

# Identification of *Ancylostoma ceylanicum* in children from a tribal community in Tamil Nadu, India using a semi-nested PCR-RFLP tool

Santosh George<sup>a,b</sup>, Saravanakumar Puthupalayam Kaliappan<sup>b</sup>, Deepthi Kattula<sup>b</sup>, Sheela Roy<sup>b</sup>, Peter Geldhof<sup>a</sup>, Gagandeep Kang<sup>b</sup>, Jozef Vercruyse<sup>a</sup> and Bruno Levecke<sup>a,\*</sup>

<sup>a</sup>Department of Virology, Parasitology and Immunology, Ghent University, Faculty of Veterinary Medicine, Merelbeke, Belgium;  
<sup>b</sup>Division of Gastrointestinal Sciences, Christian Medical College, Vellore, India

\*Corresponding author: Tel: +32 9 264 74 04; Fax: +32 9 264 74 96; E-mail: Bruno.Levecke@Ugent.be

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**Background:** It is generally assumed that hookworm infections in humans are caused by *Necator americanus* and *Ancylostoma duodenale*. However, previous studies have also reported the presence of the animal hookworm *A. ceylanicum* in human stools.

**Methods:** We determined hookworm infections in children in a tribal community in Tamil Nadu, India, using a semi-nested PCR-RFLP approach.

**Results:** The results indicate that human species account for a majority of the hookworm infections (*N. americanus* 39/41 [95%]; *A. duodenale* 6/41 [15%]), whereas the animal hookworm *A. ceylanicum* only accounts for a minority of the infections (5%; 2/41).

**Conclusions:** The results emphasize the need to consider zoonotic ancylostomiasis while developing strategies to control hookworm infections.

**Keywords:** *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, India, *Necator americanus*, Tamil Nadu, Zoonosis

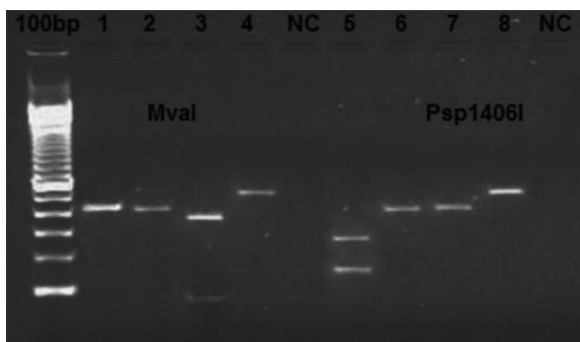
## Introduction

Recent global estimates indicate that in 2010, approximately 440 million people were infected with hookworms.<sup>1</sup> Human hookworm infections are caused by *Ancylostoma duodenale* and *Necator americanus*. Hookworm infections are also commonly found in dogs and cats (e.g., *A. braziliense*, *A. caninum* and *A. ceylanicum*), which pose a zoonotic risk to humans.<sup>2</sup> Majority of the human infections with the animal hookworms result in clinical symptoms due to migrating larvae (cutaneous larval migrans), but will not result in patent infections. An exception is *A. ceylanicum* (and to lesser extent *A. caninum*) of which adult worms can be found in the gastro-intestinal tract of humans.<sup>2</sup> To date, the role of animals as a reservoir of patent hookworm infections remains inadequately explored. This lack in epidemiological data is mainly due to the fact that hookworm infections in humans are traditionally diagnosed by demonstrating eggs in stool. However, this diagnostic strategy does not allow differentiating hookworm species, as eggs are morphologically identical. For this molecular tools are more appropriate. A recent molecular survey in Cambodia highlighted the role of animals as a reservoir for human hookworm infections, with *A. ceylanicum* contributing up to 52% of the human hookworm infections.<sup>3</sup> This high prevalence of zoonotic infections warrants further attention, particularly when current means to control hookworm infections are

mainly based on the administration of anthelmintic drugs to humans only.<sup>4</sup> To gain more insights into the role of animals as a reservoir for patent zoonotic hookworm infections, we determined hookworm infections in children from a tribal community in Tamil Nadu, India.

## Materials and methods

This study was part of an epidemiological survey conducted to: 1. estimate the prevalence of soil-transmitted helminths (STHs) (*Ascaris lumbricoides*, *Trichuris trichiura* and hookworm) among children aged 1–15 years and persons of age 16 and above; 2. study the factors associated with STH infections through a structured questionnaire; 3. identify spatial patterns in the distribution of STH infections in the tribal population of Jawadhu Hills, Tamil Nadu. The study area and design are described previously in detail.<sup>5</sup> In this survey, a total of 620 children between 1 and 15 years of age were screened for the presence of STHs using the saline wet mount microscopy method. Of them, 185 children were found excreting hookworm eggs. A subset of 50 stool samples was randomly selected from the hookworm-infected children for molecular speciation. DNA was extracted from stool samples stored at -70°C using the Qiagen stool DNA-mini kit (Qiagen, Hilden, Germany). Hookworm DNA was amplified in a semi-nested



**Figure 1.** Determination of *Ancylostoma duodenale*, *A. ceylanicum*, *A. caninum* and *Necator americanus* using PCR-RFLP analysis. Lanes 1 to 4 are PCR products of *A. duodenale*, *A. caninum*, *A. ceylanicum* and *N. americanus* digested by *Mva*I, lanes 5 to 8 are the same PCR products digested by *Psp*1406I. Lanes 1 (404 bp) and 5 (255 bp and 149 bp) represents *A. duodenale*, lanes 2 and 6 (404 bp) *A. caninum*, lanes 3 (340 bp and 64 bp) and 7 (404 bp) *A. ceylanicum*, and lanes 4 and 8 (552 bp) *N. americanus*. Lanes labelled NC represent negative controls. Each of the DNA samples were obtained from stool samples collected during this study.

PCR using the common forward primer UGHWF (5'-GTTGGGA GTATCRCCMMCK-3') and the first-round reverse primer NC1R (5'-AACAACCCTGAACCAGACGT-3') and the second-round reverse primer UGHWR (5'-ATGCGTTCAAATTTCCACCA-3'). These primers were designed to amplify parts of the internal transcriber spacer gene (ITS) 1, 2 and 5.8 s region of *A. braziliense* (GenBank accession nos. DQ359149, DQ438056, DQ438060, DQ438062, DQ438064), *A. caninum* (DQ438070, DQ438074, DQ438076, DQ438077, DQ438079), *A. ceylanicum* (DQ381541, DQ780009, DQ831519), *A. duodenale* (EU344797) and *N. americanus* (AF217891). The first-round PCR resulted in a product of 597 bp (*N. americanus*) and in a product of 449 bp (*Ancylostoma* spp.). The second PCR resulted in a product of 552 bp (*N. americanus*) and 404 bp (*Ancylostoma* spp.). Both a negative (water) and positive (hookworm DNA) control was included in each run. The amplification reactions were performed in a volume of 25  $\mu$ l containing 2.5  $\mu$ l DNA, 0.5  $\mu$ l of each primer (10  $\mu$ M), 0.5  $\mu$ l dNTP (10 mM), 1  $\mu$ l  $MgCl_2$  (25 mM), 5  $\mu$ l GoTaq Flexi buffer, 14.875  $\mu$ l PCR-grade water and 0.125  $\mu$ l GoTaq Flexi DNA polymerase. The following conditions were used: 2 min at 95°C (initial denaturation), 34 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing), 30 s at 72°C (extension), followed by a single step of 10 min at 72°C. The amplified product was detected using 1.5% agarose gel electrophoresis using ethidium bromide. The second-round PCR products for *Ancylostoma* spp. and *N. americanus* were subsequently digested using the restriction enzymes *Mva*I and *Psp*1406I at 37°C for 13 hours. *Mva*I digests PCR products of *A. ceylanicum* into two (340 bp and 64 bp), but does not digest *A. duodenale* and *A. caninum*. *Psp*1406I digests *A. duodenale* into two (255 bp and 149 bp), but does not digest *A. ceylanicum* and *A. caninum*. Figure 1 illustrates the RFLP for both enzymes on *A. duodenale*, *A. ceylanicum*, *A. caninum* and *N. americanus*.

## Results and Discussion

Of the 50 samples, 41 were found positive by PCR. Most of these infections were caused by *N. americanus* (n=39), followed by

*Ancylostoma* spp. (n=8). Further speciation of the *Ancylostoma* spp. revealed the presence of *A. duodenale* in six samples and *A. ceylanicum* in the two remaining samples. Mixed *A. duodenale* and *N. americanus* were observed in six samples. *A. ceylanicum* infections were only observed as mono-infections. Our results indicate that human hookworms account for the majority of the human hookworm infections, with *N. americanus* being the predominant species. This finding confirms previous surveys suggesting that hookworm infections in Asia are mainly due to *N. americanus*, but contrasts with molecular surveys reporting high prevalence of zoonotic *A. ceylanicum* infections. This discrepancy might be due to differences in proximity and density of animals to the study population. In Cambodia, the ratio of dogs per household was 1.4 (94/67), whereas in our study 58% (363/620) of the children had contact with dogs or cats (assessed through a questionnaire). Moreover, the higher prevalence of *A. ceylanicum* infection in the Cambodian study was observed in persons between 21 and 30 years of age,<sup>3</sup> whereas our study population was restricted to children between 1 to 15 years of age. An important limitation of this study is that we only examined human stool samples, and did not include stool samples of the sympatric dog/cat populations or samples from the environment to obtain complementary data on the transmission of zoonotic ancylostomiasis.<sup>6</sup>

In conclusion, despite the relatively low prevalence of zoonotic hookworm infections in this tribal community, our study once more highlighted the need to consider hygiene and animal health programs as part of the One Health approach for the control of this zoonosis while developing mass drug administration strategies. Moreover, it emphasizes the necessity of further exploring the impact of zoonotic hookworm transmission on public health using appropriate diagnostic tools on both stool (animals and humans) and environmental samples.

**Authors' contributions:** SG, BL and GK designed the survey; SG, SPK and SR conducted the survey; SG, DK and BL analyzed and interpreted the data; SG and BL wrote the manuscript; GK, PG and JV reviewed the manuscript. All authors have read and approved the final manuscript. BL is the guarantor of the paper.

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**Competing interests:** None declared.

**Ethical approval:** This study was approved by Institutional Review Board of Christian Medical College, Vellore, India. Written informed consent was obtained from parents/guardians of all children included in this study.

## References

- 1 Pullan RL, Smith JL, Jasrasaria R, Brooker SJ. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasit Vectors* 2014;7:37.
- 2 Bowman DD, Montgomery SP, Zajac AM et al. Hookworms of dogs and cats as agents of cutaneous larva migrans. *Trends Parasitol* 2010;26:162–7.
- 3 Inpankaew T, Schar F, Dalsgaard A et al. High prevalence of *Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, 2012. *Emerg Infect Dis* 2014;20:976–82.
- 4 Kaliappan SP, George S, Francis MR et al. Prevalence and clustering of soil-transmitted helminth infections in a tribal area in southern India. *Trop Med Int Health* 2013;18:1452–62.
- 5 WHO. Helminth control in school-age children: a guide for managers of control programmes. Geneva: World Health Organization; 2011.
- 6 Traub RJ, Pednekar RP, Cuttall L et al. The prevalence and distribution of gastrointestinal parasites of stray and refuge dogs in four locations in India. *Vet Parasitol* 2014;205:233–8.