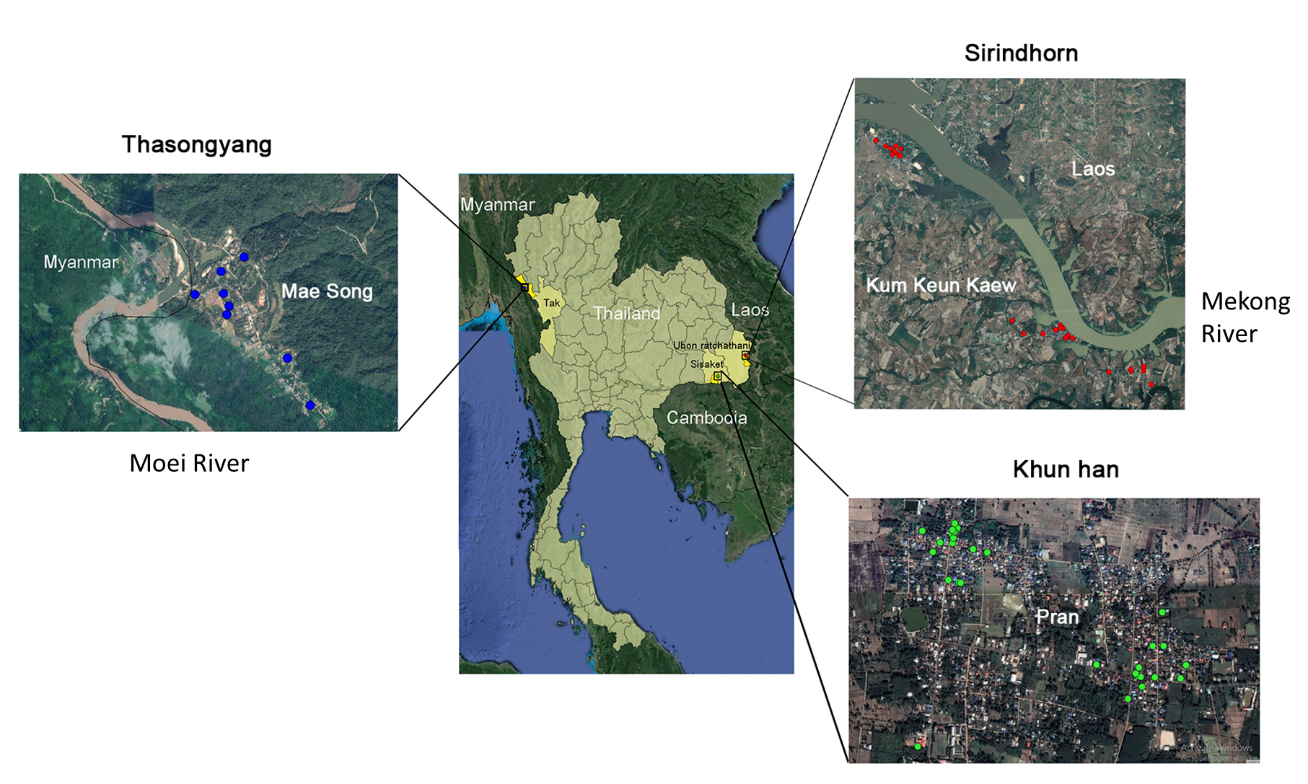
The kappa coefficient showed high levels of agreement between the qPCR and KK for *A. lumbricoides* (0.91), and *Taenia* spp. (0.86), and moderate agreement for *O. viverrini* (0.54) and hookworm spp. (0.53), while *T. trichiura* showed low agreement (0.18) (**Table 3**).

**Complementation of methods and regional differences**

Results from the KK and qPCR were combined for further prevalence analysis and considered across the three field sites (**Table 4**). The overall prevalence by Kato Katz and qPCR combined was 28.75% (95%CI: 25:01-32.48%). As shown by the individual methods, the most dominant species were *O. viverrini* (18.34%; 95%CI: 15.15-21.54), hookworm (*Ancylostoma* spp. or *N. americanus*, 6.88%; 95%CI: 4.79-8.97), *A. lumbricoides* (2.29%; 95%CI: 1.06-3.53) and *T. trichiura* (1.76%; 95%CI: 0.68-2.85).

When the KK and qPCR results were combined, within the three field sites, there was an overall prevalence of any helminth species of 28.14% in Tak, 31.00% in Ubon Ratchathani and 26.50% in Sisaket. Using the KK/qPCR combined dataset the most prevalent human helminthiases in the three individual regions surveyed were: for Tak, (Myanmar border); hookworm (17.96%; 95%CI: 12.08-23.85%), *A. lumbricoides* (7.78%; 95%CI: 3.68-11.89%) and *T. trichiura* (4.79%; 95%CI: 1.52-8.06%); for Ubon Ratchathani (Lao border): *O. viverrini* (28.00%; 95%CI: 21.72-34.28%) and *Taenia* (3.50%; 95%CI: 0.93-6.07); and for Sisaket (Cambodia border): *O. viverrini* (22.00%; 95%CI: 16.21-27.79%) and hookworm (4.00%; 95%CI: 1.26-6.74%). A complete summary of results by region is shown in **Supplementary Table 4**.

There was a relatively low level of polyparasitism in all three regions surveyed. As shown in **Table 4,** the only co-infections, as determined by KK or qPCR, consisting of two or more helminth species (in more than 1 sample) included: *A. lumbricoides* and hookworm (6 cases, prevalence 1.06%; 95%CI: 0.21-1.90); *O. viverrini* and *Taenia* (4 cases, prevalence 0.71%; 95%CI: 0-1.4); *T. trichiura* and *O. viverrini* or hookworm (both 2 cases each, prevalence 0.35%; 95%CI: 0-0.84).



**Figure 1. Location of field sites**. The Thai-Myanmar (Tak-Thasongyang), Thai- Lao (Ubon Ratchathani-Sirindhorn) and Thai-Cambodia (Sisaket- Khun han) border regions are shown. Specific field collections sites are marked for the three regions in the enlargements of regions.

**DISCUSSION**

Infection with *O. viverrini* was by far the most common species detected in this study, as shown by both the KK procedure (8.82%) and qPCR analysis (17.28%). In 2009 the national prevalence of *O. viverrini* in Thailand was reported as 8.7% based on a modified KK [[7](#_ENREF_7)]. *O. viverrini* is a food borne trematode fluke which is transmitted by consumption of raw or undercooked fish. Koi, a dish of raw, spiced fish, is a popular dish in Thailand and Lao, providing a ready means of infection with this parasite; indeed, infection with *O. viverrini* is relatively common in Thailand, Lao, Myanmar, and Cambodia [[18](#_ENREF_18)]. In addition, due to the consumption of Koi and other raw fish dishes, cholangiocarcinoma, which is induced by *O. viverrini*, is a major health problem [[2](#_ENREF_2), [7](#_ENREF_7), [11](#_ENREF_11), [19](#_ENREF_19)]. In the current study, males had a higher prevalence of any parasitic helminth infection, and of *O. viverrini* infection in particular, although this was not significant. There were no significant associations with age for *O. viverrini* or any of the other parasitic helminths.

The prevalence of the soil-transmitted helminths (STH; hookworm, *T. trichiura*, *A. lumbricoides, S. stercoralis*), and *Taenia* spp. was low; hookworm was the most prevalent at 6% (by KK), followed by *A. lumbricoides, S. stercoralis,* and *Taenia* spp. STH are endemic in Thailand, including zoonotic forms of *Trichuris* (*T. vulpis*) and the hookworm *A. ceylanicum* [[20](#_ENREF_20)]. Primers used in the current study did not differentiate between *A. duodenale* and *A. ceylanicum*, and it is therefore possible that this important zoonotic species also exists in the study areas [[7](#_ENREF_7)]. In previous surveys of preschool- and school-age children at the Thailand-Myanmar border, , the prevalence of STHs was > 20% in all preschool centres and primary schools (data from Thailand Development of Children in Remote and Poverty Area project, Ministry of Public Health, Thailand (<http://www.psproject.org/News_propagandise.htm>, in Thai). As indicated earlier, no differences in infection prevalence by age were evident in our study, but within the cohort of 567 individuals, only 52 were under 12 years of age.

Kappa analysis showed the best agreement between the qPCR and KK methods was 0.91 for *A. lumbricoides*, indicating almost perfect concordance between the two diagnostic approaches, followed by 0.86 for *Taenia* spp. (**Table 3**). The lowest kappa score, demonstrating the least agreement between the two tests, was for *T. trichiura* (0.18; **Table 3**), but the overall number of positive samples for *T. trichiura* (9 by KK, and 2 by qPCR)was low, making it difficult to draw robust conclusions from these data. One possible reason for the low number of *T. trichiura* infections positive byqPCR relates to the thickness of the *T. trichiura* egg shell, as a result of which complete lysis of the egg and DNA extraction are unlikely to be successful [[21](#_ENREF_21)]. In previous studies the addition of bead homogenisation prior to DNA extraction has been utilised to effectively extract DNA from this species [[21](#_ENREF_21)]. The addition of this step may also increase the availability of DNA from other helminth species present in the samples, potentially reducing the number of false negatives.

There was a low concordance (0.54) between the two diagnostic tests for *O. viverrini*, identification, indicating only moderate agreement between the two. The qPCR identified nearly twice as many positive samples as the KK, accounting for much of the lack of agreement evident. The KK is known to lack sensitivity, particularly in low intensity infections, [[22](#_ENREF_22), [23](#_ENREF_23)] and likely accounts for the lower prevalence of *O. viverrini* determined in this study by this method. Furthermore, the GMEPG for *O. viverrini* was a relatively low 65.49indicating that the KK missed low-grade infections that were subsequently identified by qPCR (**Table 1**).

Overall, the data presented here for Thai border communities highlight the value of molecular diagnostic tools for regional assessment of helminth prevalence in SEA as these resources provide accurate quantitative prevalence figures for helminthic diseases. Molecular methods have become increasingly common, particularly multiplex approaches, to identify a wide range of pathogens as many helminth and protozoan parasites overlap in endemic regions [[19](#_ENREF_19), [24-26](#_ENREF_24)].

The use of molecular diagnostics to obtain a more complete picture of helminthiases in SEA has proven highly effective in the Philippines where much higher levels of polyparasitism are evident and where the overall prevalence of *S. japonicum* (~91%), *A. lumbricoides* (58.17%), *T. saginata* (42.57%) and *A. duodenale* (48.07%) determined by qPCR was substantially higher than by the KK [[25](#_ENREF_25), [27](#_ENREF_27)]. The data presented here provide a contrasting profile of local helminthiases in the three Thaiborder regions surveyed compared to Palapag in the Philippines [25, 27], with the presence of *O. viverrini* and absence of active schistosomiasis being key differences. In addition, compared with the Philippines Palapag study site, we recorded low levels of polyparasitism (a small number (3.9%) of dual infections only). A 2018 study in central Thailand reported low prevalence of *S. stercoralis* (1.5%) and *O. viverrini* (0.4%) in children using the KK procedure [[10](#_ENREF_10)]. Another study in Chachoengsao Province, central Thailand, using a simple direct smear and formalin ethyl acetate concentration technique with parasite identification in positive samples confirmed using microscopy, reported an overall prevalence of 16.1% intestinal parasitic infections; STH (14.3%) were more common than protozoan infections (1.8%), and the most common intestinal parasites were hookworms (6.7%), then S. stercoralis, (5.0%), A. lumbricoides (1.3%) and T.s trichiura (1.3%) [[9](#_ENREF_9" \o "Suntaravitun, 2018 #2780)]. The findings we present here suggest that both these studies likely underestimated the prevalence of helminthiases in these two areas.

**CONCLUSIONS**

The prevalence of helminthic infections in communities of Thailand-Myanmar, -Laos and -Cambodia border regions is higher by qPCR analysis compared with previous reports based on microscopy methods alone. As a consequence, more comprehensive surveys of helminth infection prevalence and intensity using molecular methods are urgently required in these areas and should be coupled with more widespread public awareness of helminth diseases and the instigation of health educational interventions.

**ACKNOWLEDGMENTS**

Funding from the Northern Ireland Department of Economy (DfE) is noted. DPM acknowledges financial support from the National Health and Medical Research Council (NHMRC) of Australia (Grant numbers: ID613671; APP1037304; APP1098244).

The support of local public health officials in all three regions is gratefully appreciated. The excellent work of the Mahidol University field team is acknowledged.

**Table 1. Summary of prevalence and intensity levels for helminth species determined by Kato Katz combined for all three regions surveyed.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Number positive 1 | Prevalence % | 95% CI# | EPG\* | 95% CI | GMEPG\*\* | 95%CI |
| Positive any species | 104 | 18.34 | 15.15-21.54 |  |  |  |  |
| *O. viverrini* | 50 | 8.82 | 6.48-11.16 | 11.52 | 5.29-17.74 | 65.49 | 48.28-88.83 |
| Hookworm | 34 | 6 | 4.04-7.96 | 12.55 | 3.78-21.33 | 83.83 | 53.42-121.54 |
| *A. lumbricoides* | 12 | 2.12 | 0.93-3.30 | 313.77 | 0-632.24 | 2595.6 | 452.83-14878.05 |
| *T. trichiura* | 9 | 1.59 | 0.56-2.62 | 11.16 | 0-30.30 | 92.87 | 22.23-387.90 |
| *Taenia* | 9 | 1.59 | 0.56-2.62 | 71.45 | 0-149.33 | 1009.19 | 73.35-13884.48 |
| *E. vermicularis* | 4 | 0.71 | 0-1.40 | 0.4 | 0-0.96 | 39.93 | 9.60-166.09 |
| *Trichostrongyloides* | 1 | 0.18 | 0-0.52 | 0.044 | 0-0.133 | 25.51 | NA |
| 1 from 567 samples | | |  |  |  |  |  |
| \*Arithmetic eggs per gram of faeces  \*\*Geometric mean EPG  #95% confidence intervals | | |  |  |  |  |  |

**Table 2.** Summary of prevalence levels for helminth species determined by qPCR combined for all three regions surveyed.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Number positive 1 | Prevalence % | 95%CI# |
| Positive any species | 107 | 18.87 | 15.64-22.10 |
| *O. viverrini* | 98 | 17.28 | 14.16-20.41 |
| *N. americanus* | 13 | 2.29 | 1.06-3.53 |
| *A. lumbricoides* | 12 | 2.12 | 0.93-3.30 |
| *Ancylostoma spp.* | 9 | 1.59 | 0.56-2.62 |
| *S. stercoralis* | 7 | 1.23 | 0.03-2.15 |
| *T. saginata* | 6 | 1.06 | 0.21-1.90 |
| *T. trichiura* | 2 | 0.35 | 0-0.84 |
| *T. solium* | 1 | 0.18 | 0-0.52 |
| 1 from 567 samples  #95% confidence intervals | | |  |

**Table 3.** Kappa analysis for agreement of the qPCR vs Kato Katz methods.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Number positive Kato Katz 1 | Number positive qPCR 1 | Kappa |
| Hookworm2 | 33 | 21 | 0.53 |
| *A. lumbricoides* | 12 | 12 | 0.91 |
| *O. viverrini* | 50 | 98 | 0.54 |
| Taenia3 | 7 | 7 | 0.86 |
| *T. trichiura* | 9 | 2 | 0.18 |
| 1 from 567 samples | |  |  |
| 2 qPCR results from *Ancylostoma* spp. and *N. americanus* probes | | |  |
| 3 qPCR results from *T. solium* and *T. saginata* probes | |  |  |

**Table 4.** Inclusive and exclusive lists of helminth species using combined Kato Katz and qPCR data for the l three border regions collectively.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Number positive 1 | Prevalence % | 95%CI |
| Positive by Kato Katz only | 104 | 18.34 | 15.15-21.54 |
| Positive by qPCR only | 107 | 18.87 | 15.64-22.10 |
| Positive by Kato Katz or qPCR | 163 | 28.75 | 25.01-32.48 |
| Negative all species | 404 | 71.25 | 67.52-74.99 |
| *A. lumbricoides* | 13 | 2.29 | 1.06-3.53 |
| *T. trichiura* | 10 | 1.76 | 0.68-2.85 |
| *Trichostrongyloides* | 1 | 0.18 | 0-0.52 |
| *E. vermicularis* | 4 | 0.71 | 0-1.40 |
| *O. viverrini* | 104 | 18.34 | 15.15-21.54 |
| Hookworm | 39 | 6.88 | 4.79-8.97 |
| *Taenia* | 8 | 1.41 | 0.44-2.38 |
| *S. stercoralis* | 7 | 1.23 | 0.03-2.15 |
| *A. lumbricoides* only | 2 | 0.35 | 0-0.84 |
| *T. trichiura* only | 4 | 0.71 | 0-1.40 |
| *Trichostrongyloides* only | 1 | 0.18 | 0-0.52 |
| *O. viverrini* only | 95 | 16.75 | 13.67-19.84 |
| Hookworm only | 29 | 5.11 | 3.30-6.93 |
| *Taenia* only | 3 | 0.53 | 0-1.13 |
| *S. stercoralis* only | 5 | 0.88 | 0.11-.65 |
| *A. lumbricoides* + T. trichiura | 1 | 0.18 | 0-0.52 |
| *A. lumbricoides* + *E. vermicularis* | 2 | 0.35 | 0-0.84 |
| *A. lumbricoides* + Hookworm | 6 | 1.06 | 0.21-1.90 |
| *A. lumbricoides* + *S. stercoralis* | 1 | 0.18 | 0-0.52 |
| *T. trichiura* + *O. viverrini* | 2 | 0.35 | 0-0.84 |
| *T. trichiura* + Hookworm | 2 | 0.35 | 0-0.84 |
| *T. trichiura* + *S. stercoralis* | 1 | 0.18 | 0-0.52 |
| *E. vermicularis* + *O. viverrini* | 1 | 0.18 | 0-0.52 |
| *E. vermicularis* + *S. stercoralis* | 1 | 0.18 | 0-0.52 |
| *O. viverrini* + Hookworm | 1 | 0.18 | 0-0.52 |
| *O. viverrini* + *Taenia* | 4 | 0.71 | 0-1.4 |
| Hookworm + *O. viverrini* + *A. lumbricoides* | 1 | 0.18 | 0-0.52 |
| 1 from 567 samples | | |  |

**Table 5.** Prevalence of helminthiases for each of the three regions surveyed using the combined Kato Katz and qPCR data.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Tak | | | Ubon Ratchathani | | | Sisaket | | |
|  | Number positive 1 | Prevalence % | 95%CI | Number positive 2 | Prevalence % | 95%CI | Number positive 2 | Prevalence % | 95%CI |
| Positive any species | 47 | 28.14 | 21.25-35.03 | 62 | 31 | 24.53-37.47 | 53 | 26.5 | 20.33-32.67 |
| Negative all species | 120 | 71.86 | 64.97-78.75 | 138 | 69 | 62.52-75.47 | 147 | 73.5 | 67.33-79.67 |
| *A. lumbricoides* | 13 | **7.78** | 3.68-11.89 | 0 | NA | NA | 0 | NA | NA |
| *T. trichiura* | 8 | **4.79** | 1.52-8.06 | 2 | 1 | 0-2.39 | 0 | NA | NA |
| *Trichostrongyloides* | 1 | 0.6 | 0-1.79 | 0 | NA | NA | 0 | NA | NA |
| *E. vermicularis* | 2 | 1.2 | 0-2.86 | 2 | 1 | 0-2.39 | 0 | NA | NA |
| *O. viverrini* | 4 | 2.4 | 0-4.74 | 56 | **28** | 21.72-34.28 | 44 | **22** | 16.21-27.79 |
| Hookworm | 30 | **17.96** | 12.08-23.85 | 1 | 0.5 | 0-1.49 | 8 | **4** | 1.26-6.74 |
| *Taenia* spp. | 1 | 0.6 | 0-1.78 | 7 | **3.5** | 0.93-6.07 | 0 | NA | NA |
| *S. stercoralis* | 3 | 1.8 | 0-3.83 | 2 | 1 | 0-2.39 | 2 | 1 | 0-2.39 |
| Prominent species per region are in bold based on prevalence %. | | | | | |  |  |  |  |
| 1 from 167 samples | |  |  |  |  |  |  |  |  |
| 2 from 200 samples | |  |  |  |  |  |  |  |  |
| 3 from 200 samples | |  |  |  |  |  |  |  |  |

**Supplementary Table 1.** Primers used for the three multiplex quantitative PCR assays.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Assay | Target organism | Primer name | Oligonucleotide sequence (5′--3′) | Size | Target gene | Accession No. | Ref. |
| 1 | *A. lumbricoides* | AL\_FW | 5′-GTAATAGCAGTCGGCGGTTTCTT-3′ | 89 bp | ITS-1 | HQ721820.1 | [[14](#_ENREF_14)] |
| AL\_RW | 5′-GCCCAACATGCCACCTATTC-3′ |
| AL\_probe | FAM 5′-TTGGCGGACAATTGCATGCGAT-3′- BHQ1 Channel 1 |
| *S. stercoralis* | SS\_FW | 5′-GAATTCCAAGTAAACGTAAGTCATTAGC-3′ | 101 bp | 18S | AF279916.2 | [[15](#_ENREF_15)] |
| SS\_RW | 5′-TGCCTCTGGATATTGCTCAGTTC-3′ |
| SS\_probe | Cy5 5′-ACACACCGGCCGTCGCTGC-3′- BHQ2 Channel 4 |
| *T. trichiura* | TT\_FW | 5′-TCCGAACGGCGGATCA-3′ | 56 bp | ITS-1 | FM991956.1 | [[15](#_ENREF_15)] |
| TT\_RW | 5′-CTCGAGTGTCACGTCGTCCTT-3′ |
| TT\_probe | HEX 5’-TTGGCTCGTAGGTCGTT-3’- BHQ1 Channel 2 |
| 2 | *Ancylostoma spp* | AN\_FW | 5′-GAATGACAGCAAACTCGTTGTTG-3′ | 71 bp | ITS-2 | EU344797.1 | [[15](#_ENREF_15)] |
| AN\_RW | 5′-ATACTAGCCACTGCCGAAACGT-3′ |
| AN\_probe | HEX 5′-ATCGTTTACCGACTTTAG-3′- BHQ1 Channel 2 |
| *N. americanus* | NA\_FW | 5′-CTGTTTGTCGAACGGTACTTGC-3′ | 101 bp | ITS-2 | AJ001599.1 | [[15](#_ENREF_15)] |
| NA\_RW | 5′-ATAACAGCGTGCACATGTTGC-3′ |
| NA\_probe | Tex615 5′-CTGTACTACGCATTGTATAC-3′- BHQ2 Channel 3 |
| *O. viverrini* | OV\_FW | 5′-CATAAGGTTGACTAGGAAACCGGG-3′ | 330 | Repetitive DNA fragment | S80278.1 | [[16](#_ENREF_16)] |
| OV\_RW | 5′-TGTTCTCAGGCAAGTGAGTGTGCT-3′ |
| OV\_probe | FAM 5’-AATCTGCTGGCGACTGAAACATGAGG-3′- BHQ1 Channel 1 |
| 3 | *T. solium* | Tsol\_FW | 5′-ATGGATCAATCTGGGTGGAGTT-3′ | 86 bp | ITS1 | EU747662 | [[17](#_ENREF_17)] |
| Tsol\_RW | 5′-ATCGCAGGGTAAGAAAAGAAGGT-3′ |
| Tsol\_probe | HEX 5′-TGGTACTGCTGTGGCGGCGG-3′-BHQ1 Channel 2 |
| *T. saginata* | Tsag\_FW | 5′-GCGTCGTCTTTGCGTTACAC-3′ | 79 bp | ITS1 | AY392045 | [[17](#_ENREF_17)] |
| Tsag\_RW | 5′-TGACACAACCGCGCTCTG-3′ |
| Tsag\_probe | Cy5.5 5′-CCACAGCACCAGCGACAGCAGCAA-3′-BHQ2 Channel 5 |
| *S. japonicum/ S. mekongi* | Sme\_FW | 5′-GTGGAGTTGAACTGCAAGC-3′ | 264 | 28S LSU | AY157253.1.1 | [[17](#_ENREF_17)] |
| Sme\_RW | 5′-GCTCAACATTAATAGTCAAACCTG-3′ |
| Sme\_probe | Tex615 5′-ACTGACAAGCAGACCCTCACACC-3′- BHQ2 Channel 3 |

**Supplementary Table 2.** Complete collection parameters and diagnostic results for the Kato Katz and qPCR for the three field sites.

**Supplementary Table 3.** Range of CT scores for qPCR striated based on egg burdens determined by the Kato Katz counts.

|  |  |  |  |
| --- | --- | --- | --- |
| Species | Egg intensity | Ct Range | Number of samples |
| *O. viverrini* | low (1-5) | 22.63-32.09 | 32 |
| high (>5) | 21.93-25.19 | 6 |
| Hookworm (*Ancylostoma* spp. or *N. americanus*) | low (1-5) | 29.06-31.33 | 9 |
| high (>5) | 10.28-36.94 | 7 |
| *A. lumbricoides* | low <50 | 27.9-35.8 | 3 |
| high >50 | 25.75-30.6 | 7 |
| *Taenia* | low <100 | 25.25-28.80 | 3 |
| high >100 | 23.84-26.03 | 3 |

**Supplementary Table 4.** Inclusive and exclusive lists of helminth species using the combined Kato Katz and qPCR data; each of the three regions is presented separately.

**REFERENCES**

1. Silver ZA, Kaliappan SP, Samuel P, et al. Geographical distribution of soil transmitted helminths and the effects of community type in South Asia and South East Asia - A systematic review. PLoS Negl Trop Dis **2018**; 12:e0006153.

2. Gordon CA, Kurscheid J, Jones MK, Gray DJ, McManus DP. Soil-Transmitted Helminths in Tropical Australia and Asia. Trop Med Infect Dis **2017**; 2.

3. Utzinger J, Bergquist R, Olveda R, Zhou XN. Important helminth infections in Southeast Asia diversity, potential for control and prospects for elimination. Adv Parasitol **2010**; 72:1-30.

4. King CH. Health metrics for helminth infections. Acta Trop **2015**; 141:150-60.

5. Bartsch SM, Hotez PJ, Asti L, et al. The Global Economic and Health Burden of Human Hookworm Infection. PLoS Negl Trop Dis **2016**; 10:e0004922.

6. Lamberton PHL, Kabatereine NB, Oguttu DW, Fenwick A, Webster JP. Sensitivity and Specificity of Multiple Kato-Katz Thick Smears and a Circulating Cathodic Antigen Test for *Schistosoma mansoni* Diagnosis Pre- and Post-repeated-Praziquantel Treatment. PLoS Negl Trop Dis **2014**; 8:e3139.

7. Wongsaroj T, Nithikathkul C, Rojkitikul W, Nakai W, Royal L, Rammasut P. National survey of helminthiasis in Thailand. Asian Biomedicine **2014**; 8:779-83.

8. Punsawad C, Phasuk N, Bunratsami S, et al. Prevalence of intestinal parasitic infections and associated risk factors for hookworm infections among primary schoolchildren in rural areas of Nakhon Si Thammarat, southern Thailand. BMC Public Health **2018**; 18:1118.

9. Suntaravitun P, Dokmaikaw A. Prevalence of Intestinal Parasites and Associated Risk Factors for Infection among Rural Communities of Chachoengsao Province, Thailand. Korean J Parasitol **2018**; 56:33-9.

10. Assavapongpaiboon B, Bunkasem U, Sanprasert V, Nuchprayoon S. A Cross-Sectional Study on Intestinal Parasitic Infections in Children in Suburban Public Primary Schools, Saraburi, the Central Region of Thailand. Am J Trop Med Hyg **2018**; 98:763-7.

11. Jiraanankul V, Aphijirawat W, Mungthin M, et al. Incidence and risk factors of hookworm infection in a rural community of central Thailand. Am J Trop Med Hyg **2011**; 84:594-8.

12. MoPH Thailand. <http://ddc.moph.go.th/disease.php>. Accessed 12th Feb 2020.

13. Sagnuankiat S, Wanichsuwan M, Bhunnachet E, et al. Health Status of Immigrant Children and Environmental Survey of Child Daycare Centers in Samut Sakhon Province, Thailand. J Immigr Minor Health **2016**; 18:21-7.

14. Basuni M, Muhi J, Othman N, et al. A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. Am J Trop Med Hyg **2011**; 84:338-43.

15. Mejia R, Vicuna Y, Broncano N, et al. A novel, multi-parallel, real-time polymerase chain reaction approach for eight gastrointestinal parasites provides improved diagnostic capabilities to resource-limited at-risk populations. Am J Trop Med Hyg **2013**; 88:1041-7.

16. Suksumek N, Leelawat K, Leelawat S, Russell B, Lek-Uthai U. TaqMan real-time PCR assay for specific detection of *Opisthorchis viverrini* DNA in Thai patients with hepatocellular carcinoma and cholangiocarcinoma. Exp Parasitol **2008**; 119:217-24.

17. Praet N, Verweij JJ, Mwape KE, et al. Bayesian modelling to estimate the test characteristics of coprology, coproantigen ELISA and a novel real-time PCR for the diagnosis of taeniasis. Trop Med Int Health **2013**; 18:608-14.

18. Khieu V, Furst T, Miyamoto K, et al. Is *Opisthorchis viverrini* Emerging in Cambodia? Adv Parasitol **2019**; 103:31-73.

19. Traub RJ, Inpankaew T, Sutthikornchai C, Sukthana Y, Thompson RC. PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* in temple communities in Bangkok. Vet Parasitol **2008**; 155:67-73.

20. Gordon CA, McManus DP, Jones MK, Gray DJ, Gobert GN. The Increase of Exotic Zoonotic Helminth Infections: The Impact of Urbanization, Climate Change and Globalization. Adv Parasitol **2016**; 91:311-97.

21. Kaisar MMM, Brienen EAT, Djuardi Y, et al. Improved diagnosis of *Trichuris trichiura* by using a bead-beating procedure on ethanol preserved stool samples prior to DNA isolation and the performance of multiplex real-time PCR for intestinal parasites. Parasitology **2017**; 144:965-74.

22. Pontes LA, Oliveira MC, Katz N, Dias-Neto E, Rabello A. Comparison of a polymerase chain reaction and the Kato-Katz technique for diagnosing infection with *Schistosoma mansoni*. Am J Trop Med Hyg **2003**; 68:652-6.

23. Knopp S, Salim N, Schindler T, et al. Diagnostic accuracy of Kato-Katz, FLOTAC, Baermann, and PCR methods for the detection of light-intensity hookworm and *Strongyloides stercoralis* infections in Tanzania. Am J Trop Med Hyg **2014**; 90:535-45.

24. Llewellyn S, Inpankaew T, Nery SV, et al. Application of a Multiplex Quantitative PCR to Assess Prevalence and Intensity Of Intestinal Parasite Infections in a Controlled Clinical Trial. PLoS Negl Trop Dis **2016**; 10:e0004380.

25. Gordon CA, McManus DP, Acosta LP, et al. Multiplex real-time PCR monitoring of intestinal helminths in humans reveals widespread polyparasitism in Northern Samar, the Philippines. Int J Parasitol **2015**; 45:477-83.

26. Steinmann P, Zhou XN, Du ZW, et al. Occurrence of *Strongyloides stercoralis* in Yunnan Province, China, and comparison of diagnostic methods. PLoS Negl Trop Dis **2007**; 1:e75.

27. Gordon CA, Acosta LP, Gray DJ, et al. High prevalence of *Schistosoma japonicum* infection in Carabao from Samar Province, the Philippines: implications for transmission and control. PLoS Negl Trop Dis **2012**; 6:e1778.