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DNA barcoding relates *Trichuris* species from a human and a man's best friend to non-human primate sources

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Abstract. *Trichuris trichiura*, the whipworm of humans, is one of the most prevalent soil-transmitted helminths (STH) reported worldwide. According to a recent study, out of 289 STH studies in Southeast Asia, only three studies used molecular methods. Hence, the genetic assemblages of *Trichuris* in Southeast Asia are poorly understood. In this study, we used partial mitochondrial DNA (cytochrome c oxidase subunit 1 or COI) sequences for analysis. *Trichuris* grouped in a same clade with different hosts indicate the potential of cross infection between hosts. Based on COI, the adult *Trichuris* isolated from a Malaysian patient was most closely related to *Trichuris* isolated from *Papio anubis* (olive baboons) from the USA. The *Trichuris* isolated from the dog from Malaysia was genetically similar to a *Trichuris* species isolated from *Macaca silenus* (lion-tailed macaque) from Czech Republic. Both the human and dog isolated *Trichuris* grouped in clades with different hosts indicating the potential of cross infection between hosts. Specific PCR primers based on the partial COI of *T. trichiura* isolated from African green monkey and *T. serrata* were designed and successfully amplified using multiplex PCR of the pooled DNA samples. Our results suggest a complex parasite-host relationship, and support the theory of cross infection of *Trichuris* between humans and non-human primates as suggested in previous publications.

INTRODUCTION

In Malaysia, *Trichuris trichiura* is one of the most prevalent soil-transmitted helminths (STH) with occurrence documented to be over 98% in some at risk populations (Ahmed *et al.*, 2011). Among the 289 STH studies conducted within Southeast Asia, only 3 (1%) used molecular methods (i.e., PCR) to identify the species of the worm, whereas the remainder relied on egg identification (Dunn *et al.*, 2016). Most *Trichuris* spp. eggs cannot be distinguished from each other since all are of similar size, typically 'lemon' shaped and have clear, mucoid-appearing polar plugs. Therefore, the genetic assemblages of *T. trichiura* in humans and the zoonotic cross-infection between humans and non-human animals in Southeast Asia are poorly understood. DNA barcoding, the use of a short fragment of mitochondrial DNA (cytochrome c oxidase subunit 1, cox1 or COI) sequences for species identification

(Hebert *et al.*, 2003), may enable a better understanding of *T. trichiura* strains infecting humans as well as the occurrence of zoonosis. To our knowledge, DNA barcoding of endoparasites is generally small in scope with most studies performed on wildlife parasites that rarely infect humans (Ondrejicka *et al.*, 2014).

Since limited molecular data on *Trichuris* are available in Asia, especially in Southeast Asia, this study was performed with the primary aims of: 1) DNA barcoding *Trichuris* (worms and eggs) from human and non-human animals in Peninsular Malaysia; and 2) investigating the potential cross infection of *Trichuris* between human and non-human animals, as well as in between non-human animals.

MATERIALS

Sample collection

A total of 5 specimens of adult *Trichuris* worms were collected for analysis. A *Trichuris* adult worm was isolated from a middle-aged female patient suffering from gastrointestinal symptoms and underwent colonoscopy in January 2016. Two *Trichuris* adult worms (1 male and 1 female) were isolated from a cat (*Felis catus*) in St. Kitts using the same method previously reported by Ketzis *et al.* (2015). Two *Trichuris* adult worms (1 male and 1 female) were isolated from *Chlorocebus sabaeus* (African Green Monkey; AGM) on St. Kitts, isolated using the same method previously reported by Hawash *et al.* (2015) and provided under a Materials Transfer Agreement with the Behavioral Science Foundation, Caribbean Primate Laboratory. All adult worms were preserved in absolute ethanol and transported to the Department of Parasitology, Faculty of Medicine, University of Malaya.

A total of 296 fecal samples were collected from non-human animals in Malaysia and Thailand and screened for *Trichuris* eggs using formalin-ether sedimentation according to a previous study (Allen & Ridley, 1970). From Malaysia, 161 samples from dogs (70), cats (50), pigs (33), apes (3 from *Hylobates lar* (White-handed Gibbon) and 1 from *Symphalangus syndactylus* (Siamang), and raccoons (4 from *Procyon lotor*) were screened for *Trichuris*. From Thailand, a total of 135 fecal samples were screened with 55 from monkeys, 40 from pigs and 40 from goats.

DNA extraction and PCR

Genomic DNA of 5 adult *Trichuris* worms was extracted using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. Genomic DNA of stool samples containing *Trichuris* eggs was extracted using FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp., Pingtung County, Taiwan), following the manufacturer's instructions. A universal primer set that targets ~400 bp of COI gene region of Trichuridae nema-todes was retrieved from the literature (Guardone *et al.*, 2013): COIFmod (forward primer; TGRTTTTTT GGICAYCCIGARG) and COIRmod (reverse primer; CACTACATAGTADGTRTCRTG). For the AGM and cat adult *Trichuris* worms and the *Trichuris* eggs isolated from a dog, amplicons were produced by PCR in a total volume of 25 µL with 2.5 µL of each forward and reverse primer (10 µM), 12.5 µL of 2x PCR mastermix, 2.5 µL of ddH2O and 5 µL of DNA template. Thermocycling program: 95°C for 15 minutes; 40 cycles of 95°C for 30 seconds; 54°C for 1 minute; 72°C for 1 minute and a final extension of 72°C for 10 minutes. For the adult *Trichuris* worm isolated from a human, amplicons were produced by PCR in a total volume of 20 µL with 2.0 µL of each forward and reverse primer (10 µM), 10.0 µL of 2X Ex*Prime Taq* Premix (GENET BIO, Daejeon, Korea), 5.0 µL of ddH2O and 1 µL of DNA template. Thermocycling program: 95°C for 15 minutes; 40 cycles of 94°C for 30 seconds; 48°C for 1 minute; 68°C for 1 minute and a final extension of 68°C for 10 minutes. The amplicons were visualized on a 2% agarose gel stained with ethidium bromide and sent to commercial sequencing company for Sanger sequencing.

Sequence editing and analysis

Sequences were edited (primers were trimmed and ambiguous nucleotide was replaced with N using BioEdit) (Hall, 1999). These sequences are available on BOLD database (Ratnasingham & Hebert, 2007) in the public project TBAV. Eight COI sequences from Callejón *et al.* (2015) and 9 COI sequences from Dolezalova *et al.* (2015) were included as reference sequences. The COI gene region of a full mitochondria genome of *T. discolor* (Liu *et al.*, 2012c), *T. muris* (unpublished data by Holroyd *et al.*, 2015), *T. ovis* (Liu *et al.*, 2012c), *T. suis* (Liu *et al.*, 2012b), and *T. trichiura* (Liu *et al.*, 2012a; Liu *et al.*, 2012b; Hawash *et al.*, 2015) were trimmed and also included as reference sequences. All COI sequences were aligned using ClustalW in Bioedit (Hall, 1999) with a final length of 343 bp. All COI sequences were translated into amino-acid and no stop codon was observed. Maximum Likelihood tree was built and pairwise distance of the sequences was calculated in MEGA 6 (Tamura *et al.*, 2013). *Trichuris* grouped in a same clade with different hosts indicate the potential of cross infection between hosts.

Primer design and multiplex PCR

Each specific primer set targeting COI region of the *Trichuris* worm isolated from AGM (amplicon length: 370bp), and the *Trichuris* worms isolated from a cat (amplicon length: 348bp) was designed based on the sequences generated in this study (see Table 1 for primer sequences). After testing all newly designed specific primer sets on the DNA samples of *Trichuris* worms isolated from the AGM and cat, multiplex PCR was performed with total volume of 20 µL reaction; each PCR contained 0.5 µL of each forward and reverse primer, 7.5 µL of ddH2O, 14 µL of EconoTaq® Plus Green 2x Master Mix (Lucigen Corp, Parmenter St. Middleton, USA) and 2.5 µL of pooled DNA template (DNA samples of *Trichuris* worms isolated from an AGM and a cat in St. Kitts). Thermocycling program: 94°C for 15 minutes; 40 cycles of 94°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute; and final extension of 72°C for 10 minutes. The amplicons were visualized on a 2% agarose gel stained with ethidium bromide and sent to a commercial sequencing company for Sanger sequencing. Sequences produced by the multiplex PCR were matched with the sequences previously produced using the PCR with COIFmod/COIRmod.

RESULTS

Adult worms

Based on COI, the *Trichuris* of a female patient from Peninsular Malaysia (T_T05) formed a clade with the *T. trichiura* isolated from humans in China (GU385218 and NC017750), and *Papio anubis* in USA

Table 1. Animal fecal samples analyzed for *Trichuris* spp. using formalin-ether sedimentation

Animals	Number of fecal samples analyzed	Number of *Trichuris* positive samples	Number PCR amplified
Peninsular Malaysia			
Dog	70	3	1
Cat	50	0	0
Pig	33	0	0
Ape	4	0	0
Raccoon	4	0	0
Thailand			
Monkey	55	7	0
Pig	40	8	0
Goat	40	8	0
Total	296	26	1

(KT449825) (Figure 1). T_T05 was most closely related to KT449825 and the pairwise distance between them was 4.8% (Figure 2). The two *T. trichiura* from AGM (T_T03 and T_T04) were in a separate clade, but one that also included human sourced *T. trichiura* from Uganda (KT449826) and non-human primate sourced *T. trichiura* from the Czech Republic (JF690963) and Denmark (KT449824) (Figure 1). The pairwise distance between T_T03 and T_T04 was 0.0%. The pairwise distance between T_T04 and KT449826 was 0.3% (Figure 2). The two adult *Trichuris serrata* (i.e., T_S01 and T_S02) did not group with any other *Trichuris* spp. The pairwise distance between T_S01 and T_S02 was 0.0% (Figure 2).

Trichuris eggs

Out of 296 animal fecal samples, 26 samples were positive for *Trichuris* eggs based on morphological identification (e.g., size, shape and internal structure) (Table 1). Due to low yields of *Trichuris* eggs in 25 of the samples, only one dog fecal sample from a Malaysian animal shelter was successfully PCR amplified (i.e., T_S06). The *Trichuris* (T_S06) was genetically similar to a *Trichuris* species isolated from a non-human primate (*Macaca silenus*; JF690966) from Czech Republic (Dolezalova *et al.*, 2015) (Figure 1). The pairwise distance between T_S06 and JF690966 was 0.6% (Figure 2).

Primers for multiplex PCR

Specific PCR primers based on the partial COI of *T. trichiura* isolated from AGM and *T. serrata* were designed and successfully amplified using multiplex PCR of the pooled DNA samples (see Table 2 for primer sequences). The sequences amplified by the multiplex PCR were 100% matched with the sequences previously amplified using the PCR with COIFmod/COIRmod.

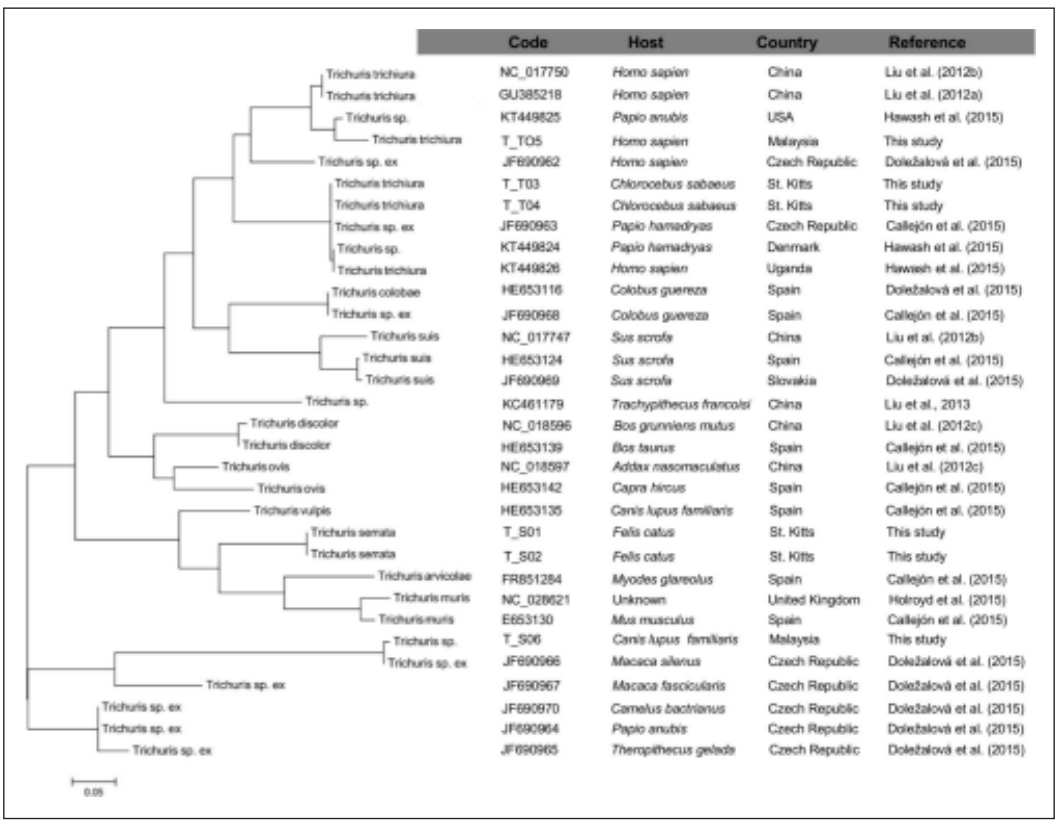

Figure 1. Revised as attached.

Num.	Trichuris	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	Trichuris_arvicolae_(FR851284)																															
2	Trichuris_colobae_(HE653116)	0.396																														
3	Trichuris_discolor_(HE653139)	0.483	0.354																													
4	Trichuris_discolor_(NC018596)	0.480	0.364	0.010																												
5	Trichuris_muris(E653130)	0.216	0.416	0.427	0.433																											
6	Trichuris_muris_(NC028621)	0.237	0.449	0.468	0.474	0.051																										
7	Trichuris_ovis_(HE653142)	0.439	0.300	0.212	0.221	0.422	0.429																									
8	Trichuris_ovis_(NC018597)	0.424	0.302	0.170	0.180	0.399	0.431	0.150																								
9	Trichuris_serrata_(T_S01)	0.264	0.364	0.369	0.382	0.275	0.284	0.349	0.337																							
10	Trichuris_serrata_(T_S02)	0.264	0.364	0.369	0.382	0.275	0.284	0.349	0.337	0.000																						
11	Trichuris_sp._(KC461179)	0.478	0.352	0.380	0.382	0.488	0.517	0.374	0.352	0.482	0.482																					
12	Trichuris_trichiura_(GU385218)	0.401	0.279	0.310	0.305	0.389	0.441	0.386	0.382	0.459	0.459	0.320																				
13	Trichuris_trichiura_(NC017750)	0.401	0.279	0.310	0.305	0.389	0.441	0.386	0.382	0.459	0.459	0.320	0.000																			
14	Trichuris_sp._(KT449824)	0.376	0.332	0.353	0.359	0.414	0.472	0.331	0.366	0.423	0.423	0.298	0.216	0.216																		
15	Trichuris_sp._(KT449825)	0.414	0.314	0.324	0.319	0.390	0.429	0.420	0.379	0.445	0.445	0.355	0.050	0.050	0.219																	
16	Trichuris_sp._(T_S06)	0.927	0.822	0.780	0.788	0.900	0.964	0.787	0.782	0.695	0.695	0.930	0.786	0.786	0.709	0.760																
17	Trichuris_sp._ex_(JF690962)	0.381	0.257	0.340	0.335	0.374	0.432	0.317	0.340	0.437	0.437	0.327	0.170	0.170	0.205	0.181	0.729															
18	Trichuris_sp._ex_(JF690963)	0.376	0.326	0.348	0.353	0.414	0.472	0.336	0.360	0.423	0.423	0.293	0.212	0.212	0.003	0.214	0.709	0.201														
19	Trichuris_sp._ex_(JF690964)	0.408	0.351	0.321	0.323	0.367	0.410	0.339	0.287	0.359	0.359	0.403	0.389	0.389	0.403	0.377	0.736	0.347	0.398													
20	Trichuris_sp._ex_(JF690965)	0.468	0.436	0.389	0.383	0.423	0.472	0.417	0.365	0.441	0.441	0.445	0.436	0.436	0.445	0.423	0.846	0.407	0.439	0.037												
21	Trichuris_sp._ex_(JF690966)	0.908	0.790	0.764	0.771	0.882	0.945	0.770	0.764	0.680	0.680	0.928	0.784	0.784	0.707	0.758	0.006	0.700	0.707	0.721	0.829											
22	Trichuris_sp._ex_(JF690967)	0.624	0.561	0.509	0.503	0.611	0.711	0.548	0.520	0.568	0.568	0.550	0.456	0.456	0.455	0.488	0.569	0.378	0.455	0.334	0.376	0.546										
23	Trichuris_sp._ex_(JF690968)	0.396	0.000	0.354	0.364	0.416	0.449	0.300	0.302	0.364	0.364	0.352	0.279	0.279	0.332	0.314	0.822	0.257	0.326	0.351	0.436	0.790	0.561									
24	Trichuris_sp._ex_(JF690970)	0.408	0.351	0.321	0.323	0.367	0.410	0.339	0.287	0.359	0.359	0.403	0.389	0.389	0.403	0.377	0.736	0.347	0.398	0.000	0.037	0.721	0.334	0.351								
25	Trichuris_suis_(HE653124)	0.310	0.274	0.326	0.339	0.405	0.408	0.309	0.281	0.376	0.376	0.370	0.255	0.255	0.304	0.309	0.688	0.311	0.299	0.397	0.496	0.673	0.536	0.274	0.397							
26	Trichuris_suis_(JF690969)	0.323	0.285	0.328	0.341	0.408	0.393	0.311	0.282	0.390	0.390	0.372	0.266	0.266	0.316	0.311	0.694	0.323	0.311	0.400	0.500	0.679	0.553	0.285	0.400	0.006						
27	Trichuris_suis_(NC017747)	0.334	0.280	0.382	0.377	0.436	0.426	0.352	0.340	0.428	0.428	0.363	0.318	0.318	0.316	0.341	0.665	0.307	0.321	0.389	0.487	0.651	0.537	0.280	0.389	0.099	0.099					
28	Trichuris_trichiura_(KT449826)	0.376	0.332	0.353	0.359	0.414	0.472	0.331	0.366	0.423	0.423	0.298	0.216	0.216	0.000	0.219	0.709	0.205	0.003	0.403	0.445	0.707	0.455	0.332	0.403	0.304	0.316	0.316				
29	Trichuris_trichiura_(T_T03)	0.376	0.326	0.348	0.353	0.414	0.472	0.336	0.360	0.423	0.423	0.293	0.212	0.212	0.003	0.214	0.709	0.201	0.000	0.398	0.439	0.707	0.455	0.326	0.398	0.299	0.311	0.321	0.003			
30	Trichuris_trichiura_(T_T04)	0.376	0.326	0.348	0.353	0.414	0.472	0.336	0.360	0.423	0.423	0.293	0.212	0.212	0.003	0.214	0.709	0.201	0.000	0.398	0.439	0.707	0.455	0.326	0.398	0.299	0.311	0.321	0.003	0.000		
31	Trichuris_trichiura_(T_T05)	0.491	0.362	0.413	0.407	0.436	0.480	0.482	0.438	0.527	0.527	0.391	0.081	0.081	0.273	0.048	0.802	0.214	0.268	0.436	0.511	0.800	0.519	0.362	0.436	0.365	0.367	0.396	0.273	0.268	0.268	
32	Trichuris_vulpis_(HE653135)	0.266	0.397	0.338	0.351	0.277	0.320	0.368	0.308	0.233	0.233	0.454	0.397	0.397	0.362	0.418	0.658	0.333	0.362	0.355	0.432	0.643	0.626	0.397	0.355	0.314	0.316	0.369	0.362	0.362	0.362	0.490

Figure 2. Revised as attached on COI sequences.

Table 2. Primers used for DNA barcoding of *Trichuris*

Primer	Sequence (5'-3')
TSCOI_F	GCATTAGAAAGTGATACGCC
TSCOI_R	TCTTGTATTACCCGCATTCG
TTCOI_F	AGTATAAGGTCTAGCGAGGC
TTCOI_R	TACATTTTAGTGCTCCCAGC

DISCUSSION

The results of this study, with different clades containing both human and non-human primate *Trichuris*, support the hypothesis of multiple *Trichuris* species or strains in humans and non-human primates (Ravasi *et al.*, 2012; Ghai *et al.*, 2014; Dolezalova *et al.*, 2015; Hawash *et al.*, 2015). Both *Trichuris* species or strains seen in the human and dog are first reports for Malaysia and suggest the potential of more cryptic *Trichuris* species to be identified in Malaysia. The genetic distance between T_T05 and the *T. trichiura* isolated from *Papio anubis* in USA (KT449825) was 4.8%, which is more than 2.0%. According to Ratnasingham & Hebert (2013), the genetic difference within a described species rarely exceed 2.0%. Hence, T_T05 might be a cryptic species. However, genetic markers like mitochondrial COI gene and nuclear ITS2 (internal transcribed spacer 2) gene, sometimes are unable to identify evolutionary young sister species, potentially because the genetic markers are polymorphic or poorly differentiated (Avise, 2000; Lukhtanov *et al.*, 2015a, b, 2016). To confirm whether T_T05 is a cryptic species, more studies should be conducted, for example whole genome sequencing, morphological and ecological studies.

Based on the results, *T. trichiura* (TT_05) may pose a risk of cross-infection between humans and non-human primates. *Papio anubis* (olive baboon) is not in the checklist of wild primates in Malaysia (Azlan, 2006) nor even listed as a species in Southeast Asia (Roos *et al.*, 2014). However, this primate does exist in Malaysian zoos although no *Trichuris* were found in six olive baboons examined (Lim *et al.*, 2008). In the case of the dog, previous studies on *Trichuris* in dogs in Peninsular Malaysia have relied on morphological identification of eggs in feces with the infections declared as *Trichuris vulpis* (Ngui *et al.*, 2014; Zain *et al.*, 2015; Jia-Chi *et al.*, 2016), except for Tun *et al.* (2015) who only declared the genus in the dog. In this study, the *Trichuris* isolated from a dog was not *Trichuris vulpis*, but genetically similar to a *Trichuris* species isolated from *Macaca silenus* (lion-tailed macaque; an endangered species) which, like *Papio anubis*, does not occur in Malaysia (Roos *et al.*, 2014).

Considering that neither *Macaca silenus* nor *Papio anubis* are wild primates in Malaysia (Azlan, 2006) and Southeast Asia (Roos *et al.*, 2014), there are a few possibilities as to how these *Trichuris* species were transmitted to the human and the dog in this study. Macaques and baboons infected with *Trichuris* may be legally or illegally imported into Peninsular Malaysia with the *Trichuris* eventually transmitted to the human and the dog in this study. If this happened, these *Trichuris* species, which are not native to Peninsular Malaysia, could be considered as invasive alien species (IAS). International Union for Conservation of Nature (IUCN) defines IAS as non-native species that settled in a habitat and subsequently threatens native biodiversity as an agent of change (Westphal *et al.*, 2008). If the dog has become a true host of this non-human primate *Trichuris*, this switching of hosts might be due to climate change (Brooks and Hoberg, 2007), which can cause shifts in host or parasite ranges, eventually resulting in disease emergence (Harvell *et al.*, 2002). Another possibility is these *Trichuris* species or strains are native to Malaysia and to these hosts but not previously identified, until now, due to the lack of genetic analysis of *Trichuris* in the region. Further studies are needed to determine which hypotheses is correct.

The PCR primers designed based on *T. serrata* and *T. trichiura* (from AGM) could be used individually or together (multiplex PCR) with more multiplex PCR primers to detect a wider range of *Trichuris*

spp. in the environment (e.g., water and soil samples) and to better understand host specificity. This also could be useful for clinical diagnosis of *Trichuris* infection in humans.

CONCLUSION

The results of this study, while limited since based on only one dog and one human sample, suggest a complex parasite-host relationship. While *T. vulpis* has limited zoonotic potential, the ability of dogs to serve as hosts of non-human primate *Trichuris* and the ability of non-human primate *Trichuris* to infect humans, could change the role of dogs as a zoonotic source of infection for humans. Also, *T. trichiura* from Peninsular Malaysia may be able to infect both humans and non-human primates. More sampling of *Trichuris* from dogs, humans, and non-human primates (captive or wild) in Malaysia and its neighboring countries should be conducted to strengthen the hypothesis of the zoonotic cross-infection of *T. trichiura* between humans and non-human primates, as well as the cross-infection of a *Trichuris* sp. between dogs and non-human primates. Understanding these transmission patterns will ultimately be of benefit in developing and accessing interventions to decrease human, non-human primate, and dog infections.

Competing interests

The authors have no competing interests to declare.

Author contributions

G.J.B.M. and V.N. designed the experiment. C.J.S., P.B. and T.M. carried out sample collection, G.J.B.M., J.K.K., P.J., T.T.C. and N.S. performed laboratory (microscopic and molecular) work. G.J.B.M. and A.Y. designed the primers. G.J.B.M. carried out sequence editing and analysis. All authors contributed to writing the manuscript and agreed in submitting for publication.

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