Short communication

Genetic characterization of the partial mitochondrial cytochrome oxidase c subunit I (cox 1) gene of the zoonotic parasitic nematode, Ancylostoma ceylanicum from humans, dogs and cats

Romano Ngui, Mohammed A.K. Mahdy, Kek Heng Chua, Rebecca Traub, Yvonne A.L. Lim

Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
School of Veterinary Science, The University of Queensland, Gatton, Queensland 4343, Australia
Department of Parasitology, Faculty of Medicine, Sana’a University, Sana’a, Yemen
Research department, University of Science and Technology, Sana’a, Yemen

ABSTRACT

Ancylostoma ceylanicum is the only zoonotic hookworm species that is able to produce patent infections in humans with the majority of cases reported in South East Asia. Over the past few years, there have been an increasing number of studies investigating the prevalence of this parasitic zoonosis using molecular diagnostic tools and a single genetic locus as marker for species identification. As there can be limitations in using a single genetic locus for epidemiological studies and genetic discrimination, the complementary use of a more variable locus will provide additional evidence to support the zoonotic exchange of hookworm species between humans and animals. In the present study, the cytochrome c oxidase subunit 1 (cox 1) sequence of A. ceylanicum from positive human and animal faecal samples were determined and compared with published reference sequences. Phylogenetic analysis demonstrated that isolates of A. ceylanicum were divided into two clusters, one consisting 3 human isolates and the other comprising 19 isolates of human and animal origin from different geographical locations within Malaysia. The two groups of A. ceylanicum could be distinguished from one another through five fixed nucleotide differences at locations 891, 966, 1008, 1077 and 1083. The detection of genetically distinct groups and considerable level of genetic variation within the cox 1 sequence of A. ceylanicum might suggest potential haplotype-linked differences in zoonotic, epidemiological and pathobiological characteristics, a hypothesis that still needs further investigation.

1. Introduction

Hookworms are blood-sucking nematodes of the Ancylostomatidae family that infect both humans and animals. Necator americanus and Ancylostoma duodenale are the two most commonly found hookworm species in humans (Chan et al., 1994). Besides anthropogenic hookworms, humans may also harbor patent infections with Ancylostoma ceylanicum, a zoonotic hookworm that utilize dogs and cats as their natural definitive hosts. Zoonotic ancylostomiasis caused by A. ceylanicum has been reported in many regions of Asia and Southeast Asia (Conlan et al., 2012; Jiraanankul et al., 2011; Ngui et al., 2012a,b; Sato et al., 2010; Traub et al., 2008).

Recently, an influx of studies has investigated the prevalence of this parasitic zoonosis using molecular tools. These advanced tools are mainly used to detect and characterize infection directly from eggs in human and animal feces particularly targeting the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA as the genetic marker for hookworm species identification (Conlan et al., 2012; Jiraanankul et al., 2011; Mahdy et al., 2012; Ngui et al., 2012a,b; Sato et al., 2010; Traub et al., 2008). This locus has a lower mutation rate and is repetitive due to less sequence variation among or between populations, which makes it suitable to be used as a species-specific marker (Hoste et al., 1995; Romstad et al., 1998; Stevenson et al., 1995).

Although the ITS region provides a highly sensitive tool for the detection and differentiation of hookworm DNA at the species level (Traub et al., 2004), it fails to provide sufficient genetic resolution at a sub-species level (Hoste et al., 1995; Romstad et al., 1998; Stevenson et al., 1995). Mitochondrial DNA genes have more
intraspecific sequence variation because of their maternal inheritance and relatively high evolutionary rates (Avise et al., 1987) making them suitable for population genetic studies (Blouin, 2002; Hu et al., 2002). The cytochrome c oxidase subunit 1 (cox 1) gene has been successfully utilized to demonstrate finer intra-species level structure of many strongyloid nematodes, including hookworm (Hu et al., 2002; Hawdon et al., 2001; Zhan et al., 2001).

Our previous study indicated that almost a quarter of hookworm-positive individuals were infected with A. ceylanicum (Ngui et al., 2012a) in communities in which this species of hookworm was highly endemic in dogs (73.9%) and cats (26.1%) (Ngui et al., 2012b). Hookworm species were genetically characterized based on variation of the ITS-2 region of nuclear rDNA in the latter. In this study we provide further genetic discrimination of these isolates of A. ceylanicum harbored by humans, dogs and cats at the cox 1 gene to provide further evidence of zoonotic exchange of hookworm species between humans and animals.

2. Materials and methods

Human, dog and cat fecal samples that were positive for A. ceylanicum based on PCR targeting the second internal transcribed spacer (ITS-2) ribosomal gene as reported previously (Ngui et al., 2012a,b), were used in this study. In brief, the extracted genomic DNA was subjected to polymerase chain reaction (PCR) assay targeting the A. ceylanicum mitochondrial cytochrome oxidase dase c subunit 1 (cox 1) gene using a forward primer JB3 (5'-TTTTTTGGCCATCTCATGTTAT'-3') and a reverse primer JB4.5 (5'-TAAGAAAGAACATAATGAAAATG-3') (Bowles et al., 1992), verified to be sufficiently conserved for hookworm by sequencing (Hu et al., 2002). Positive amplicons generated from each sample were subjected to bidirectional sequencing. The sequences obtained were then manually aligned and the consensus sequence was traced for each sample. Neighbor-Joining (NJ) algorithm with Kimura 2 parameter analysis and maximum parsimony (MP) phylogenetic analysis were conducted using MEGA4 program (Tamura and Kumar, 2002). Details on methodology are provided in the Supplemental Information section.

3. Results

A total of 36 genomic DNA fecal samples extracted from humans, dogs and cats in which A. ceylanicum infection was previously confirmed were successfully amplified by an optimized PCR targeting the cox 1 gene. Of the 36 samples, only 22 samples (i.e., 12 dogs, 8 humans and 2 cats) were successfully sequenced at the cox 1 gene. All sequences from this study representing humans, dogs and cats together with four reference sequences obtained from GenBank database were used to examine the relationship between the A. ceylanicum isolates using Neighbor-Joining (NJ) (Fig. 1) and maximum parsimony (MP) phylogenetic analysis (Fig. 2, provided in the Supplemental Information).

The tree was distinctly separated into 3 clusters with the A. ceylanicum isolates grouped together, and genetically distinct from A. caninum isolates (GenBank accession numbers NC012305 and FJ483518) and A. duodenale isolates (GenBank accession numbers NC003415 and AJ417718). Within A. ceylanicum, the cluster was divided into two clades, one consisting of 3 human isolates, the other comprising of 19 isolates sourced from both humans and animals origin from different geographical locations (Fig. 1 and Supplementary Fig. 2). This separation was strongly supported by bootstrap analysis (99% for both NJ and MP methods). The two groups of A. ceylanicum could be distinguished from each other by five fixed nucleotide differences at locations 891, 966, 1008, 1077 and 1083 with the highest number of nucleotide variations being noted at location 1083 involving adenine, thymine and guanine bases (Table 1, provided in the Supplemental Information).

Of these, four fixed nucleotide differences at locations 966, 1008, 1077 and 1083 were recorded in human strains, could be distinguished from each other based on their geographical locations. Similarly, the A. ceylanicum strains of dogs isolated from different geographical locations could also be distinguished from one another by four fixed nucleotide differences at locations 891, 1008, 1077 and 1083. In cats, the two A. ceylanicum strains from different geographical locations (i.e., Gurney and Pangsun) could be distinguished from one another by two fixed nucleotide differences at locations 891 and 1088. Strains of A. ceylanicum from the same geographical location (i.e., Gurney) isolated from different hosts (i.e., humans and dogs) clustered together within the same group providing evidence to show that strains of A. ceylanicum are being circulated between human and animal hosts within endemic foci of hookworm infection. However, given the small number of A. ceylanicum isolates in the present study, no statistical significance was observed when comparing each strain based on their geographical locations.

All 22 cox 1 sequences in this study were subjected to multiple alignments against the complete mitochondria genome of A. caninum and A. duodenale to investigate the intra-species and inter-species genetic diversity of A. ceylanicum. Altogether 59 different mismatches were recorded within the genus representing transversions (Tv), transitions (Ts) or multiple substitution events (Table 1, provided in the Supplemental Information). Within each A. ceylanicum, A. caninum and A. duodenale species, the percentage of transversions (62.7%; 37/59) was higher than transitions (55.9%; 33/59). However, the Tv:Ts ratio was not statistically significant as assessed by Pearson’s chi-square test ($\chi^2$ test) ($P > 0.05$) (data not shown). Other mismatches were also observed although rarely by comparison, for example G and T (9 Tv) and transversion of A and C (1 Ts). These mismatches at different locations allowed the discrimination between Angylostoma species. For example, mismatches at positions 858, 939, 966, 996, 999 and 1083 separated A. ceylanicum, A. caninum and A. duodenale from each other. Mismatches at locations 729, 738, 819, 822, 837, 858, 867, 909, 912, 918, 924, 945, 948, 969, 975, 981, 987, 1018, 1026, 1036 and 1062 allowed differentiation of A. ceylanicum with A. caninum and A. duodenale.

4. Discussion

In this study, sequences of the cytochrome c oxidase subunit 1 (cox 1) were successfully amplified and sequenced for A. ceylanicum isolates representing strains from different geographical locations and hosts within Malaysia. Of the 36 A. ceylanicum isolates subjected to PCR at the cox 1 gene, only 22 samples were successfully sequenced. Failure of PCR amplification on the 14 samples could be caused by low concentration of parasite DNA due to the low intensity of infection for the respective samples. In addition, it was also thought to be associated with the low sensitivity of the PCR assay. In this study, we used the existing primer set developed by Bowles et al. (1992) and Hu et al. (2002). The reported works did not state the sensitivity of their PCR assays. Thus, it is difficult to estimate the lowest detectable concentration in which parasite DNA can be detected for our study.

Sequence analysis showed that the A. ceylanicum strains could be divided into two distinct groups, one consisting of human isolates whilst the other consisting of a mixture of human and animal isolates from different geographical locations. These two groups of A. ceylanicum had differences at five fixed nucleotide differences in the cox 1 sequence at locations 891, 966, 1008, 1077 and 1083, which could be distinguished from one another. This might suggest that the two groups within A. ceylanicum represent
genetically distinct haplotypes rather than cryptic species. However, this hypothesis requires further statistical investigation given the small number of *A. ceylanicum* isolates in the current work. Similarly, two distinct groups were detected within *A. duodenale* from humans from Zhejiang Province in China, which suggested that the two groups within *A. duodenale* represent genetically distinct subpopulation, rather than cryptic species (Hu et al., 2002). Likewise, study conducted in dogs in Townsville, Australia revealed that two genetically distinct groups were detected within *A. caninum* from these dogs (Prociv and Croese, 1996). Previous morphological and clinical studies in Australia have indicated that *A. caninum* is not only specific to dogs, but also capable of infecting other hosts including cats and humans causing eosinophilic enteritis (Croese et al., 1994; Loukas et al., 1992).

In addition, some of the *A. ceylanicum* strains from both human and animal host within the same geographical location (e.g., Gurney) clustered together within the same group. This provides evidence to show that dogs and humans share genetically similar genotypes of *A. ceylanicum* within the same geographical location. These results add weightage to confirm our findings in the previous study that demonstrated individuals who had close contact with dogs and cats were eleven times more likely to be infected with *A. ceylanicum* (Ngui et al., 2012b). Additionally, zoonotic ancylostomiasis caused by *A. ceylanicum* in humans has also been reported in other South East Asia countries including Thailand (Jiraanankul et al., 2011; Traub et al., 2008) and Laos PDR (Conlan et al., 2012; Sato et al., 2010). Therefore, it is tempting to postulate that certain genetically distinct groups within *A. ceylanicum* can selectively infect non-canine host such as humans. However, further statistical investigation is required to prove this hypothesis since our sample numbers were limited. Perhaps such pattern of variability within *A. ceylanicum* may have arisen due to secondary contact between allopatrically evolved populations or subpopulations in different or non-overlapping geographical areas (Avise et al., 1987), which may have arisen due to host movement from other geographical locations where this species are endemic.

The detection of considerable level of mitochondrial genetic variation within *cox 1* sequence of *A. ceylanicum* might suggest different biological, epidemiological (e.g., transmission) and disease characteristic (i.e., pathogenic effects) linked to different genotypes. However, there is presently no evidence to support this hypothesis, warranting further investigation using other loci and

---

Fig. 1. Phenogram of the *A. ceylanicum* sequences of *cox 1* constructed using Neighbor-Joining (Nj). Sequences from the present study and reference sequences from GenBank are indicated. Figure at nodes represent the degree of bootstrap support based on 1000 bootstrapped trees (Tamura and Kumar, 2002). Only bootstrap values >50% are shown. The isolates were labeled according to the host and geographical locations. All sequences generated in this study were deposited and available in the NCBI, GenBank (accession numbers: KC247727 to KC247745).
further isolates as genetic markers. In addition, further studies investigating the epidemiology, transmission dynamics and clinical significance of *A. ceylanicum* in an endemic community with hookworm disease will be beneficial in unraveling the true significance of this zoonosis in humans. As all the *A. ceylanicum* strains in human were isolated from asymptomatic individuals (Ngui et al., 2012b), it would also be of particular interest to compare the genetic make-up of *A. ceylanicum* in symptomatic human infected with this zoonotic species with those from dogs or cats from the same geographical areas. In conclusion, the detection of genetically distinct groups and considerable level of genetic variation within the *cox 1* sequence of *A. ceylanicum* as reported in the present study might suggest potential haplotype-linked differences in zoonotic, epidemiological and pathobiological characteristics, a hypothesis that still need further investigation. Addressing these questions should offer valuable insight into aspects of *A. ceylanicum* or other hookworm transmission, thus having implication on how disease control measures can be instituted.

**Conflict of interest**

The authors have no conflict of interest concerning the work reported in this paper.

**Acknowledgements**

The authors would like to sincerely thank Prof. Robin Gasser from University of Melbourne for his critical input and counsel during the initial preparation of this manuscript. This study was funded by the E-Science (16-02-03-6034) and UM/MoHE High Impact Research (H-20001-00-E000061) grants.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2013.06.003.

**References**


