Original article

Trichuris vulpis and *T. trichiura* infections among schoolchildren of a rural community in northwestern Thailand: the possible role of dogs in disease transmission

Pannatat Areekul, Chaturong Putaporntip, Urassaya Pattanawong, Prasert Sitthicharoenchai, Somchai Jongwutiwes
Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Background: Trichuriasis is an important soil-transmitted helminth infection caused by *Trichuris trichiura*. About one-tenth of the world population may be infected. Incidentally, *T. vulpis* or dog whipworm has been reported to infect humans based on the egg size. However, an overlapping egg dimension occurs between *T. trichiura* and *T. vulpis* leading to the potential for misdiagnosis.
Objective: Develop a PCR method to differentiate *T. trichiura* and *T. vulpis* eggs in stool samples and to investigate the prevalence of both whipworms in humans and dogs in a rural community in Thailand.
Materials and methods: We determined and compared the small subunit ribosomal RNA sequences of both species of whipworms for developing species-specific PCR diagnosis. After validation of the method, we conducted a cross-sectional survey at Ta Song Yang District in Tak Province, northwestern Thailand in 2008. Stool samples were randomly recruited from 80 schoolchildren (36 males, 44 females) and 79 dogs in this community.
Results: Fifty-six individuals harbored *Trichuris* eggs in their stools. The PCR-based diagnosis revealed that 50 cases were infected with *T. trichiura* and six (10.7%) were co-infected with both *T. trichiura* and *T. vulpis*. Although the dimension of *Trichuris* eggs provided some diagnostic clues for species differentiation, a remarkable variation in the length of these whipworm eggs was observed among samples that could lead to misdiagnosis.
Conclusion: Both *T. trichiura* and *T. vulpis* eggs were detected in stool samples of dogs that roamed around this community, highlighting the potential reservoir role of dogs in the transmission of both human and dog whipworms in this population.

Keywords: *Trichuris vulpis*, *Trichuris trichiura*, PCR, Thailand

Trichuriasis is one of the most important soil-transmitted helminth infections with a wide geographic distribution, especially in tropical and subtropical regions where public sanitation and living conditions are substandard. It has been estimated that 604-795 millions of the world population are infected with *Trichuris trichiura* and more than three billion people are at risk of infection [1]. The highest prevalence of trichuriasis has been confined to Central Africa, Southern India, and Southeast Asia. Infections in children in some endemic areas may surpass 90% [2-6]. Although the majority of infected individuals remain asymptomatic, a significant number of trichuriasis patients, especially children with longstanding massive infections, have dysenteric syndrome presenting with chronic mucous diarrhea, rectal prolapse, anemia from chronic blood loss and iron deficiency, clubbing of fingers, protein-energy malnutrition, and growth retardation [7-10]. More importantly, deficits in cognitive function and stunting have been observed in childhood trichuriasis, hindering educational achievement and psychomotor

Correspondence to: Chaturong Putaporntip, Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: p.chaturong@gmail.com

development [11-13]. It has also been assessed that *trichuriasis* is responsible for 1.6 – 6.4 disability-adjusted life years [14-16].

Infection of *T. trichiura* is acquired through ingestion of infective embryonated eggs from contaminated soil, vegetables, and food products. Upon passage to small intestine, the first larval stage is released from egg. It then undergoes multiple molting processes before maturation. The adult stage usually develops in 30-90 days after infection and mainly inhabits the cecum where the anterior part of the worm burrows into the mucosal surface. It has been estimated that adult life span of *T. trichiura* is one-two years and the egg production per female worm per day is around 2,000-30,000 [17].

Definite diagnosis of trichuriasis relies on identification of characteristic *T. trichiura* eggs measures 50-54 μm by 22-23 μm in stool samples [18]. Incidentally, the larger egg size, measured 70-80 μm by 30 - 42 μm with prominent but relatively small bipolar mucoid plugs, belonging to *T. vulpis* or dog whipworm. It has been detected in human stool samples, suggesting zoonotic transmission [19-20]. However, variation in egg size occurs in *T. trichiura*. They can be classified into two groups: the smaller or regular size eggs being <64 μm in length and the larger ones being >64 μm [21]. Although *T. vulpis* eggs are usually larger than *T. trichiura* eggs, morphometric study has revealed an overlapping length between eggs of these nematodes. This could mislead a diagnosis of *Trichuris* species based on egg dimension. To date, it is unclear how often *T. vulpis* infects human, especially in disease endemic areas due to a lack of a feasible, sensitive, and specific diagnostic tools. Meanwhile, applications of PCR-based methods have been successfully deployed to differentiate morphologically indistinguishable diagnostic stages of parasites such as eggs of *Taenia solium* and *T. saginata,* eggs of *Opisthorchis viverrini, Clonorchis sinensis* and *Haplorchis taichui*, and blood stages of *Plasmodium knowlesi* and *P. malariae* [22-25]. Undoubtedly, differentiation of *T. trichiura* eggs and *T. vulpis* eggs in stool samples has epidemiological and clinical relevance in terms of prevention and control. Although molecular based methods have been developed to identify helminths in the genus *Trichuris*, no attempts have been done to differentiate *T. trichiura* eggs from *T. vulpis* eggs in clinical samples [26-28].

In this study, we developed a PCR method targeting the small subunit ribosomal RNA (SSU rRNA) gene for the specific detection of *T. trichiura* and *T. vulpis* in stool samples. The method was so highly sensitive that a single egg of either species could be detected. By applying this PCR-based method to a cross-sectional survey in a rural community of Thailand, we found co-existence of *T. trichiura* and *T. vulpis* in human stool samples. Dogs in the same endemic area harbored eggs of both species, suggesting that dogs could serve as important reservoirs for both parasites.

Material and methods
Cloning and sequencing of the SSU rRNA and ITS-1 regions

Genomic DNA of *T. trichiura* was obtained from an adult worm recovered from an infected Thai patient. After washing in sterile water, it was homogenized prior to DNA extraction using QIAamp DNA tissue kit following the manufacturer's instruction (Qiagen, Hilden, Germany). Likewise, *T. vulpis* was isolated from an infected dog in Bangkok and genomic DNA was prepared using the same protocol. To obtain the SSU rRNA gene of *T. vulpis*, we designed the PCR primers based on interspecific conserved sequences of the available SSU rRNA and 5.8S rRNA genes from various species of *Trichuris*. Sequences of the SSU rRNA gene of *T. trichiura* (GenBank accession number DQ118536), *T. suis* (GenBank accession numbers AY851265 and AY856093) and *T. muris* (GenBank accession number AF036637) were aligned by using the Clustal X program with the default option. Likewise, alignment was performed for the 5.8 rRNA genes of *T. vulpis*, *T. suis* and *T. muris* (GenBank accession numbers AM234616, AM229670 and AM299407, respectively). Amplification of the DNA fragment spanning the SSU rRNA, ITS-1 and 5.8S rRNA regions was done by semi-nested PCR. The forward primer for primary PCR was TT18SF0 (5'-ACGGTGAAGCCGCGAATGGCTC-3') and the reverse outer primer TT5.8SR0 (5'-GTTTGATCGAC GCTGCAATGTG-3'). Secondary PCR contained the same forward primer TT18SF0 and the reverse inner primer TT5.8SR1 (5'-GCAATGTGCGTTCAAAGT ATTC-3'). DNA amplification was carried out in a total volume of 30 μL of the reaction mixture containing template DNA, 2.5 mM $MgCl_2$, 300 mM each deoxynucleoside triphosphate, 3 μL of 10x LATaq PCR buffer, 0.3 μM of each primer and 1.25 units of LATaq

DNA polymerase (Takara, Seta, Japan). Thirty-five cycles (96°C for 20 seconds, 55°C for 30 seconds, and 62°C for three minutes) were performed for primary and secondary PCR. The PCR products were separated in 1% agarose gel; stained with ethidium bromide; and imaged under a UV transilluminator. The semi-nested PCR products were excised from agarose gel, purified by using a QIAquick PCR purification kit (Qiagen), and ligated into pGEM-T-Easy Vector (Promega, Madison WI, USA). After incubation at 4°C overnight, the reaction mixture was precipitated, dissolved in 10 µL of double-distilled water, and transformed into *Escherichia coli* strain JM109. DNA sequences were determined bi-directionally from nested PCR-purified templates and from 10 recombinant subclones of each sample. Sequencing analysis was performed on an ABI3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, USA). Singleton substitution was re-determined using PCR products from two independent amplifications from the same DNA template. Sequences have been deposited in the GenBank™ Database under the accession numbers GQ352547 - GQ352558.

Phylogenetic relationship

Phylogenetic tree was constructed using the Neighbor-Joining algorithms based on the maximum composite likelihood method [29]. Reliability of clustering patterns in a phylogenetic tree was performed by bootstrapping using 1000 pseudoreplicates as implemented in MEGA 4.0 program [29]. Sequences and their GenBank accession numbers included in the analysis were the SSU rRNA genes of *T. trichiura* (accession numbers DQ118536 and GQ352547), *T. vulpis* (GQ352556), *T. muris* (AF036637), *T. suis* (AY851265), *Eucoleus dispar* (EU004821) and *Trichinella spiralis* (U60231).

Sequence conservation and divergence

To define interspecific sequence conservation and divergence at the SSU rRNA and ITS-1 loci between *T. trichiura* and *T. vulpis*, average number of nucleotide substitution per site (Dxy) between species was computed as implemented in the DnaSP (version 5) program [30]. A sliding window of 100 nucleotides with step size of 25 nucleotides was plotted along the sequence alignment. Sites with alignment gaps were not counted in the window length.

Development of *T. trichiura* and *T. vulpis* PCR assays

Outer primers for primary PCR were derived from interspecies conserved regions of the SSU rRNA gene of the genus *Trichuris*: HTT18SF0 (5'-TCTTGATTCAGTGGGTAGTGG -3') and HT 18SR0 (5'-CTTACTGGGAATTCCTCGTTC-3'). The forward inner primers specific to *T. trichiura* and to *T. vulpis* for secondary PCR were HTT 18SF (5'-AGCGCTCCGCGGAGCACCT-3') and HTV18SF(5'-CGCCCCTTGGAGCAGCA-3'), respectively, whereas the reverse inner primer was derived from a conserved sequence, HT18SR (5'-CTGTCCCAGTCACGAGAAC-3') (**Fig. 1**).

Amplification was carried out in a total volume of 20 µL of the reaction mixture containing template DNA, 2.5 mM MgCl$_2$, 300 mM each deoxynucleoside triphosphate, 2 µL of 10X ExTaq PCR buffer, 0.3 µM of each primer, 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan) and 3 µL of DNA template. Semi-nested PCR assays for *T. trichiura* and *T. vulpis* detection were carried out in separate reactions using 1 µL of products from primary PCR amplification as DNA template. The thermal cycling profiles for both primary and secondary PCR contained a pre-amplification denaturation at 94°C for one minute, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 s and extension at 72°C for one minute and a final elongation at 72°C for five minutes.

Sensitivity and specificity of the PCR assays

Stool samples containing large eggs (>64 µm in length) of *T. vulpis* from three dogs and small eggs (<64 µm) of *T. trichiura* from three infected humans were recruited for analysis. Classification of egg size was after Yoshikawa et al. [21]. Eggs were concentrated by formalin-ether concentration technique (FECT) as described by Garcia et al. except that formalin solution was substituted with 0.85% NaCl solution [31]. After discarding supernatant in the final wash, the pellet was transferred into a 1.5 mL microcentrifuge tube, and washed thrice by adding 1 mL of sterile water and centrifuged at 2,500 rpm for three minutes. An aliquot of 100 µL containing approximately 20 *T. vulpis* eggs were transferred onto a clean glass slide (25.4 mm x 76.2 mm) without applying a coverslip. For each stool sample, a series of 1, 5, and 10 eggs were subsequently transferred onto other clean glass slides by micropipette
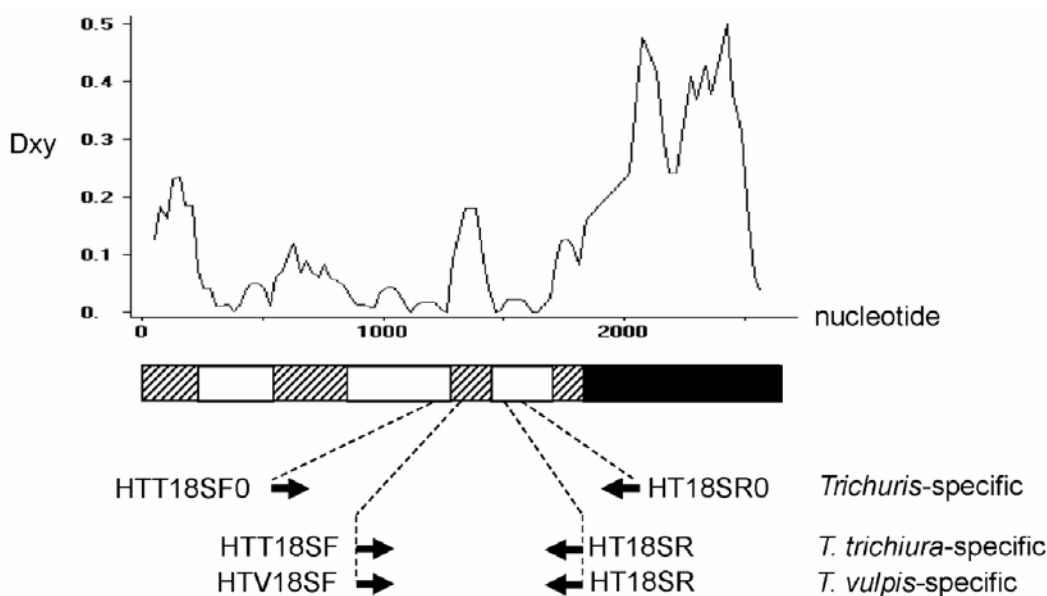
Fig. 1 Plots of average number of nucleotide substitution per site between *T. trichiura* and *T. vulpis* (Dxy) in a sliding window of 100 nucleotides with step size of 25 nucleotides along the SSU rRNA and ITS-1 sequences. Nucleotides represent centered positions. Corresponding scheme of the regions is shown underneath the plot. Upper diagonal and unfilled blocks are interspecific variable and conserved regions in the SSU rRNA gene, respectively. Filled block denotes the ITS-1 region. Locations of primers for primary PCR (*Trichuris*-specific) and secondary PCR (*T. trichiura*- and *T. vulpis*-specific) are shown underneath the scheme.

manipulation under light microscope (Olympus BX51, Tokyo, Japan). Prior to DNA extraction, *Trichuris* eggs were mechanically disrupted by directly pressing 10 μL pipette tip on individual eggs until all eggs were seen ruptured as monitored by visual inspection under 40x or 100x magnifications of light microscope. Solution containing known number of ruptured eggs was subject to DNA extraction using QIAamp DNA tissue kit. DNA of each sample was re-suspended in 50 μl of TE buffer (10 mM Tris.HCl, 1 mM EDTA). Five μL of DNA extracted from each sample were used as templates for the PCR assays developed in this study. To validate the consistency of the test, nested PCR assays were repeated 6 times for each sample, comprising a series of DNA templates extracted from 1, 5, and 10 eggs.

Genomic DNA previously extracted and kept frozen at -40°C from *Ascaris lumbricoides*, *Necator americanus*, *Strongyloides stercoralis*, *Capillaria philippinensis*, *Trichinella spiralis*, *Opisthorchis viverrini*, *Haplorchis taichui*, *Taenia saginata*, *Giardia lamblia*, *Pentatrichomonas hominis*, *Entamoeba histolytica, Blastocystis hominis* and human DNA were included for comparative analysis.

PCR and test for inhibitor

Detection of *T. trichiura* and *T. vulpis* was done by the PCR methods developed in this study using 5 μL of DNA template in a total volume of 20 μL. Test for PCR inhibitors was done by adding 3 μL of genomic DNA of *T. trichiura* and *T. vulpis* to DNA template of each tested sample and subject to the same amplification conditions.

Study area

A community in Ta Song Yang District, Tak Province, northwestern Thailand, bordering Myanmar was chosen for study. This is because our previous cross-sectional survey in April 2008 revealed that more than 90% of schoolchildren in this community (n=720) harbored one or more species of parasites in their stools leading to a high prevalence of malnutrition, diarrhea, and anemia. Although all infected individuals were treated with antiparasitic drugs, repeated survey six months later has shown a minimal reduction in the prevalence rate, i.e., 85% remained infected. This community is located 426 km northwest of Bangkok and occupies 355 km^2 with a population density of approximately 24 persons/km^2. Most areas are

mountainous and filled with forests. The Mei River marks the western side of Thailand. It is also where tap water supply was obtained. After a few days of sedimentation in reservoirs, the water is distributed without further treatment. Most parents of these schoolchildren earned their livings by agriculture such as growing rice, vegetables and raising domesticated animals. During the rainy season, villagers travel into the jungle for long period to cut the bamboo shoots for sale. They often return with febrile malaria attack. About half of these households had latrines. This community comprised of approximately 8,618 people. Although most of the dogs in this community had owners, they were always allowed to roam and defecate in almost any places. These dogs were given a regular yearly vaccination against rabies but none were given any other care including antiparasitic treatment.

Sample sizes

Stool samples were randomly recruited from 80 schoolchildren (36 males, 44 females, 9.5±3.0 years (mean age±SD)) and 79 dogs in this community in October 2008. The ethical aspects of this study have been approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

Stool examination

Aliquots of fresh stool samples from both humans and dogs were subject to direct smear and FECT to examine for parasites. The remaining samples were kept on ice and transported to a laboratory at Chulalongkorn University for further parasitological and molecular analysis.

Morphometry of *Trichuris* eggs

Egg dimension was obtained by measuring at least 30 eggs from each sample under 400x magnification of light microscope (Olympus BX51, Tokyo, Japan). The length and width of eggs were determined from the tips of both mucoid plugs and the outer borders of eggshells, respectively.

DNA extraction

Prior to DNA purification, disruption of the egg shell to release the parasite cellular material was performed by either or both of the following methods.

Method 1: Direct pressing. The procedure was as described for DNA preparation from individual eggs except that at least 50 eggs from each sample were included. DNA was subsequently extracted by using QIAamp DNA Tissue kit (Qiagen).

Method 2: Bead beating. Sediment from saline-ether concentration technique was adjusted to 300 µl with sterile water. Glass beads (0.2 g, 425-600 um diameter, Sigma, St. Louise, USA) were added and the mixture was shaken vigorously by vortexing for one hour at maximum speed. After a brief spin at 2,000 rpm, the liquid phase was transferred to a new microcentrifuge tube and DNA was extracted by using QIAamp Stool Mini kit (Qiagen) following the manufacturer's protocol.

Results

The SSU rRNA and ITS-1 sequences of T. trichiura and T. vulpis

PCR products spanning the SSU rRNA and ITS-1 genes of *T. trichiura* and *T. vulpis* generated by primers TT18SF0 and TT58SR1 yielded products of approximately 2,580 and 2,367 bp, respectively. Direct sequencing of these PCR fragments has shown superimposed signals at some positions on an electropherogram, suggesting heterogeneity among each unit of the RNA cistron. Subcloning and sequencing of these PCR products revealed three and five distinct sequences for *T. trichiura* and *T. vulpis,* respectively. The SSU rRNA sequences of *T. trichiura* in this study and that in the GenBank database (DQ118536) contained 24 nucleotide substitutions and nine insertions/deletions while nine nucleotide substitutions without insertions/deletions were observed in the sequences of *T. vulpis*. However, the nucleotide diversity and its standard error at this locus of *T. vulpis* were slightly greater than that of *T. trichiura*, i.e. 0.0033±0.0009 and 0.0029±0.0006, respectively. The ITS-1 region of *T. trichiura* contained the AGC repeats with variation in repeat units among sequences. The average number of nucleotide substitution per site between *T. trichiura* and *T. vulpis* (Dxy) was most pronounced in the ITS-1 region while three domains in the SSU rRNA gene were conserved (see **Fig. 1**). The neighbor-joining tree based on the SSU rRNA sequences has shown that *T. trichiura* was closely related to *T. suis* while *T. vulpis* was remarkably related to *T. muris* than *T. trichiura* (**Fig. 2**).
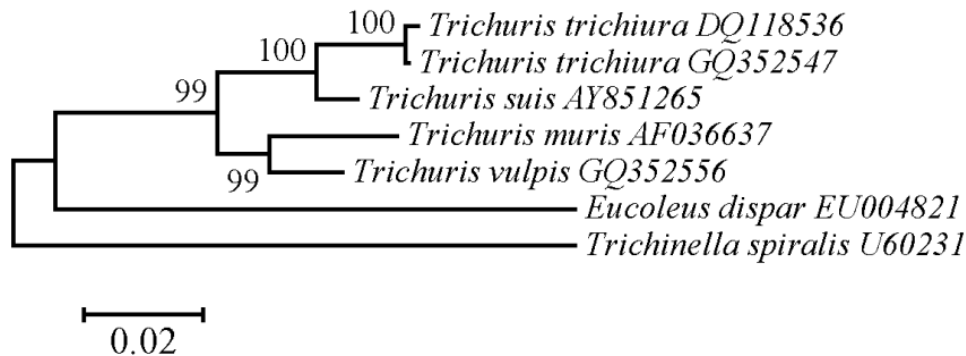
Fig. 2 Neighbor-joining tree inferred from the SSU rRNA region of *Trichuris trichiura*, *T. suis*, *T. muris*, *T. vulpis*, *Eucoleus dispar* and *Trichinella spiralis*. GenBank accession numbers or clone names are indicated next to the scientific names of nematodes. The optimal tree with the sum of branch length = 0.29897918 is shown. Bootstrap values from 1000 pseudoreplicates are shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site [29].

Development of T. trichiura- and T. vulpis-specific PCR assays.

Locations of the diagnostic PCR primers targeting the SSU rRNA genes of *T. trichiura* and *T. vulpis* are depicted in **Fig. 1**. The PCR assays could amplify DNA extracted from stool samples containing eggs of *T. trichiura* or *T. vulpis,* giving specific products of 212 bp or 207 bp, respectively (**Fig. 3**).

The sensitivity of the PCR assays developed in this study was determined using DNA template extracted from a series of 1, 5 and 10 *Trichuris* eggs in stool samples from three infected dogs and three human cases by direct disruption of individual eggs prior to DNA purification. The PCR assays could amplify target DNA of respective species without cross-species amplification. In order to observe the consistency of the DNA extraction and PCR amplification, the procedures were repeated six times for each sample. In total of 36 assays, both *T. trichiura*- and *T. vulpis*-specific nested PCR tests could detect as few as single eggs of the respective species in 22 amplifications. The negative results could be due to the presence of PCR inhibitor rather than the loss of DNA material during template preparation because addition of positive control DNA template yielded only one positive result in the previously 14 negative tests. However, the PCR methods could reproducibly amplify as few as five eggs in the samples
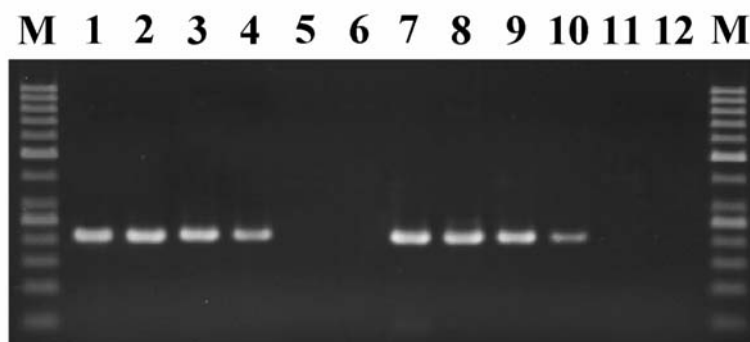

Fig. 3 Sensitivity and specificity of nested PCR for diagnosis of *Trichuris trichiura* and *T. vulpis* targeting the SSU rRNA gene. Lanes M, 1-6 represent amplified products using *T. trichiura*-specific primers (HTT18SF and HT18SR) from DNA template extracted from 20, 10, 5 and 1 *T. trichiura* egg, respectively, negative stool sample and 20 *T. vulpis* eggs. Lanes 7-12 represent amplified products using *T. trichiura*-specific primers (HTT18SF and HT18SR) from DNA template extracted from 20, 10, 5 and 1 *T. vulpis* egg, respectively, negative stool sample and 20 *T. trichiura* eggs.

and consistent results were obtained on DNA templates extracted from 10 eggs (**Fig. 3, Table 1**). No amplification was found when DNA from other helminthes, protozoan or human was used as template, indicating no cross-reactivity of the primers in this study.

Prevalence of parasites in humans in a cross sectional survey

Stool examination of 80 individuals at Ta Song Yang District by FECT revealed that 76 (95.0%) samples contained one or more species of parasites. It is noteworthy that soil-transmitted helminth infections were highly prevalent in this community in which trichuriasis contributed the highest prevalent (70.0%), followed by ascariasis (47.5%) and hookworm infection (17.5%). Other intestinal parasites found in this population included *Opisthorchis*-like (6.3%), *Enterobius vermicularis* (5.0%), *Taenia* spp. (1.3%), *Strongyloides stercoralis* (1.3%), *Giardia lamblia* (17.5%), *Entamoeba histolytica*/*dispar* (12.5%), *Blastocystis hominis* (82.5%), *Entamoeba coli* (46.3%), *Endolimax nana* (25%), *Entamoeba hartmanni* (21.3%), *Iodamoeba buetschlii* (5.0%), *Chilomastix mesnili* (3.8%), and *Sarcocystis* spp. (1.3%).

Validation of DNA extraction methods and prevalence of T. trichiura and T. vulpis in humans

Because the quality and quantity of DNA extraction procedures from parasite eggs or protozoan cysts could influence the outcome of PCR-based detection, DNA extraction methods by direct pressing and by bead beating were compared using 46 out of 80 human stool samples from Ta Song Yang District in Tak Province. Of randomly chosen 18 *Trichuris*-positive samples, all gave positive results for the PCR assays developed in this study when *Trichuris* eggs were disrupted by direct pressing with pipette tip on individual egg prior to DNA purification whereas the bead beating method gave positive results in eight *Trichuris*-positive samples. Out of 28 *Trichuris*-negative samples by microscopy, none gave positive results when DNA was extracted by the bead beating method. Therefore, the diagnostic efficacy of PCR assays when applied to DNA extraction by the bead beating method yielded a sensitivity of 44.4% (95.0% confidence interval [CI], 67.5%, 100%) and specificity of 100% (95%CI, 58.0%, 85.0%). The false negative results from DNA extraction by this method could mainly stem from the presence of PCR inhibitors during sample preparation because addition of control *Trichuris* DNA into 10 false PCR-negative samples

Table 1. Sensitivity of nested PCR detection of *Trichuris trichiura* and *T. vulpis*.

Sample	Host	Primers	Number of positive tests in six tests		
			1 egg*	5 eggs	10 eggs
D56	Dog	TT18SF-T18SR	0	0	0
		TV18SF-T18SR	1	6	6
D58	Dog	TT18SF-T18SR	0	0	0
		TV18SF-T18SR	6	6	6
D59	Dog	TT18SF-T18SR	0	0	0
		TV18SF-T18SR	5	6	6
TST137	Human	TT18SF-T18SR	4	6	6
		TV18SF-T18SR	0	0	0
TST236	Human	TT18SF-T18SR	0	6	6
		TV18SF-T18SR	0	0	0
TST377	Human	TT18SF-T18SR	6	6	6
		TV18SF-T18SR	0	0	0

*Number of eggs used in DNA extraction by directly pressing pipette tip on individual eggs to break the egg shell prior to purification procedures.

has resulted in one positive and nine negative tests. Therefore, we deployed DNA extraction method by the direct pressing method for determination of *Trichuris* species in the remaining 28 *Trichuris*-positive samples by microscopy albeit samples without detectable eggs could not be tested by this procedure. Results have shown that all 56 *Trichuris* positive samples by microscopy were positive by the PCR method using primers specific to *T. trichiura* (TT18SF and T18SR) while six of these samples (10.7%) also gave positive results with the primers specific to *T. vulpis* (TV18SF and T18SR), indicating mixed species infections.

Microscopic detection of parasites in dog stool samples

Examination of 79 dog stool samples from Ta Song Yang District revealed that 58 dogs (73.4%) were infected with one or more species of parasites, which included hookworm (34.2%), *Ascaris lumbricoides* (22.8%), *Physaloptera* spp. (12.7%), *Trichuris* spp. (21.5%), *Toxocara canis* (15.2%), *Spirometra* spp. (6.3%), *Isospora* spp. (6.3%), *Taenia* spp. (2.5%), *Giardia lamblia* (2.5%), *Entamoeba* spp. (2.5%), and *Paragonimus kellicotti* (1.3%).

PCR-based detection of dog stool samples

DNA extraction by the direct pressing method prior to purification was also applied to determine species of *Trichuris* eggs in dog stool samples. Of 17 *Trichuris*-positive samples examined by microscopy, 14 gave positive results by the PCR method in which four samples gave products for *T. vulpis*-specific primers (TT18SF and T18SR) while the remaining 10 samples yielded positive tests for *T. trichiura*-specific primers (TT18SF and T18SR). Three of the *Trichuris*-positive samples by microscopy that did not yield products in the PCR assays could likely stem from the presence of inhibitors in these samples because no PCR products were generated when positive control DNA of *T. trichiura* or *T. vulpis* was applied in the reactions. Taken together, the PCR method developed in this study could determine species of *Trichuris* in 82.4% of those positive by FECT (**Table 2**). No mixed infections of *T. trichiura* and *T. vulpis* were observed in dog stools in this study.

Morphometry of Trichuris eggs in dog stools

PCR-proven *T. vulpis* eggs (n=120) from four dogs had an overall average length (±SD) of 81.8 (±2.4) µm (range 75.0-87.5 µm), an average width (±SD) of 40.0 (±1.6) µm (range 38.8-48.8 µm) and an average dimension index (length by width ratio) of 2.05 (range 2.0-2.1). The distribution of the mean length and the mean width of each sample were also variable. The remaining *T. trichiura*-positive samples had an average egg length (±SD) of 56.5 (±4.0) µm (range 49.5-77.5 µm), an average width (±SD) of 27.8 (±1.6) µm (range: 24.5-35.5 µm) and an average dimension index of 2.0 (range: 1.9-2.2) that fall within the dimension of *T. trichiura*. Therefore, an overlapping egg length of 2.5 µm was noted between these species in dog stool samples (**Fig. 4A**).

Morphometry of Trichuris eggs in human stool.

Trichuris eggs (n=1680) from stool samples of 56 infected individuals has shown that the mean length and the mean width showed a striking variation among samples and could be classified into three categories based on the minimum and the maximum length within samples: (i) small eggs (minimum length <50 µm and maximum length <64 µm) found in 22 samples, (ii) eggs with overlapping size (minimum length <50 µm and maximum length >64 µm) contributed the majority of the samples (n=33), and (iii) large eggs (minimum length >50 µm and maximum length >64 µm)

Table 2. Egg length and PCR assays of *T. trichiura* and *T. vulpis* in 56 human stools.

Egg size*	Mean ± SD (µm)	Range (µm)	Number (%)	PCR assays (%)	
				T. trichiura	*T. vulpis*
Small	56.3 ± 2.3	47.5 – 63.0	22 (39.3)	20 (90.9)	2 (9.1)
Overlapping	58.7 ± 5.6	47.5 – 82.0	33 (58.9)	29 (87.9)	4 (12.1)
Large	70.6 ± 4.6	58.3 – 76.8	1 (1.8)	1 (100)	-

*Small eggs: minimum length <50 µm and maximum length < 64 µm. Eggs with overlapping size: minimum length <50 µm and maximum length > 64 µm. Large eggs: minimum length > 50 µm and maximum length > 64 µm.

characteristic of *T. vulpis* detected in one sample. The distribution of egg length in each category was exemplified in **Fig 4B**. Although small eggs were morphologically typical for *T. trichiura*, results from PCR assays revealed that 9.1% of eggs in this category belonged to *T. vulpis*. Likewise, the majority of eggs (87.9%) with overlapping length between eggs of *T. trichiura* and *T. vulpis* were *de facto* single infection of the former species. It is noteworthy that a stool sample proven to contain only *T. trichiura* eggs by the PCR tests displayed the characteristic eggs akin to those of *T. vulpis* with an average length (±SD) of 70.6 (±4.6) μm (range 58.0-77.0 μm), suggesting that species differentiation of *Trichuris* based on egg size *per se* could be at times unreliable (see **Table 2**).

Discussion

In general, morphology-based identification of parasites in clinical specimens is the most practical diagnostic method in routine laboratory service and in epidemiological study. However, diagnostic stages of some parasites do not permit definite species identification because of shared morphological features between different genus or species. Recent application of molecular tools to diagnosing parasites in clinical samples has rendered correct differentiation of parasite species that possesses ambiguous morphology and even unveils endemicity of some parasites previously thought to be non-existence in certain geographic areas [24, 25]. Correct identification of *Trichuris* species has clear medical consequences in terms of disease transmission, prevention, and control. Although specific identification of *T. trichiura* in stool specimens can be based on its characteristic egg, variation in egg dimension has complicated differentiation of *T. trichiura* from *T. vulpis* because an overlapping dimension occurs between these species. Yoshikawa and colleagues measured uterine eggs of 30 adult females of
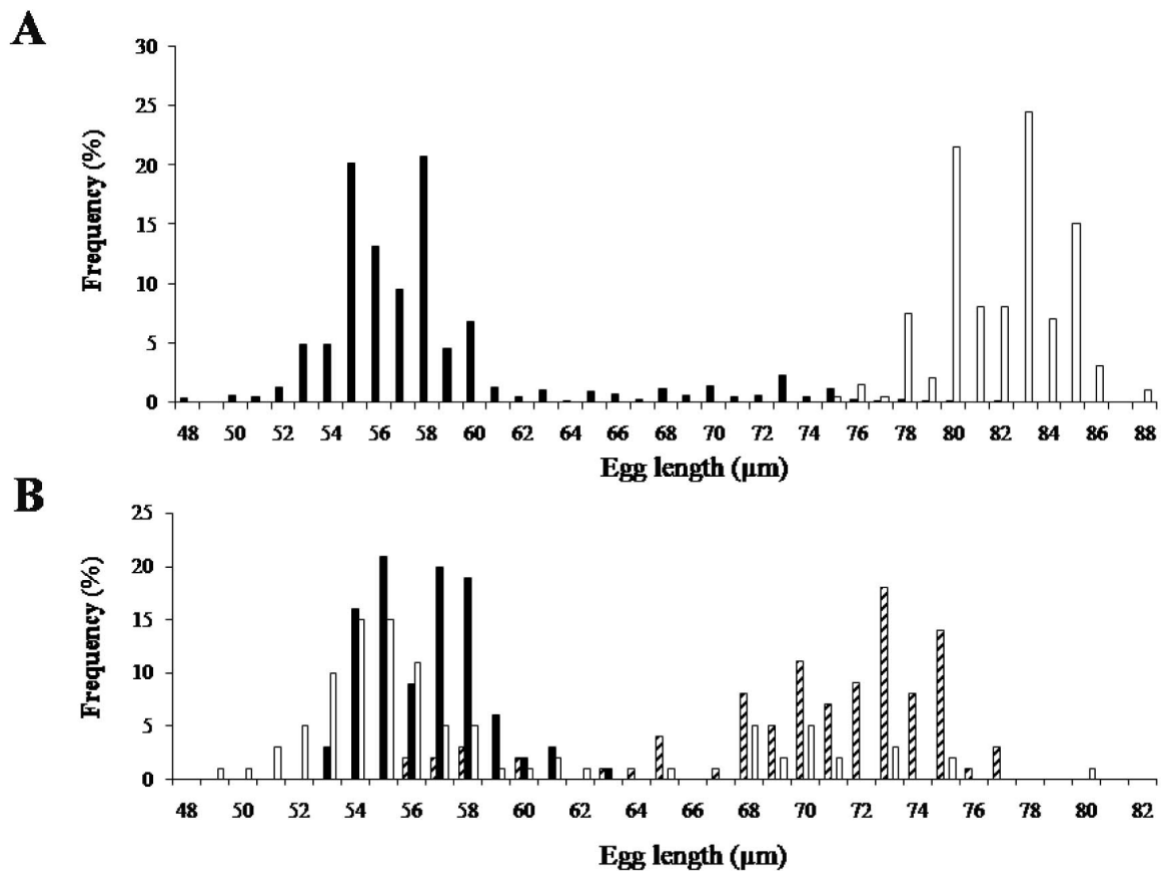

Fig. 4 (**A**) Distribution of egg length of PCR-proven *Trichuris trichiura* (filled bars, n=220) and *T. vulpis* (unfilled bars, n=220). (**B**) Distribution of egg length of *Trichuris* found in three children classified as small eggs (filled bars, n=100), eggs with overlapping size (unfilled bars, n=100) and large eggs (upward diagonal bars, n=100).

T. trichiura isolated from a patient and found that 14 harbored only typical smaller eggs (length <64 μm), 15 had two populations of eggs, one of smaller size and the other larger size (length > 64 μm), and one worm possessed only larger eggs [21]. Consistently, our study has supported the presence of such variation in a human population at Tak Province with similar frequency distribution. Importantly, the present study has further unveiled that some *T. vulpis* eggs could resemble those of *T. trichiura* in having small eggs while the presence of two populations of eggs in some samples could be due to mixed infections. Therefore, differentiation of *Trichuris* species based on egg dimension was not completely accurate and may not be a feasible means for epidemiological purposes.

Meanwhile, almost all reported *T. vulpis* infections in humans were solely based on the presence of large eggs in stool samples [19, 20]. On the other hand, histological and immunological methods have been applied to diagnose *T. vulpis* as a causative agent of visceral larva migrans in humans [32, 33]. However, morphological similarity between *T. vulpis* and other *Trichuris* species in tissue sections render it difficult to identify the worm species unequivocally while cross-reactive antigens occur between species within the same genus of nematodes [34, 35]. Although we did not evaluate the diagnostic efficacy of our PCR assays for tissue samples, the method should be applicable to other sources of samples provided that DNA template was appropriately prepared.

A random amplified polymorphic DNA analysis has been described to diagnose *T. trichiura* eggs in stool samples [27]. However, genetic material co-extracted from fecal samples could potentially interfere with the pattern of the amplified products and could mask correct diagnosis. In the present study, our PCR assays could simultaneously and unequivocally differentiate *T. vulpis* and *T. trichiura* using DNA extracted from eggs in the presence of other genetic material co-purified from stool samples. The PCR assays developed in this study could be reproducibly performed with high sensitivity and specificity as tested in a cross-sectional survey. However, one of the major hurdles in successful PCR amplification using DNA extracted from fecal samples as template is the inefficiency of DNA extraction procedures and the presence of PCR inhibitors. Removal of PCR inhibitors has shown a remarkable improvement in the success rate of PCR diagnosis of *Opisthorchis*-like eggs in fecal samples [36]. Although

attempts to remove PCR inhibitors have not been done in this study, disruption of *Trichuris* eggshell by direct pressing of micropipette tip to individual eggs followed by DNA purification yielded an overall 70 positive PCR tests from 73 *Trichuris*-positive human and dog stool samples. The low sensitivity of the bead beating method could be due to either low efficiency of eggshell breaking or PCR inhibitors from stool materials while very limited amount of stool content was transferred to the DNA extraction kit with high certainty of eggshell disruption by the direct pressing method. Nevertheless, PCR method cannot substitute microscopy detection of parasite eggs in fecal samples because of several constraints such as time, equipment required, expertise of personnel and budget that were usually limited in routine practice in disease endemic areas. On the other hand, our PCR assays for differentiation of *T. trichiura* and *T. vulpis* are of diagnostic value for molecular epidemiology purpose.

Canine trichuriasis is prevalent among dogs living in the community in Ta Song Yang District, accounting for 21.5% of all dog stool samples examined in this study akin to the previous survey in Bangkok showing an infected rate of 20.5% [37]. A lower prevalence was reported in a survey in the city of Naples in Italy where *T. vulpis* eggs were recovered in 10.1% of canine fecal samples [38]. In a remote tea-growing community of Assam in India, trichuriasis and ascariasis were prevalent among dogs, responsible for 25% and 31%, respectively. PCR-RFLP analysis spanning ITS-1, 5.8S rRNA and ITS-2 has shown that all *Ascaris* in dogs shared identical restriction profiles with those found in humans. This indicates the roles of dogs in disease transmission in the study community [39]. Furthermore, it is also suggested that canine trichuriasis in the same survey is caused by *T. trichiura* based on morphology and dimension of eggs despite no detail information regarding the range of egg dimension of each sample [39]. On the other hand, eggs dimension of *Trichuris per se* could mislead species identification because eggs of *T. trichiura* may at times resemble those of *T. vulpis* and mixed infections between both species were common as demonstrated in this study. Interestingly, *T. ovis* and *T. skrjabini*, causing ovine trichuriasis, also possess two different egg sizes, reflecting a common feature of some species within this genus [40]. Meanwhile, in the present study we did not allocate human subjects who donated their stool samples into those who owned and those who did not own dogs because the dogs in

this community seem to be semi-domesticated and were allowed to roam throughout the community, providing almost similar risks of acquiring pathogens from them. Importantly, our molecular diagnosis unequivocally confirmed the presence of *T. trichiura* in dogs in the study population, implying the reservoir roles of dogs in maintenance and transmission of this important nematode. However, it remains to be investigated whether *T. suis* and *T. muris* that are phylogenetically closely related to *T. trichiura* and *T. vulpis*, respectively, could established overt infections and complete their life cycles in their related species of hosts. It is an issue that could be of medical concern when *T. suis* was used in iatrogenic infection as an immunomodulator in the treatment of inflammatory bowel diseases [41].

Conclusion

We have determined the nucleotide sequence of the SSU rRNA genes and ITS-1 of both *T. trichiura* and *T. vulpis* and deployed the sequences for PCR-based diagnosis of these whipworms. The presence of these whipworms in both humans and dogs in a rural community of Thailand would highlight the role of dogs as reservoir hosts for disease transmission to humans.

Acknowledgements

References

1. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet. 2006; 367:1521-32.

2. Albonico M, Chwaya HM, Montresor A, Stolfzfus RJ, Tielsch JM, Alawi KS, Savioli L. Parasitic infections in Pemba Island school children. East Afr Med J. 1997; 74:294-8.

3. Hadju V, Abadi K, Stephenson LS, Noor NN, Mohammed HO, Bowman DD. Intestinal helminthiasis, nutritional status, and their relationship; a cross-sectional study in urban slum school children in Indonesia. Southeast Asian J Trop Med Public Health. 1995; 26:719-29.

4. Needham C, Kim HT, Hoa NV, Cong LD, Michael E, Drake L, Hall A, Bundy DA. Epidemiology of soil-transmitted nematode infections in Ha Nam Province, Vietnam. Trop Med Int Health. 1998; 3:904-12.

5. Norhayati M, Zainudin B, Mohammod CG, Oothuman P, Azizi O, Fatmah MS. The prevalence of *Trichuris*, *Ascaris* and hookworm infection in Orang Asli children. Southeast Asian J Trop Med Public Health. 1997; 28: 161-8.

6. Stephenson LS, Latham MC, Kurz KM, Kinoti SN, Brigham H. Treatment with a single dose of albendazole improves growth of Kenyan schoolchildren with hookworm, *Trichuris trichiura*, and *Ascaris lumbricoides* infections. Am J Trop Med Hyg. 1989; 41:78-87.

7. Layrisse M, Aparcedo L, Martinez-Torres C, Roche M. Blood loss due to infection with *Trichuris trichiura*. Am J Trop Med Hyg. 1967; 16:613-9.

8. Ramdath DD, Simeon DT, Wong MS, Grantham-McGregor SM. Iron status of schoolchildren with varying intensities of *Trichuris trichiura* infection. Parasitology.1995; 110:347-51.

9. Robertson LJ, Crompton DW, Sanjur D, Nesheim MC. Haemoglobin concentrations and concomitant infections of hookworm and *Trichuris trichiura* in Panamanian primary schoolchildren. Trans R Soc Trop Med Hyg. 1992; 86:654-6.

10. Stephenson LS, Latham MC, Ottesen EA. Malnutrition and parasitic helminth infections. Parasitology. 2000; 121:S23-38.

11. Cooper ES, Bundy DA, Henry FJ. Chronic dysentery, stunting, and whipworm infestation. Lancet. 1986; 8501:280-1.

12. Nokes C, Bundy DA. Does helminth infection affect mental processing and educational achievement? Parasitol Today. 1994; 10:14-8.

13. Watkins WE, Pollitt E. Stupidity or worms: do intestinal worms impair mental performance? Psychol Bull. 1997; 121:171-91.

14. Chan MS. The global burden of intestinal nematode infections: fifty years on. Parasitol Today. 1997; 13: 438-43.

15. Murray CJL, Lopez AD. The global burden of disease: a comprehensive assessment of mortality and morbidity from disease, injuries, and risk factors in 1990 and projected to 2020. 1st ed. Cambridge (MA): Harvard University Press; 1996.

16. World health report: Annex Table 2: Deaths by cause, sex, and mortality stratum in WHO regions. World Health Organization; 2002.

17. Bundy DA, Cooper ES. *Trichuris* and trichuriasis in humans. Adv Parasitol. 1989; 28:107-73.

18. Beaver PC, Jung RC, Cupp EW. Aphasmid nematodes (Adenophorea). In: Beaver PC, Jung RC, Cupp EW, editors. Clinical Parasitology, 9th ed. Philadelphia:Lea and Febiger; 1984. p.231-52.

19. Dunn JJ, Columbus ST, Aldeen WE, Davis M, Carroll KC. *Trichuris vulpis* recovered from a patient with chronic diarrhea and five dogs. J Clin Microbiol. 2002; 40:2703-4.

20. Kenney M, Yermakov V. Infection of man with *Trichuris vulpis*, the whipworm of dogs. Am J Trop Med Hyg. 1980; 29:1205-8.

21. Yoshikawa H, Yamada M, Matsumoto Y, Yoshida Y. Variations in egg size of *Trichuris trichiura*. Parasitol Res. 1989; 75:649-54.

22. Lovis L, Mak TK, Phongluxa K, Soukhathammavong P, Sayasone S, Akkhavong K, et al. PCR diagnosis of *Opisthorchis viverrini* and *Haplorchis taichui* infections in a Lao Community in an area of endemicity and comparison of diagnostic methods for parasitological field surveys. J Clin Microbiol. 2009; 47:1517-23.

23. Mayta H, Gilman RH, Prendergast E, Castillo JP, Tinoco YO, Garcia HH, et al. Cysticercosis Working Group in Peru. Nested PCR for specific diagnosis of *Taenia solium* taeniasis. J Clin Microbiol. 2008; 46:286-9.

24. Putaporntip C, Hongsrimuang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, et al. Differential prevalence of *Plasmodium* infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. J Infect Dis. 2009; 199:1143-50.

25. Traub RJ, Macaranas J, Mungthin M, Leelayoova S, Cribb T, Murrell KD, et al. A new PCR-based approach indicates the range of *Clonorchis sinensis* now extends to central Thailand. PLoS Negl Trop Dis. 2009; 3:e367.

26. Cutillas C, de Rojas M, Ariza C, Ubeda JM, Guevara D. Molecular identification of *Trichuris vulpis* and *Trichuris suis* isolated from different hosts. Parasitol Res. 2007;100:383-9.

27. Martinez EM, Correia JA, Villela EV, Duarte AN, Ferreira LF, Bello AR. Random amplified polymorphic DNA analysis of DNA extracted from *Trichuris trichiura* (Linnaeus, 1771) eggs and its prospective application to paleoparasitological studies. Mem Inst Oswaldo Cruz. 2003; 98:S59-62.

28. Oliveros R, Cutillas C, de Rojas M, Arias P. Characterization of four species of *Trichuris* (Nematoda: Enoplida) by their second internal transcribed spacer ribosomal DNA sequence. Parasitol Res. 2000; 86:1008-13.

29. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA. 2004; 101:11030-5.

30. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25:1451-2.

31. Garcia LS. Macroscopic and microscopic examination of fecal specimens. In: Garcia LS, editor. Diagnostic Medical Parasitology 4th ed. Washington, DC: ASM Press; 2001. p.741-85.

32. Masuda Y, Kishimoto T, Ito H, Tsuji M. Visceral larva migrans caused by *Trichuris vulpis* presenting as a pulmonary mass. Thorax. 1987; 42:990-1.

33. Sakano T, Hamamoto K, Kobayashi Y, Sakata Y, Tsuji M, Usui T. Visceral larva migrans caused by *Trichuris vulpis*. Arch Dis Child. 1980; 55:631-3.

34. Coulter JB, Jewsbury JM, Beesley WN, Bailey W. Visceral larva migrans and *Trichuris vulpis*. Arch Dis Child. 1981; 56:406.

35. Kradin RL, Badizadegan K, Auluck P, Korzenik J, Lauwers GY. Iatrogenic *Trichuris suis* infection in a patient with Crohn disease. Arch Pathol Lab Med. 2006; 130:718-20.

36. Duenngai K, Sithithaworn P, Rudrappa UK, Iddya K, Laha T, Stensvold CR, et al. Improvement of PCR for detection of *Opisthorchis viverrini* DNA in human stool samples. J Clin Microbiol. 2008; 46:366-8.

37. Inpankaew T, Traub R, Thompson RC, Sukthana Y. Canine parasitic zoonoses in Bangkok temples. Southeast Asian J Trop Med Public Health. 2007; 38: 247-55.

38. Rinaldi L, Biggeri A, Carbone S, Musella V, Catelan D, Veneziano V, et al. Canine faecal contamination and parasitic risk in the city of Naples (southern Italy). BMC Vet Res. 2006; 2:29.

39. Traub RJ, Robertson ID, Irwin P, Mencke N, Thompson RC. The role of dogs in transmission of gastrointestinal parasites in a remote tea-growing community in northeastern India. Am J Trop Med Hyg. 2002; 67: 539-45.

40. Jansen J. Abnormal eggs of *Trichuris ovis* (Abildgaard 1795) (Nematoda: Trichuridae). Z Parasitenkd. 1984; 70:827-8.

41. Elliott DE, Summers RW, Weinstock JV. Helminths and the modulation of mucosal inflammation. Curr Opin Gastroenterol. 2005; 21:51-8.