Hookworm infections among migrant workers in Malaysia: Molecular identification of *Necator americanus* and *Ancylostoma duodenale*

Norhidayu Sahimin⁵, Yvonne Ai Lian Lim⁶, Benacer Douadi⁶, Mohd Khairul Nizam Mohd Khalid⁴, John-James Wilson⁴, Jerzy M. Behnke⁶, Siti Nursheena Mohd Zaina⁵,⁎

⁵ Institute of Biological Science, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia
⁶ Department of Parasitology, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia
⁷ Molecular Diagnostics and Protein Unit, Specialised Diagnostics Centre, Institute for Medical Research, 50588, Kuala Lumpur, Malaysia
⁸ International College Beijing, China Agricultural University, Beijing 100083, PR China
⁹ School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

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**ABSTRACT**

Ongoing urbanisation of the working population as well as cross-border migration of workers particularly into large cities has contributed to the development and growth of urban slums. These deprived areas are conducive for the transmission of intestinal pathogens including hookworm. The aim of this study was to determine both the prevalence and species identity of hookworm infections among the migrant worker community in Malaysia. A total of 388 faecal samples were collected from migrant workers between September 2014 and August 2015, representing workers from five employment sectors: construction, manufacturing, agriculture and plantations, food services and domestic services. Faecal samples were examined by microscopy and positive samples were subjected to molecular analysis. A total of 51 samples (13.1%) were positive by microscopy for hookworm infections. A two-step PCR based method amplifying a fragment of the 28S rRNA-ITS2 region was used to identify infections by *Necator americanus* and *Ancylostoma* spp. PCR products positive for *Ancylostoma* spp. were sequenced bidirectionally, and sequences analysed through BLAST and phylogenetic analysis. Samples containing *Ancylostoma duodenale* were further characterized by amplification and sequencing a fragment of cytochrome c oxidase subunit 1 (*cox1*) gene. PCR amplicons were successfully obtained from 42 (82.4%) of 51 samples, with 81.0% (34 of 42) identified as *Necator americanus*, 16.7% (7 of 42) as *Ancylostoma* spp. and 2.4% (1 of 42) as mixed infections of both species. All eight *Ancylostoma* spp. were confirmed to be *Ancylostoma duodenale* and this is the first time *A. duodenale* was reported in Malaysia. Samples containing *A. duodenale* from Nepalese and Indonesian workers shared high-similarity and were distinct compared to sequences from other countries. This study highlights the prevalence of hookworm infections among migrant workers living in Malaysia. Our findings underscore the necessity of screening migrant workers for hookworm infections, particularly those working in food-related services and industries.

**1. Introduction**

The robust economic growth of Malaysia has led to an increase in demand for low-skilled workers, primarily in employment sectors such as construction, manufacturing, plantation, food services and domestic services. Migrant workers have arrived in Malaysia from other South East Asian countries (Indonesia, Cambodia, Vietnam, the Philippines and Myanmar) and South Asian countries (Nepal, India and Bangladesh) both legally and illegally (Ministry of Human Resources Malaysia, 2015; Bardan, 2014). The number of migrant workers entering Malaysia from neighbouring countries has almost doubled over the last decade from 1.06 million in 2002 to 2.07 million in 2014 (Bardan, 2014). In addition to workers arriving from abroad, there has been ongoing urbanisation of the local working population (Schneider et al., 2015). The increasing number of workers in urban areas has contributed to cramped living conditions. These deprived areas are conducive for the transmission of intestinal pathogens including hookworm. Despite compulsory medical screening for foreigners prior to entering the Malaysian workforce, screening for parasitic infections is often lacking or inadequate (Sahimin et al., 2016).

Neglected intestinal parasitic infections (IPIs), such as soil-transmitted helminths (STHs), have been recognized as one of the major
causes of illness, especially among disadvantaged communities (Ngui et al., 2011; Sinniah et al., 2014). The World Health Organization (WHO) included STHs among 17 important neglected tropical diseases, which together account for infections in more than 1.5 billion people, or 24% of the world’s population. Human hookworm infection is an infection generally attributed to one of two principal species: Necator americanus and Ancylostoma duodenale (Phosuk et al., 2013). Other hookworm species can also cause human infection such as Ancylostoma ceylanicum which was estimated to infect between 19 and 73 million people (Traub, 2013). The importance of A. ceylanicum was also evident in a recent survey in Asia that ranked this species as the second most common hookworm species infection human (Traub et al., 2008; Jiraanankul et al., 2011; Conlan et al., 2012; Ngui et al., 2012b; Ipankaev et al., 2014).

Mild hookworm infections in adults are usually asymptomatic, however symptoms such as itchy rash, bloody stools and abdominal pain may appear in heavy hookworm infection. Other common symptoms include anaemia, vitamin A deficiency, stunted growth, malnutrition, intestinal obstruction and impaired development. It is estimated that currently up to 600 million people are infected with hookworm infections worldwide (World Health Organization, 2015).

Hookworm infection in humans is currently diagnosed through the identification of parasite eggs in the patient’s faeces, a method which is technically simple and low cost. However, this method is hampered by morphological similarities between the eggs of N. americanus, A. duodenale, other species of Ancylostoma and other strongylid nematodes including species in the genera Oesophagostomum and Trichostrongylus. The goal of the present study was to determine both the prevalence and species identity of hookworms causing infection among the migrant worker community in Malaysia. Our findings will highlight the extent of hookworm infections as well as the risk factors among migrant workers.

2. Materials and methods

2.1. Recruitment of study participants, data collection and ethical clearance

According to government regulations, foreign workers in professions classified as “low skilled or semi-skilled” can only be legally employed in Malaysia in five working sectors: construction, manufacturing, agriculture and plantations, food services, and domestic services. Participants were recruited from various agencies and companies in Malaysia from September 2014 to August 2015. Participants were asked to complete a questionnaire in order to collect participant information, including: socio-demographic data, migration history, environmental health, life-style habits, recent episodes of illness, occupational health and safety, and any history of taking anthelmintic drugs. The interview process was performed through an interpreter if the participants had difficulty in understanding Malay or English languages. All participants were fully informed of the nature of the study in order to enable maximum co-operation and the completion of consent forms.

Ethical clearance was obtained from the ethics committee, University Malaya Medical Centre (UMMC), Malaysia prior to commencement of the study (Reference number: MECID NO: 20143-40). All adults provided written, informed consent to participate in the study. Furthermore, all individual tested positive for hookworm infections were notified through their respective employers.

2.2. Sample collection and microscopic analysis of faecal samples

After consent was obtained and the questionnaire completed, each individual was provided with a sterile plastic container marked with a specific identification number and the name of the participant. The participant was instructed to scoop a thumb size faecal sample into the container, ensuring that the sample was not contaminated with urine. The plastic container contained 2.5% potassium dichromate solution for sample preservation. Samples were brought to the Institute of Biological Science, Faculty of Science, University of Malaya for analysis. Approximately 1–2 g of each faecal sample were mixed with 7 ml formalin and 3 ml ethyl acetate and centrifuged for 5 min at 2500 rpm. After centrifugation a drop of pellet was taken and stained with Lugol’s iodine on a clean glass slide. Slides were examined under a light microscope at 10x magnification to screen for the presence of hookworms and other intestinal parasites (Sahimin et al., 2016).

2.3. Molecular analysis

Genomic DNA was extracted from faecal samples found to be positive for hookworms by microscopic screening. Approximately, 250–500 mg of each faecal sample were used for DNA extraction, using a NucleoSpin® Soil kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer’s instructions. The extracted DNA was stored at −20 °C until further analysis.

A two-step semi-nested PCR was used for DNA amplification. For the first amplification, the forward primer, NC1 (5′- AGG TCT GTG TCA GGG TTC TT – 3′), and reverse primer, NC2 (5′- TTA GGG TTC TT CCT CGG CT – 3′), were used to amplify a region of internal transcribed spacer 2 and 28S ribosomal RNA (ITS2-28S rRNA) which accounted for approximately 310 bp in Ancylostoma spp. and approximately 420 bp in N. americanus(Gasser et al., 1993; Ngui et al., 2012a). The PCR was performed using Maxime PCR PreMix Kit (i-Taq) (iNTRON Biotechnology, Inc.) in a 20 μl reaction. The reaction contained i-Taq™ DNA polymerase (2.5U), deoxynucleoside triphosphate (dNTPs) (2.5 mM each), 1X reaction buffer, gel loading buffer (1x), DNA template (2 μl), primers (10 pM each) and ultrapure water. The PCR conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. Known samples containing N. americanus and Ancylostoma spp. genomic DNA were included as positive controls.

Samples showing successful PCR amplification based on agarose gel electrophoresis were subjected to a second PCR. The second PCR included two forward primers, NA (5′- ATG TGC ACG TTA TTC ACT – 3′) (for N. americanus) (Verweij et al., 2001), AD1 (5′- CGA CTT TAG AAC GTT TCG GC – 3′) (for Ancylostoma spp.) (de Gruijter et al., 2005) and NC2 as a common reverse primer. All 3 primers were included in a single reaction for simultaneous amplification of N. americanus and Ancylostoma spp. The recipe for the second PCR was identical to the first PCR except that 2 μl of first PCR products were used as the template. The cycling conditions for the second PCR were 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The expected PCR product size was 250 bp for N. americanus and 130 bp for Ancylostoma spp.

PCR products for Ancylostoma spp. (i.e. ~130 bp) were purified using a MEGAquick-spin™ Total Fragment DNA Purification Kit (iNTRON Biotechnology, 2011, Korea) according to the manufacturer’s protocol. Bidirectional DNA sequencing was performed by a local commercial sequencing company (First BASE, Pte. Ltd., Singapore). Sequences were viewed using Sequence Scanner software version 1.0 (Applied Biosystems, USA). Forward and reverse sequences were manually aligned and consensus sequences were created using BioEdit (www.mbio.ncsu.edu). The similarity between consensus sequences and previously published sequences of hookworm was compared using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast). All consensus sequences were deposited in GenBank under accession numbers KX650194- KX650201. A neighbor-joining tree was constructed with MEGA6 (Tamura et al., 2013) and evaluated with 1000 bootstrap re-samples.

I. Microscopic Screening

After the microscopic examination, faecal samples were collected from 110 workers.

2. DNA Extraction

DNA was extracted from the faecal samples using Nucleospin® Soil kit. After DNA extraction, PCR was performed using Maxime PCR PreMix Kit (i-Taq) (iNTRON Biotechnology, Inc.) in a 20 μl reaction. The reaction contained i-Taq™ DNA polymerase (2.5U), deoxynucleoside triphosphate (dNTPs) (2.5 mM each), 1X reaction buffer, gel loading buffer (1x), DNA template (2 μl), primers (10 pM each) and ultrapure water. The PCR conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. Known samples containing N. americanus and Ancylostoma spp. genomic DNA were included as positive controls.

3. Sequencing

The sequencing process was performed using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast). All consensus sequences were deposited in GenBank under accession numbers KX650194- KX650201.
2.4. PCR, DNA sequencing and phylogenetic analysis of cox1 gene of *A. duodenale*

Samples that were positive for *A. duodenale* on the basis of the two-step PCR above were further characterized by analysis of cox1 gene. Primers, AceyCOX1F (5’-GCT TTT GGT ATT GTA AGA CAG-3’) and AceyCOX1R (5’-CTA ACA TAA TAA TCA TG-3’), were used to amplify a 377-bp of cox1 gene (according to Inpankaew et al., 2014). The PCR recipe followed the first PCR above. The cycling conditions were 94 °C for 2 min, followed by 30 cycles at 94 °C for 20 s, 50 °C for 10 s, 72 °C for 50 s, and a final extension at 72 °C for 5 min. Purification of PCR-positive samples and bidirectional DNA sequencing was performed. Sequence analysis followed the procedure described above. All Cox1 gene sequences were deposited in GenBank under accession numbers (KY400020-KY400026).

2.5. Statistical analysis

Prevalence was analysed using maximum likelihood techniques based on log linear analysis of contingency tables using the software package SPSS (Version 22). Infection was considered as a binary factor (presence/absence of parasites). Analysis was conducted with the intrinsic factors: sex (2 levels: males and females), age (5 age classes: those < 25 years old, 25-34 years old, 35-44 years old, 45-54 years old and those > 54 years) and nationality (5 countries: Indonesia, Bangladesh, Myanmar, India and Nepal). Other extrinsic factors analysed include employment sector (5 sectors: construction, manufacture, plantation, food service and domestic), years of residence in Malaysia (2 categories: less than 1 year and more than 1 year), accommodation type (3 types: hostel provided by the employer, construction site and own/rented house) and level of education (4 levels: primary school, secondary school, university and no formal schooling).

3. Results

3.1. Prevalence, species identification and phylogenetic analysis

Of 388 stool samples examined microscopically, 51 samples (13.1%) were found to be positive for hookworms. Prevalence of other intestinal parasites detected in this study were *A. lumbricoides* (43.3%), *E. histolytica/dispar* (11.6%), *Giardia* sp. (10.8%), *T. trichura* (9.5%), *Cryptosporidium* sp. (3.1%), *H. nana* (1.8%) and *E. vermicularis* (0.5%) (Sahimin et al., 2016). PCR amplicons were obtained from 42 of the 51 (82.4%) of the microscopically positive hookworm samples.

The two-step PCR revealed that of the 42 PCR-positive samples, 81.0% (34/42) were *N. americanus*, 16.7% (7/42) were *Ancylostoma* spp. and the remaining 2.4% (1/42) had a mixed infection of both species, giving respectively a prevalence of 9.0% for *N. americanus* (n = 35; CL95 = 6.2–11.9%) and 2.1% for *Ancylostoma* spp. (n = 8; CL95 = 0.6–3.5%) based on in the combined microscopy plus PCR detection approach. When screened by PCR, all 337 samples found to be negative by microscopy showed no amplification. BLAST analysis confirmed that all eight *Ancylostoma* spp. positive samples were *Ancylostoma duodenale*.

Consensus sequences from all eight *A. duodenale* samples together with six sequences representing four *Ancylostoma* species obtained from GenBank and one sequence of *Necator americanus* used as an outgroup, were included in a neighbour-joining analysis (Fig. 1).

3.2. Neighbour-joining analysis of cox1 gene sequences of *A. duodenale*

PCR amplification of cox1 gene sequences was successful for seven out of eight *A. duodenale* samples. One positive sample failed to amplify (AS3) possibly due to insufficient *A. duodenale* DNA for a single-step PCR of the cox1 gene. When a neighbour-joining analysis was performed, the consensus sequences showed very few differences between them. The samples obtained from the participants (migrant workers living in Malaysia) clustered according to the participants country of origin (Nepal and Indonesia) but were more similar to each other than to any of the sequences obtained from GenBank (representing Australia, China and Japan samples) (Fig. 2).

3.3. Combined prevalence of *N. americanus* and *A. duodenale* by risk factors

Overall there was no significant difference between prevalence in males (males = 8.5%) [CL95 = 5.70–11.30] and females (females = 2.3% [CL95 = 0.80–3.80]), but there was a significant effect of age (*χ^2^ = 27.72, P < 0.001). Prevalence was highest in the youngest age class and none of the 39 participants in the oldest age class were infected (for age classes 1–5, prevalence = 6.7%, 2.1%, 2.1%, 0% and 0% respectively). Prevalence did not differ significantly between the five nationality classes. Furthermore, none of the extrinsic factors affected prevalence of hookworms significantly.

3.4. Intrinsic factors associated with *N. americanus* and *A. duodenale* infections

Infections with *N. americanus* and *A. duodenale* were analysed statistically in relation to sociodemographic factors. In the minimum sufficient model identified by the backwards stepwise selection procedure that included sex, age and nationality, only age (*χ^2^ = 19.174, P < 0.001, df = 4) (Table 1) was found to be significantly associated with *N. americanus* infections. Meanwhile, analyses of *A. duodenale* infections did not find any of the three intrinsic factors to be statistically significant (Table 1).

3.5. Extrinsic factors associated with *N. americanus* and *A. duodenale* infections

Of the four extrinsic factors considered (employment sectors, years of residence in Malaysia, type of accommodation and level of education), none of the factors were found significantly affect the prevalence of *N. americanus* infections. However, one factor was found to significantly affect the prevalence of *A. duodenale* infections, i.e. years of residence in Malaysia. Infection was significantly and positively associated with those who recently arrived in Malaysia (*χ^2^ = 5.578, P = 0.018, df = 1).

4. Discussion

Malaysia is classified as an upper middle income country (World Bank, 2016) whose economy has transformed into an emerging multi-sector economy. Since the 1970s, the Malaysian economy has been facilitated largely by imported migrant workers. Malaysia has a higher standard of living compared with most other countries in the South East Asian and South Asian region. Slightly over 10% of the participants (migrant workers living in Malaysia) screened in this study were positive for hookworms which was consistent with previous studies in Malaysia conducted among Orang Asli (Nor Aini et al., 2007; Hakim et al., 2007; Lim et al., 2009; Anuar et al., 2014; Sinniah et al., 2014), rural communities (Sinniah et al., 2014), urban squatters (Sinniah et al., 2014), and aboriginal children (Al-Mekhla et al., 2008), with infection rates ranging between 3.0% to 12.8%.

However, all previous studies employed techniques that were unable to identify the species of hookworm and therefore it is vital to employ molecular methods and sequencing to overcome this limitation (Grujiter et al., 2005). Nonetheless, despite the high sensitivity of molecular methods, we found a small number of microscopically positive samples to be negative by PCR. One possibility is that the eggs observed by microscopy were not from hookworms but other strongyloid nematodes such as *Trichostrongyulus* spp whose DNA is not amplified by the
Fig. 1. A neighbour-joining tree based on partial ITS2-28S rRNA sequences of hookworm species constructed using MEGA6 program. Numbers above branches represent the percentage of 1000 bootstrap pseudo-resampled trees that contained the branch. GenBank accession numbers are provided after species names.

Fig. 2. A phylogenetic tree based on 337 bp partial cox1 gene sequences of A. duodenale. Numbers above branches represent the percentage of 1000 bootstrap replication trees in that branch. Accession numbers indicate sequences from GenBank.
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previous worldwide studies highlighting Americanus primers used. The presence of dominant human hookworm species (Gruijter et al., 2005; Ngui et al., Americanus (87.2%; 41/47) to be the predominant hookworm species, followed by (Romstad et al., 1998). Most studies across Asia have also highlighted infections in eight communities in rural areas of Peninsular Malaysia, suggesting infections originated from the participants' country of origin where infections are endemic due to lack of footwear, poverty, poor hygiene practices and inadequate sanitation services (Rai et al., 1997; Sahimin et al., 2016). Many studies have been conducted in Nepal on the prevalence of intestinal helminths and protozoa among the community (Devleesschauwer et al., 2014) however there are only limited studies identifying the species of hookworm in the region (Rai et al., 1997; Navitsky et al., 1998). In an annual report of hookworm infections (3.8%–10.7%) among residents of Nepal in 1997, a higher predominance of A. duodenale (67.0%) was reported compared to N. americanus (33.0%) (Rai et al., 1997). Likewise, A. duodenale was found to be endemic and highly prevalent among pregnant women in the rural plains of Nepal. All cultured hookworm larvae were identified as A. duodenale (Navitsky et al., 1998). In a study in Indonesia, A. duodenale was less commonly isolated compared to N. americanus (Wibawa and Satoto, 2016), however, mixed infection of A. duodenale and N. americanus were generally reported (Margono, 2003). A recent study in Flores Island, Indonesia, likewise reported a higher prevalence of N. americanus (51.7%) compared to A. duodenale (3.7%) (Wiria et al., 2015).

Infections of hookworm species are important to note because infections with A. duodenale can result in more severe pathological effects and produce intense symptoms with fewer worms compared to N. americanus (Bogitsh and Cheng, 1999). For example, on a per worm basis A. duodenale infections cause approximately five times greater blood loss resulting in a higher degree of iron deficiency than N. americanus infections (Pawlowski et al., 1991).

Transmission of intestinal nematode infections within the community is dependent on human behaviour, particularly during eating and primers used.

Most of the hookworm infections in this study were caused by N. americanus (81%; 35 of 43 infections) and this finding is consistent with previous worldwide studies highlighting N. americanus as the predominant human hookworm species (Gruijter et al., 2005; Ngui et al., 2012a). Sequence comparison with reference sequences in GenBank confirmed that all eight Ancylostoma spp. detected were A. duodenale (100% similarity) and therefore our study is the first to report the presence of A. duodenale in Malaysia.

A previous study in Malaysia by Ngui et al. (2012a) reported N. americanus (87.2%; 41/47) to be the predominant hookworm species, followed by A. ceylanicum (23.4%; 11/47) and reported no A. duodenale infections in eight communities in rural areas of Peninsular Malaysia. Similarly, another species-specific study in Sarawak (East Malaysia) found infections with N. americanus but no A. duodenale was detected (Romstad et al., 1998). Most studies across Asia have also highlighted the higher predominance of N. americanus infections compared to A. duodenale. In southern Thailand, a high predominance of N. americanus (99.9%) was reported among school children infected with hookworms (32.7%; 481/1473) compared to A. duodenale (0.1%) (Anantaphruti et al., 2002). Another study in a rural community in central Thailand recorded 92.0% N. americanus infection compared to 2.0% A. duodenale (Jiraanankul et al., 2011) and a study in northern Vietnam also recorded high predominance of N. americanus (95% of subjects infected with hookworms) infections (Verle et al., 2003). Conversely, a study in a rural area of Laos found a higher prevalence of Ancylostoma spp. infection (9.4%) compared to N. americanus (5.9%), however the species of Ancylostoma was not reported (Sato et al., 2010). In West Bengal, India (Pal et al., 2007) and Hainan Province, China (Gandhi et al., 2001) studies have also reported the predominance of N. americanus with a prevalence of 42.8% and 60.0%, respectively. A recent study from a tribal area in Tamil Nadu, India found the presence of N. americanus (100%), followed by A. caninum (16.8%) and A. duodenale (8.4%) from subjects with positive hookworm samples (83.2%; 119/143) (George et al., 2016).

In the present study, A. duodenale infected workers were from Indonesia and Nepal. Of these, six were newly arrived workers in Malaysia, suggesting infections originated from the participants' country of origin where infections are endemic due to lack of footwear, poverty, poor hygiene practices and inadequate sanitation services (Rai et al., 1997; Sahimin et al., 2016). The prevalence of N. americanus in Malaysia, as reported in the present study, is 87.2% (41/47) compared to 8.4% from subjects with positive hookworm infections (83.2%; 119/143) (George et al., 2016). In the present study, A. duodenale infected workers were from Indonesia and Nepal. Of these, six were newly arrived workers in Malaysia, suggesting infections originated from the participants' country of origin where infections are endemic due to lack of footwear, poverty, poor hygiene practices and inadequate sanitation services (Rai et al., 1997; Sahimin et al., 2016). The prevalence of N. americanus in Malaysia, as reported in the present study, is 87.2% (41/47) compared to 8.4% from subjects with positive hookworm infections (83.2%; 119/143) (George et al., 2016).
deorientation, personal hygiene, cleanliness and most importantly failure to use footwear, particularly when crossing damp, contaminated soil. The high prevalence of parasitic infections among the migrant community sampled in this study provides an insight into the conditions under which the participants live, and reflects the availability of environmental sanitation as well as the socioeconomic status of this sector of the population in Malaysia (World Health Organization, 2015). Although there was a significant effect of age on the prevalence of hookworm infections in this study, the participants were all adults with the youngest being 21 years old. High disease prevalence observed amongst the participants suggests that infections may have been acquired due to the lack of sanitation and clean water in their country of origin and transmission is compounded by behavioural factors such as failure to use footwear, and poor hygiene practice that continue to persist after entry into Malaysia. Therefore, not only is screening necessary but there is a need for workers to be further educated on good hygiene practices and knowledge of disease transmission.

In conclusion, the present study has identified the prevalence of hookworm infections among migrant workers in Malaysia. We have also demonstrated the predominance of *N. americanus* among infected workers and provided the first evidence for *A. duodenale* infections in Malaysia. Our study has identified in the case of *N. americanus* age as a risk factor and in the case of *A. duodenale*, period of residence in Malaysia. Screening of migrant workers, especially using molecular methods as presented here, is strongly recommended particularly for those working in food sectors and related industries.

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### References


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