Parasitology

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Molecular identification of *Ancylostoma ceylanicum* in the Philippines

Oyime P. Aula[1,2], Donald P. McManus[1], Kosala G. Weerakoon[3], Remigio Olveda[4,†], Allen G. Ross[5,6], Madeleine J. Rogers[1,2] and Catherine A. Gordon[1]

[1]Molecular Parasitology Laboratory, QIMR Berghofer Medical Research Institute, Queensland, Australia; [2]School of Chemistry and Molecular Biology, The University of Queensland, Queensland, Australia; [3]Department of Parasitology, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Sri Lanka; [4]Immunology Department, Research Institute of Tropical Medicine, Manila, the Philippines; [5]Menzies Health Institute Queensland, Gold Coast, Australia and [6]icddr b, Dhaka, Bangladesh

Research Article

†Deceased 2020

Cite this article: Aula OP, McManus DP, Weerakoon KG, Olveda R, Ross AG, Rogers MJ, Gordon CA (2020). Molecular identification of *Ancylostoma ceylanicum* in the Philippines. *Parasitology* **147**, 1718–1722. https://doi.org/10.1017/S0031182020001547

Received: 25 June 2020
Revised: 4 August 2020
Accepted: 14 August 2020
First published online: 24 August 2020

Key words:
Ancylostoma ceylanicum; Ancylostoma duodenale; Necator americanus; Philippines; real-time PCR

Author for correspondence:
Catherine A. Gordon,
E-mail: Catherine.Gordon@qimrberghofer.edu.au

Abstract

Hookworms are some of the most widespread of the soil-transmitted helminths (STH) with an estimated 438.9 million people infected. Until relatively recently *Ancylostoma ceylanicum* was regarded as a rare cause of hookworm infection in humans, with little public health relevance. However, recent advances in molecular diagnostics have revealed a much higher prevalence of this zoonotic hookworm than previously thought, particularly in Asia. This study examined the prevalence of STH and *A. ceylanicum* in the municipalities of Palapag and Laoang in the Philippines utilizing real-time polymerase chain reaction (PCR) on stool samples previously collected as part of a cross-sectional survey of schistosomiasis japonica. Prevalence of hookworm in humans was high with 52.8% ($n = 228/432$) individuals positive for any hookworm, 34.5% ($n = 149/432$) infected with *Necator americanus*, and 29.6% ($n = 128/432$) with *Ancylostoma* spp; of these, 34 were PCR-positive for *A. ceylanicum*. Considering dogs, 12 ($n = 33$) were PCR-positive for *A. ceylanicum*. This is the first study to utilize molecular diagnostics to identify *A. ceylanicum* in the Philippines with both humans and dogs infected. Control and elimination of this zoonotic hookworm will require a multifaceted approach including chemotherapy of humans, identification of animal reservoirs, improvements in health infrastructure, and health education to help prevent infection.

Introduction

Soil-transmitted helminths (STH) cause around 2 billion infections worldwide; these include hookworms which, as of 2010, accounted for an estimated 438.9 million infections worldwide with 70% of these occurring in Asia (Pullan *et al.*, 2010). There are a number of zoonotic hookworm species parasitic in dogs and cats, including *Ancylostoma caninum, A. braziliensis* and *A. ceylanicum* which cause a range of symptoms in humans. Historically, it was believed that *N. americanus* and *A. duodenale* were responsible for all human hookworm infections (de Silva *et al.*, 2003; Bethony *et al.*, 2006); however, it is now known that *A. ceylanicum* is also an important hookworm of humans, particularly in South East Asia (SEA) (Traub *et al.*, 2008; Bradbury and Traub, 2016). Morphologically these species are similar but there are molecular assays available that distinguish *A. duodenale* from *A. ceylanicum* (Palmer *et al.*, 2007; Traub *et al.*, 2008; Jiraanankul *et al.*, 2011; Inpankaew *et al.*, 2014). Precise determination of the species of hookworm causing the human infection is important for control as *A. ceylanicum* can be transmitted by cats and dogs as well as by infected humans (Yoshida *et al.*, 1968; Setasuban *et al.*, 1976; Traub, 2013).

The only previous report identifying *A. ceylanicum* in the Philippines is from 1968 and utilized microscopy-based diagnosis (Velasquez and Cabrera, 1968). More recently, molecular-based copro-diagnostics have determined a relatively high prevalence of *A. ceylanicum* in SEA in both humans and dogs. In Cambodia, 52% of human hookworm infections and >90% of infected dogs were shown due to *A. ceylanicum* (Inpankaew *et al.*, 2014). Molecular studies have identified *A. ceylanicum* in humans in Thailand (Traub *et al.*, 2008; Jiraanankul *et al.*, 2011), Laos (Conlan *et al.*, 2012), Malaysia (Ngui *et al.*, 2012*b*), and the Solomon Islands (Bradbury *et al.*, 2017) and in Australian dogs (Palmer *et al.*, 2007).

To date, there had been no molecular identification of *A. ceylanicum* in the Philippines. Here we report on the prevalence of *A. ceylanicum* in humans and dogs resident in the municipalities of Palapag and Laoang in Northern Samar of that country utilizing a real-time PCR (qPCR) assay on previously collected stool samples.

Methods

Ethics statement

This study was approved by the QIMR Berghofer Medical Research Institute (QIMRB) Human Ethics Committee and the Institutional Review Board of the Research Institute for Tropical

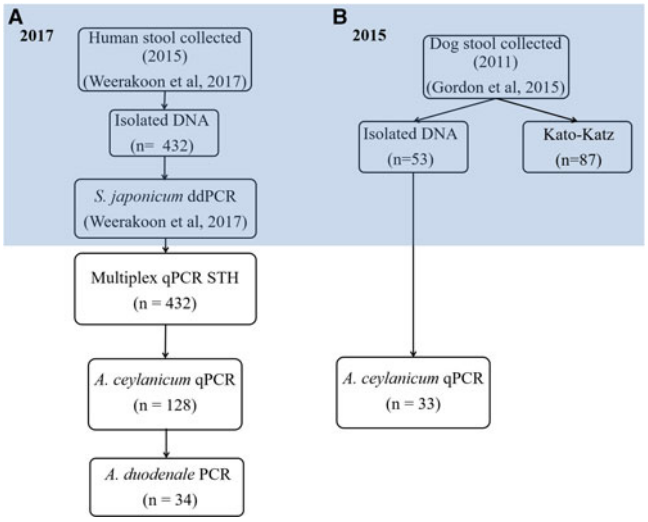

Fig. 1. Workflow for qPCR analysis for *Ancylostoma ceylanicum* in this study. **A.** Stool samples were collected initially for the detection of *S. japonicum* by ddPCR and qPCR (shaded blue) (Weerakoon *et al*., 2017). Extracted DNA was stored at −20°C until utilized in the current study for a multiplex STH and *A. ceylanicum* qPCR. **B.** Dog stool samples were collected and DNA extracted as reported in a previous study in 2011 (Gordon *et al*., 2015*a*, 2015*b*); the DNA was stored at −20°C until analysed in the current study using the *A. ceylanicum* qPCR. Of the dog stool DNA samples available, 33 had sufficient DNA quality and quantity to proceed with the *A. ceylanicum* qPCR assay.

Medicine (RITM), Manila. Informed written consent was received for all study participants; consent from minors was signed for by a legal parent or guardian before the commencement of the study. Individual identification numbers (IDN) were assigned to each participant. Participants were aged 4–72 years. Informed written consent was received from all animal owners in the study area and ethical approval for the animal work was provided by the Ethics Committee of the RITM and the QIMRB Animal Research Ethics Committee. This study was performed in accordance with the recommendations of the Australian code of practice for the care and use of animals for scientific purposes, 2004.

Study design

Human stool DNA samples used in this study were originally collected as part of a previously undertaken cross-sectional survey on *Schistosoma japonicum* in humans and bovines in the Philippines (Weerakoon *et al*., 2017). Briefly, the survey was performed in 18 barangays (villages) in the municipalities of Palapag and Laoang in Northern Samar in 2015 (Weerakoon *et al*., 2017). Stool samples, collected from 452 individuals, with age and gender data collected at the same time, were stored in 80% (v/v) ethanol before being transported to the QIMRB for DNA extraction and a ddPCR assay for *S. japonicum* identification (Weerakoon *et al*., 2017).

Dog DNA samples used here were originally collected in 2011 for an earlier study on *S. japonicum*, although the samples were not used in that particular study (Gordon *et al*., 2015*a*, 2015*b*). Briefly, a cross-sectional survey involving animals was undertaken in six barangays in the Palapag municipality. Stool samples were sought from all dog owners in the study area with age and gender data collected from owners and stored in 80% ethanol before being transported to the QIMRB for DNA extraction in 2011. A total of 87 dog stool samples were collected and subjected to the Kato-Katz (KK) method as part of the original study. Of these, 53 had sufficient stool for DNA extraction, completed in 2011; one sample was discarded as the DNA quality and quantity were low. DNA quality and quantity were re-checked in 2018 using a NanoDrop 1000 prior to qPCR analysis; 33 dog stool samples had sufficient DNA quality and quantity to proceed with the *A. ceylanicum* qPCR assay.

The workflow for the qPCR analysis is shown in Fig. 1.

Study area

The Palapag and Laoang barangay area, where stool samples were collected, has been described in detail elsewhere (Olveda *et al*., 2014; Papier *et al*., 2014; Gordon *et al*., 2015*a*, 2015*b*, 2015*c*; Olveda *et al*., 2016; Ross *et al*., 2017; Weerakoon *et al*., 2017; Weerakoon *et al*., 2018). Briefly, the area is of low socio-economic status and many households in the study barangays (villages) lacked toilets or sewerage systems and running water (Ross *et al*., 2017). The barangays had been subjected to annual mass drug administration (MDA) for schistosomiasis with praziquantel (PZQ), and albendazole for STH amongst school-aged children (Ross *et al*., 2015; DepED, 2015*a*, 2015*b*; Inobaya *et al*., 2018). Human stool samples were obtained by handing out labelled stool cups which were collected over 1 week in each barangay. Stool samples were stored in 80% (v/v) ethanol for subsequent DNA isolation and molecular analysis. Ages of participants ranged from 4 to 72 years; the mean age in the study population was 33. Gender was evenly distributed; 53% of study participants were male.

Dog ages were collected from questionnaires given to all human participants (Gordon *et al*., 2015*a*; Gordon *et al*., 2015*b*). Apart from two animals of unknown age, the ages of the dogs ranged from 1 to 14 years, with the mean age being 3 years; 48% of the dogs were male.

Multiplex qPCR for STH

A multiplex qPCR to amplify hookworm (*Ancylostoma* spp., *N. americanus*), and *A. lumbricoides* DNA, utilizing previously described primers and probes (Gordon *et al*., 2015*c*), was carried out on the collected stool samples.

Reaction mixes with a final volume of 13 μL were prepared to contain 8 μL of GoTaq® qPCR Master Mix (Promega, Madison, USA), 0.8 μL of H_2O, appropriate amounts of forward and reverse primers and probes for *A. lumbricoides*, *N. americanus* and *Ancylostoma* spp. (Table 1), and 2 μL of template DNA. The qPCR was run on a 5-plex Corbett RotorGene 6000 (Qiagen, Hilden, Germany) with the following cycling conditions; 98°C for 2 minutes which was followed by 40 cycles at 98°C for 20 seconds, 74°C for 20 seconds, 58°C for 30 seconds and a final extension at 72°C for 5 minutes.

Genomic DNA of each of the STH analysed was used as positive controls in the qPCR assays, with nuclease-free water used as a negative control. A 1:10 DNA dilution series for each positive control was used to prepare the standard curve [35].

Ancylostoma ceylanicum qPCR

Human and dog DNA samples shown positive for *Ancylostoma* spp. were subjected to a singleplex qPCR with specific primers and probe to identify *A. ceylanicum* as previously described [43]. Reaction mixes with a final volume of 7 μL were prepared to contain 3.5 μL of GoTaq® qPCR Master Mix (Promega, Madison, USA), 0.71 μL of H_2O, appropriate amounts of primer and probe for *A. ceylanicum* (Table 1), and 2 μL of template DNA. The qPCR was run on a 5-plex Corbett RotorGene 6000 (Qiagen) with the following cycling conditions: 50°C for 2 minutes followed by 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, and 59°C for 60 seconds.

A positive control, comprising cloned copies of *A. ceylanicum* G-block gene fragments (Integrated DNA Technologies,

https://doi.org/10.1017/S0031182020001547 Published online by Cambridge University Press

Table 1. Primers and probes used in multiplex qPCR for STH and for *A. ceylanicum* qPCR

Organism	Target	Reference	Probe /quencher	Sequence (5′ – 3′)	Final concentration (nM)
Ascaris lumbricoides	ITS1	(Basuni *et al*, 2011; Taniuchi *et al*, 2011)	*FAM/ZEN*	GTAATAGCAGTCGGCGGTTTCTT	60
				GCCCAACATGCCACCTATTC	60
				TTGGCGGACAATTGCATGCGAT	100
Ancylostoma spp.[a]	ITS2	(Basuni *et al,* 2011; Taniuchi *et al*, 2011)	*Cy5/BHQ2* (LNA)	GAATGACAGCAAACTCGTTGTTG	200
				ATACTAGCCACTGCCGAAACGT	200
				ATC + GTTTA + C + C + GA + CTTTAG	200
Necator americanus	ITS2	(Basuni *et al*, 2011; Taniuchi *et al*, 2011)	*HEX/BHQ1* (LNA)	CTGTTTGTCGAACGGTACTTGC	200
				ATAACAGCGTGCACATGTTGC	200
				CT + GTA + CTA + C + G + CATT + GTATAC	100
Ancylostoma ceylanicum	repeat	(Papaiakovou *et al*, 2017)	*FAM/ZEN*	CAAATATTACTGTGCGCATTTAGC	125
				GCGAATATTTAGTGGGTTTACTGG	1000
				CGGTGAAAGCTTTGCGTTATTGCGA	125
Ancylostoma duodenale	repeat	(Pilotte *et al*, 2016)	*Cy5/ZEN*	GTATTTCACTCATATGATCGAGTGTTC	200
				GTTTGAATTTGAGGTATTTCGACCA	200
				TGACAGTGTGTCATACTGTGGAAA	200

[a]Amplifies *A. duodenale* and *A. ceylanicum*

Melbourne, Australia), and nuclease-free water as a negative control, were run in tandem for each qPCR assay. A 1:10 dilution series of the positive control was used to prepare the standard curve.

Ancylostoma duodenale qPCR

Human DNA samples, positive for *A. ceylanicum,* were subject to a singleplex qPCR with specific primers and probe to identify *A. duodenale* as previously described [43]. Reaction mixes with a final volume of 8 μL were prepared to contain 3.5 μL of GoTaq® qPCR Master Mix (Promega), 0.71 μL of H_2O, appropriate amounts of primer and probe for *A. ceylanicum* (Table 1), and 3 μL of template DNA. The qPCR was run on a 5-plex Corbett RotorGene 6000 (Qiagen) with the following cycling conditions; 95°C for 15 minutes followed by 40 cycles at 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 30 seconds.

Clones of *A. duodenale* G-block gene fragments (Integrated DNA Technologies), used as a positive control, and nuclease-free water as negative control were run for each qPCR assay. A 1:10 dilution series of the positive control was used to prepare the standard curve.

Statistical analysis

Data analysis was carried out using Microsoft Excel (Microsoft; LA, USA, 2010) and SAS (version 9.4). A sample was considered positive if it had a cycle threshold (Ct) value less than 35 for the multiplex analysis and less than 32 for the singleplex *A. ceylanicum* qPCR. Prevalence and 95% confidence intervals (95%CI), and levels of significance were calculated in SAS. 95% CI was calculated using a standard formula based on the binomial and log-normal distribution (prevalence). Significance was calculated using general estimating equations.

Results

Hookworm accounted for the highest STH prevalence in the human subjects with 52.8% (228/432; 95% CI 48.05–57.50) of individuals infected with either *Ancylostoma* spp. or *N. americanus* (Table 2). Of these, 34.5% (149/432; 95% CI 30.00–38.99) were infected with *N. americanus* and 29.6% (128/432; 95% CI 25.31–33.95) with *Ancylostoma* spp. Of those positive for *Ancylostoma* spp., 26.6% (34/128; 95% CI 18.81–34.32) were positive for *A. ceylanicum*, (Table 2). Of those positive for *A. ceylanicum* 9 were also positive for *N. americanus*, 11 were positive for *A. duodenale,* and 10 were positive for all three hookworm species (Table 2).

The prevalence of hookworm in dogs determined by the KK was 33.3% (29/87; 95% CI 23.23–43.44). Of the dog stool samples subjected to the *A. ceylanicum* qPCR assay, 36.4% (12/33; 95% CI 19.04–3.69) were positive.

Discussion

The prevalence of human hookworm in the Palapag and Laoang study area in the Philippines was lower (52.8%) than previously reported (82.3%) in 2013 (Gordon *et al*., 2015*c*), likely due to implementation (in 2015) of the national school deworming program (DepED, 2015*b*; Peñas *et al*., 2018). Mass drug administration (MDA) with albendazole of school-aged children for STH has been carried out bi-annually in the Philippines since 2016; community MDA is not provided (DepED, 2015*b*; DepED, 2016). In our 2013 survey from the same area, we did not identify any infections due to *N. americanus* but in the current study, it accounted for the majority (34.5%) of the hookworms present. This may be due to the smaller number of villages surveyed in the earlier study.

The Palapag/Laoang study area is predominantly rural, it is isolated, and it is of low socioeconomic status; there is little in the way of septic or water treatment and roughly 40% of people do not have access to a water-sealed toilet while the remainder use pit latrines which can become flooded during the rainy season (Ross *et al*., 2015). In most cases drinking water is collected from wells a few feet deep, or from natural springs; occasionally, river water is used for bathing and washing. Poor hygiene, food safety and sanitation are significant risk factors for infection with STH, including hookworm, which makes the study site a prime transmission area for these intestinal worms. This is the first molecular

https://doi.org/10.1017/S0031182020001547 Published online by Cambridge University Press

Table 2. Prevalence of *Ascaris* and hookworm in human subjects from Palapag and Laoang determined by qPCR

Species	N	No. Positive	Prevalence (%)	95% CI
Ascaris lumbricoides	432	76	17.6	14.0–21.2
Ancylostoma spp.	432	128	29.6	25.3–34.0
Necator americanus	432	149	34.5	30.0–39.0
Positive any	432	258	59.7	55.1–64.5
Ancylostoma ceylanicum	128	34	26.6	18.8–34.3
A. duodenale	34	16	61.8	44.6–79.0
Hookworm – all species	432	228	52.8	48.1–57.0
A. duodenale only	228	64	28.1	22.2–34.0
N. americanus only	228	100	43.9	37.4–50.4
A. ceylanicum only	228	4	0.9	0–1.8
A. duodenale + *N. americanus*	228	30	13.2	8.7–17.6
N. americanus + *A. ceylanicum*	228	9	4.0	1.4–6.5
A. duodenale + *A. ceylanicum*	228	11	4.8	2.0–7.6
N. americanus + *A. ceylanicum* + *A. duodenale*	228	10	4.4	1.7–7.1

study reporting on the prevalence of *A. ceylanicum* in the Philippines although this hookworm species was identified morphology in 1968 (Velasquez and Cabrera, 1968).

Similar to other reports from SEA, the current study showed a higher human infection prevalence of *N. americanus* than *A. ceylanicum* (Ngui *et al*., 2012*a*; Inpankaew *et al*., 2014). The prevalence of *A. ceylanicum* in Palapag and Laoang adds to growing concerns that this hookworm is widespread in SEA and is likely to be present in other areas of the Philippines (Velasquez and Cabrera, 1968; Ngui *et al*., 2014).

Dog stool samples, collected in the study area in 2011, were here subjected to qPCR for hookworm presence. Although not reported, the study undertaken in Palapag in 2015 (Gordon *et al*., 2015*a*, 2015*b*) identified 29 of 87 dogs from six barangays as being KK-positive for hookworm. Only a small number (33) of these dog stool DNA samples were available for analysis in the current study but we successfully identified 12 animals infected with *A. ceylanicum*. Increased knowledge of the zoonotic transmission of *A. ceylanicum* is important in terms of the epidemiology of the parasite and in hookworm control generally; the provision of hookworm treatment to humans only will not prevent infection from dogs which freely roam the barangays. The significant health problem of zoonotic diseases caused by domestic animals – in this case, dogs – cannot be overemphasized. Infected dogs contaminate the external environment with hookworm eggs giving rise to larvae that can readily infect humans. Elimination of zoonotic diseases is impossible without interventions that also target animal hosts which act as reservoirs of infection.

The sequencing and identification of *A. ceylanicum* haplotypes will be important to determine the extent of cross-infection between humans and dogs in the Philippines and elsewhere (Ngui *et al*., 2013; Inpankaew *et al*., 2014). In addition, the identification of other canine hookworm spp., with zoonotic potentials, such as *A. brazilliensis,* will also be important in any future STH-focused surveys.

Concluding remarks

We have successfully employed a molecular approach to unambiguously identify infections of *A. ceylanicum* in humans and dogs in the Philippines. Hookworm control programs that adopt a One Health approach will more likely succeed by targeting this zoonotic hookworm both in humans and in dogs. However, this One Health approach will only be effective if residents are appropriately educated about the importance of using correct hygiene practice and undertaking responsible pet ownership. As well, local authorities need to increase health infrastructure that promotes improved sanitation, safe water supply, and enhanced hygiene practices in endemic areas prevalent with hookworm infection.

Supplementary material. To view supplementary material for this article, please visit https://dx.doi.org/10.1017/S0031182020001547.

Funding. DPM receives Program Grant funding (APP1132975) for his research on neglected tropical and zoonotic diseases from the National Health and Medical Research Council of Australia

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https://doi.org/10.1017/S0031182020001547 Published online by Cambridge University Press