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Molecular species identification of *Trichuris trichiura* in African green monkey on St. Kitts, West Indies

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ABSTRACT

The population of African green monkeys (AGM, *Chlorocebus aethiops sabaeus*) on St. Kitts, West Indies is believed to be as large as or greater than the human population. Interactions with humans are frequent and the pathogens carried by AGM, such as *Trichuris* spp., may pose a risk to humans. The objectives of this study were to assess the use of molecular methods for diagnosing *Trichuris* spp. in AGM and compare its DNA sequences to those of *Trichuris* spp. found in other non-human primates and humans. Fecal samples were collected from trapped and individually housed AGM between January and December 2015 and analysed using fecal flotation with Sheather's sugar flotation solution and PCR amplification and DNA sequencing of 18S rRNA and ITS2 fragments. Phylogenetic analysis was performed. 91% (81/89) and 55.4% (31/56) were *Trichuris* spp. positive by fecal flotation and PCR, respectively. Both AGM-NADH1 gene and *T. trichiura*-18S rRNA gene showed no variations in sequence and were 100% identical to corresponding sequences deposited in GenBank. Nevertheless *Trichuris* ITS2 showed some diversities among 12 sequences, which was < 5%. Phylogenetic analysis of ITS2 put *Trichuris* spp. in Kittitian AGM into the same clades of *T. trichiura* found in human and other non-human primates in many other geographical regions. These data confirm that AGM are reservoirs for *T. trichiura* in humans. We suggest a one health approach to curtail enteric parasitic infections in human populations in the insular country.

1. Introduction

African green monkeys (AGM, *Chlorocebus aethiops sabaeus*), introduced into St. Kitts during the 17th century, occupy a unique insular ecological niche. St. Kitts, which is encircled by the Caribbean and Atlantic Oceans at 17°20′N62°45′W, is slightly under 69 mile^2 (Kittitian Government, 2017). With an estimated population density of 250–500 AGM per square mile, AGM come into contact with humans frequently by their routine visit to outdoor dining areas, home gardens and agricultural land. Also, some are kept as pets and used in the tourism industry as an attraction and for tourists to hold and be photographed with. It is highly possible that pathogens including enteric parasites such as *Trichuris* spp. (whipworms) are shared and transmitted between humans and AGM.

Trichuris trichiura is one of the most common soil-transmitted helminth (STH) species harbored by one billion people worldwide (Stephenson et al., 2000; World Health Organization, 2013). Heavy *T. trichiura* infections may lead to *Trichuris* dysentery syndrome which includes chronic dysentery, rectal prolapse, anemia, and poor growth among children (Stephenson et al., 2000).

In general, the helminthic species in the genus *Trichuris* have strong host specificity. Humans, dogs and swine are the definitive hosts of *T. trichiura, T. vulpis* and *T. suis*, respectively. Although human infections with *T. vulpis* and *T. suis* have been documented, they are rare (Dunn et al., 2002; Kenney and Eveland, 1978; Kenney and Yermakov, 1980; Marquez-Navarro et al., 2012; Nissen et al., 2012; Singh et al., 1993). *Trichuris* spp. in non-human primates (NHP) have been a topic of debate. Do they carry the human whipworm *T. trichiura* or their own species? Ghai and colleagues had analysed internal transcribed spacer (ITS) 1 sequences from *Trichuris* spp. originated from humans, chimpanzee (*Pan troglodytes*) and seven diurnal monkey species in western Uganda. The latter included black-and-white colobus (*Colobus guereza*), blue monkeys (*Cercopithecus mitis*), grey-cheeked mangabeys (*Lophocebus albigena*), l'hoest monkeys (*Cercopithecus lhoesti*), olive baboons

Abbreviations: AGM, African green monkeys; BSF, Behavioural Science Foundation; EPG, eggs per gram of feces; STH, soil-transmitted helminth; IACUC, Institutional Animal Care and Use Committee; ITS, internal transcribed spacer; NHP, non-human primates; RUSVM, Ross University School of Veterinary Medicine; SNP, single nucleotide polymorphism

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(*Papio anubis*), red colobus (*Procolobus rufomitratus*), and red-tailed guenons (*Cercopithecus ascanius*). They found three co-circulating *Trichuris* groups, of which one was detected only in humans and another was circulating among all screened NHP and humans (Ghai et al., 2014). South African chacma baboons (*Papio ursinus*) harbored two distinct *Trichuris* genotypes that were also found in humans in various geographic regions as revealed by ITS1-5.8S-ITS2 (Ravasi et al., 2012). ITS sequences derived from fecal eggs isolated from NHP (*Colobus guereza kikuyensis* and *Nomascus* gabriellae) that had been cultured and developed to the infectious eggs were almost identical to each other, and were clearly different from that of *T. suis* (Cutillas et al., 2009). Another study using ITS of adult whipworms recovered from captive Japanese macaques (*Macaca fuscata*) and grivets (*Chlorocebus aethiops*) found that specimens from *M. fuscata* may be distinct from, but related to, *T. trichiura*, whereas a close relationship was suggested between the subclade formed by the specimens from *C. aethiops* and the subclade formed by *T. suis* (Cavallero et al., 2015). In an analysis of mitochondrial genome Hawash and colleagues found a complex of cryptic species harbored by NHP with some species being able to infect both humans and NHP. NHP used in their analysis included the François' leaf-monkey in China, the olive baboon in USA, and the hamadryas baboon in Denmark (Hawash et al., 2015). Liu and colleagues concluded that the François' leaf-monkey (langur)-derived whipworm was a separate species from that of humans by comparison of the complete mitochondrial genome and ITS-1 and ITS-2 of *Trichuris* spp. (Liu et al., 2013). Indeed, two new species from NHP have been recently described. They are *T. colobae* and *T. ursinus* isolated from *Colobus guereza kikuyensis* and *Papio ursinus Keer*, respectively (Callejón et al., 2017; Cutillas et al., 2014).

Many NHP harbor *Trichuris* spp. making them a reservoir for human whipworm infections if the species and strain are the same. AGM population on St. Kitts is equal to the number of human residents. They frequently contact humans as mentioned earlier. These unique features render investigation of *Trichuris* spp. in AGM a necessary component for understanding *Trichuris* epidemiology. The major aim of the current study was to characterize *Trichuris* spp. in AGM using molecular techniques and phylogenetic analysis. Our data confirmed that *Trichuris* spp. harbored by AGM were the same species as *T. trichiura* in human.

2. Materials and methods

2.1. Ethics

All animal related procedures were performed under standard operating procedures or protocols approved by the Behavioural Science Foundation (BSF) Institutional Animal Care and Use Committee (IACUC) or the Ross University School of Veterinary Medicine (RUSVM) IACUC. All samples were collected from AGM undergoing procedures for other studies with no AGM trapped, sedated or euthanized specifically for this study.

2.2. Sample collection and fecal egg counts

Fecal samples were collected from 70 AGM that were trapped throughout St. Kitts and individually housed at BSF between January and December 2015. Fecal samples were placed into a cooler with ice packs immediately after collection and transferred to RUSVM the same day. Samples were refrigerated (2–6 °C) until fecal flotation analysis (within 7 days). The remaining feces was frozen (− 20 °C or − 80 °C) until DNA extraction.

Fecal samples and adult *Trichuris* spp. worms also were collected from 19 AGM that were euthanized as part of other studies. After all procedures were completed for which euthanasia was required, the large intestine was removed. Feces were collected from the distal end and preserved as previously described. The intestine was then opened and soaked in saline for 2–3 h. The saline and intestinal contents were poured over a ≤ 100 μm sieve and the intestine was gently washed over the sieve. Adult worms were collected from the sieve and the number of adult worms recorded for each AGM.

Fecal samples were analysed using flotation by double centrifugation with Sheather's sugar flotation solution (specific gravity 1.27–1.28) (Zajac and Conboy, 2012). One or two grams of feces were used and all *Trichuris* spp. eggs counted to determine the eggs per gram of feces (EPG).

2.3. DNA extraction and quantification

Total DNA was individually extracted from fecal samples using the QIAamp DNA Stool Kit (QIAGEN, Hilden, Germany) by following the supplier's protocol with an initial incubation temperature of 95 °C to break the hardy eggs of *Trichuris* spp. Approximately 0.2 g of feces of each sample were used during DNA extraction. Final elution of DNA was performed in 30 μl elution buffer.

DNA concentration and quality were assayed using the Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland). DNA was stored at − 20 °C until use. Various volumes of DNA solution ranging from 1 μl to 20 μl were used as templates in a final volume of 25 μl for each PCR depending upon individual DNA concentrations. Samples with a DNA concentration lower than 5 ng/μl were not analysed by PCR. A minimum of 100 ng DNA template was used for each PCR reaction. PCR reagents used included HotStart Taq Plus 2 × Master Mix (QIAGEN) for samples with a DNA concentration ≥ 10 ng/μl, and *Taq* DNA polymerase (TaKaRa, Clontech, CA, USA) for samples with a DNA concentration between 5 and 10 ng/μl. All primers were synthesized by IDT (IA, USA) and used in a final concentration of 1 μM in PCR. They were: a) AGM-NADH1, forward-5′CGCCCTAATGGAGCCCTACGAGCCG3′, reverse-5′TAGAAAGATTGTAGTTGTTAGGGCG3′ with an expected size of amplicon 348 bp (Compton et al., 2012); b) *Trichuris* sp.-18S rRNA, forward-5′CTGCGAGGATTGACAGATCA3′, reverse-5′GTACAAAGGGC AGGGACGTA3′, 498 bp (Phuphisut et al., 2014); c) *Trichuris* sp.-ITS2, forward-5′CTCGTAGGTCGTTGAAGAAC3′, reverse-5′TTAGTTTCTTTTC CTCCGCT3', ~680 bp (Nissen et al., 2012). AGM-NADH1 was used as a quality control to rule out that inhibitors in the feces interfered with PCR amplification. The samples were rerun if no NADH1 fragment was produced. The samples were deemed unsuitable due to PCR inhibitors and excluded from data analysis if the rerun was repeatedly negative.

PCR was performed in a thermal cycler (Mastercycler Nexus Gradient, Eppendorf, NY, USA) for 35 cycles of 95 °C for 30 s, 55 °C (primer pairs a and b) or 50 °C (primer pair c) for 1 min and 72 °C for 2 min with a final extension at 72 °C for 10 min following initial 95 °C for 2 min. The amplicons were visualized via electrophoresis in a 1.2% agarose gel. DNA fragments of expected sizes were cut out from agarose gel and eluted using QIAquick Gel Extraction Kit or directly extracted with the QIAquick PCR Purification Kit (QIAGEN). The purified PCR products were directly sequenced in both directions using the same PCR primers (Macrogen, Korea).

2.4. DNA sequences

Nucleotide sequence data reported in this paper are available in the GenBank™ databases under the accession numbers: KX961633-KX961653.

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the free software MEGA (Version 5.2.2) (MEGA, 2017; Tamura et al., 2011). Both Maximum Likelihood Tree and Neighbor Joining Tree were performed under 1000 bootstrap replications with complete deletion of gabs. Both yielded the same results. However only the Maximum Likelihood Tree was presented.