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# Trichuriasis in Human Patients from Côte d'Ivoire Caused by Novel Species *Trichuris incognita* with Low Sensitivity to Albendazole/Ivermectin Combination **Treatment**

## **Appendix**

### **DNA Extraction Protocol for Fecal Samples**

#### **Washing**

1) Aliquot 500  $\mu$ L (250  $\mu$ L, 2×) of homogenized fecal sample into the PowerBead Pro tube (from PowerFecal Pro Kit). You should first remove the beads from the bead tube and transfer them to another tube before this step. (Aliquoting the sample in  $2 \times 250 \mu L$  volume ensures adequate homogenization and capture of as much feces as possible in the total 500  $\mu$ L of starting material).

2) To the sample (500  $\mu$ L) in the PowerBead Pro tube, add 1 mL molecular grade water, briefly vortex and spin in the mini centrifuge at 13,400 rpm ( $\approx$ 12,000  $\times$  *g*) for 5 minutes by using an Eppendorf Mini Spin Centrifuge.

3) Discard 500  $\mu$ L of supernatant and add 500  $\mu$ L ddH<sub>2</sub>O. Spin again for the same amount of time and at the same speed.

4) Discard 1,000 µL of supernatant from the previous step. Do not add more water. Spin again for 2 minutes at the same speed.

5) After spinning, carefully discard ≈250–300 µL of supernatant, depending on the nature and volume of your pellet.

6) After washing, you should now have a final volume of 200–250 µL of feces in water in your tube. Add 500  $\mu$ L of CD1 solution (PowerFecal Pro Kit), transfer the beads back to this tube, vortex well and carry out the optimization steps.

#### **Mechanical Disruption Steps,** ×**3**

1) Snap freeze the tubes by dipping them in liquid nitrogen for ≈10 seconds or until they are completely frozen.

2) Heat at 100°C at 750 rpm for 15 minutes in a thermoblock.

3) Use the Mini Bead Beater (MBB 96) to carry out bead beating for 3 minutes. Vortex after this step.

4) Repeat steps 1 through 3 three times.

#### **DNA Extraction using the Powerfecal Pro Kit**

1) After optimization, add the remaining 300 µL of CD1 solution, bringing the total volume of CD1 solution to  $800 \mu L$ . (The sample after optimization becomes very frothy, be careful while opening the caps and adding the remaining volume of CD1)

2) Secure the PowerBead Pro Tube horizontally on a Vortex Adaptor and vortex at maximum speed for 10 minutes.

3) Centrifuge the PowerBead Pro Tube at  $15,000 \times g$  for 2 minutes (centrifugation time changed compared with actual protocol).

4) Transfer the supernatant to a clean 2 mL microcentrifuge tube (provided). Transfer ≈**800–850** µ**L of supernatant** (depending on the nature and volume of the pellet).

5) Add 200 µL of Solution CD2 and vortex for 5 seconds.

6) Centrifuge at  $15,000 \times g$  for 1 minute. Avoiding the pellet, transfer up to **800 µL of** 

**supernatant** (depending on the volume of your pellet) to a clean 2 mL microcentrifuge tube (provided).

7) Add 600 µL of Solution CD3 and vortex for 5 seconds.

8) Load 650  $\mu$ L of the lysate onto an MB Spin Column and centrifuge at 15,000  $\times$  *g* for 1 minute.

9) Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.

10) Carefully place the MB Spin Column into a clean 2 ml collection tube (provided). Avoid splashing any flow-through onto the MB Spin Column.

11) Add 500  $\mu$ L of Solution EA to the MB Spin Column. Centrifuge at 15,000  $\times$  *g* for 1 min.

12) Discard the flow-through and place the MB Spin Column back into the same 2 mL Collection Tube.

13) Add 500  $\mu$ L of Solution C5 to the MB Spin Column. Centrifuge at 15,000  $\times g$  for 1 minute.

14) Discard the flow-through and place the MB Spin Column into a new 2 mL collection tube (provided).

15) Centrifuge at up to  $15,000 \times g$  for 2 minutes. Carefully place the MB Spin Column into a new 1.5 mL elution tube (provided).

16) Add 100 µL of Solution C6 to the center of the white filter membrane. **Incubate at room temperature for 1 minute.**

17) Centrifuge at  $15,000 \times g$  for 1 minute. Discard the MB Spin Column. Store the DNA at  $-20$ °C.

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\*Fecal egg counts were calculated by using quadruplicate Kato-Katz analyses for each sample. EPG, eggs per gram; ID, identification, Lao PDR, Lao People's Democratic Republic.

**Appendix Table 2.** Primers used for fecal DNA metabarcoding of *Trichuris* spp. loci



**Appendix Table 3.** *Trichuris* spp. sequences from GenBank used for phylogenetic analyses of ribosomal and mitochondrial loci\*





#### **Appendix Table 4.** Genetic diversity of the rDNA loci



\*Number of samples sequenced. ASV, amplicon sequence variant; Hd, ASV heterozygosity; ITS-1, internal transcribed spacer 1; ITS-2, internal transcribed spacer 2; π, nucleotide diversity.

**Appendix Table 5.** Average pairwise nucleotide identity between *Trichuris* sp. from *Colobus* monkeys (n = 2), *T. suis* (n = 3), and *T. incognita* (n = 8) clades

	Nucleotide identity for cox-1. %			Nucleotide identity for all protein coding genes, %		
	Trichuris sp. Colobus			Trichuris sp., Colobus		
<b>Species</b>	monkey	T. suis	T. incognita	monkey	T. suis	incoanita
Trichuris sp., Colobus	$99.6*$	77.8	80.2	$98.1*$	71.6	73.1
monkey						
T. suis	77.8	$93.1*$	78.9	71.6	91.7	71.7
T. incognita	80.2	78.9	$99.4*$	73.1	71.7	$94*$

\*Indicates average pairwise nucleotide identity between samples of the same species/clade.



**Appendix Figure 1.** Gel electrophoresis results from PCR amplicon generation. Gel electrophoresis of PCR-amplified ITS-1, ITS-2 and mitochondrial genes *cox-1, nad-1,* and *nad-4* from patient fecal samples collected from Côte d'Ivoire, Laos, and Pemba Island, Tanzania. Species-specific primers designed by using *T. trichiura* reference sequences (Genbank accession nos. NC\_017750, GU385218, AP017704, and KT449825) successfully generated 32 *cox-1*, 35 *nad-1*, and 36 *nad-4* amplicons from samples collected in Laos and 29 *cox-1*, 28 *nad-1*, and 19 *nad-4* amplicons from samples collected in Pemba Island. No amplicons were generated from any of the 22 samples collected in Côte d'Ivoire. ITS-1, internal transcribed spacer 1; ITS-2, internal transcribed spacer 2.



**Appendix Figure 2.** Read depth and frequency of *Trichuris* spp. ASVs of *cox-1, nad-1,* and *nad-4* genes from patient fecal samples collected from Laos, and Pemba Island, Tanzania. A–C) ASVs were identified by amplicon sequencing of *cox-1* (A), *nad-1* (B), and *nad-4* (C) gene markers. Histograms indicate read depths of each gene sequence. Bar graphs indicate relative frequencies of different ASVs of *Trichuris* mitochondrial markers amplified from each region. Lower bar plots represent the relative abundance of the ASVs present in each sample from Laos and Pemba . Metabarcoding data were generated from 27 (Laos) and 25 (Pemba) samples for the 430-bp *cox-1* amplicon, from 32 (Laos) and 27(Pemba) samples for the 470-bp *nad-1* amplicon, and from 28 (Laos) and 12 (Pemba) samples for the 446-bp *nad-4* amplicon. Paired-end reads from each of the 3 gene markers were merged and concatenated to produce average mapped read depths of 10,300 (range 1,355–32,600) reads for *cox-1*, 26,490 (range 10,791– 56,330) reads for *nad-1*, and 36,350 (range 21,936–55,346) reads for *nad-4*.



**Appendix Figure 3.** Alpha diversity at the mitochondrial DNA loci. A–C) Shannon-Wiener Index values, were determined for *Trichuris* spp. *cox-1* (A), *nad-1* (B), and *nad-4* (C) amplicon sequence variants generated by sequencing fecal sample DNA from patients in Laos, and Pemba Island, Tanzania. Horizontal lines within boxes indicate medians; box tops and bottoms indicate upper (third) and lower (first) quartiles; error bars (whiskers) indicate minimum and maximum values. Pairwise *t*-tests were used to calculate p values. D) Genetic diversity observed at the 3 mitochondrial loci. ASV, amplicon sequence variant; N, number of samples; NASV, number of ASVs; Hd, ASV heterozygosity; π, nucleotide diversity.



**Appendix Figure 4.** Maximum-likelihood phylogenetic trees of mitochondrial *cox-1, nad-1,* and *nad-4* amplicon sequence variants (ASVs) from patient fecal samples collected in Laos and Pemba Island, Tanzania. Trees indicate *cox-1* (A), *nad-1* (B), and *nad-4* (C) ASVs amplified from fecal samples from patients in Laos and Pemba Island as well as additional *Trichuris* reference sequences from pigs, humans, and nonhuman primates in GenBank. Gamma-distributed rate variation, general time reversible model was chosen for the mitochondrial markers. *Trichinella spiralis* (GenBank accession: no. NC002681) was used as the outgroup. Each tip of the tree is an ASV or a sequence from GenBank, and the color represents the geographic region in which the ASV was found. Trees not to scale.



**Appendix Figure 5.** Multidimensional metric analysis indicating beta diversity of *cox-1, nad-1,* and *nad-4* amplicon sequence variant (ASV) data generated by amplicon sequencing of patient fecal DNA samples from Laos and Pemba Island, Tanzania. A–C) Principal coordinate analysis plots of mitochondrial *cox-1* (A)*, nad-1* (B)*,* and *nad-4* (C) loci from samples collected from Laos and Pemba Island. Multidimensional metric analysis was performed by using the Bray-Curtis dissimilarity matrix, which calculates distances according to the abundance of ASVs, and the Jaccard index, which calculates distances according to the presence or absence of ASVs. Each point is an individual patient sample, and the points are colored according to the geographic region in which the samples were collected. PCo, principal coordinates.



**Appendix Figure 6.** Haplotype network of *Trichuris* amplicon sequence variants (ASVs) of mitochondrial markers generated by amplicon sequencing of patient fecal samples from Laos and Pemba Island, Tanzania. A–C) Statistical parsimony haplotype networks are indicated for mitochondrial *cox-1* (A), *nad-1* (B), and *nad-4* (C) ASVs. Colored circles indicate the region, and size of each circle indicates the ASV frequency. Numbers on connecting lines indicate the number of nucleotide differences between adjacent haplotypes. Haplotype networks show the major high frequency ASVs differed between the 2 regions for all 3 markers and most low-frequency ASVs were unique to either region.



**Appendix Figure 7.** Sequence alignments of ITS-1 amplicon sequence variants from patient fecal samples collected in Côte d'Ivoire with whole-genome sequences from 8 adult worms obtained through an expulsion study. A) Multiple sequence alignments of the 406-bp ribosomal internal transcribed spacer (ITS) 1 sequences generated from the *Trichuris* population in Côte d'Ivoire by using amplicon sequencing of fecal samples and ITS-1 sequences extracted after whole-genome sequencing of adult worms. Dissimilarities are highlighted by black lines or different colors. B) Distance matrix displaying the percentage identity among the ITS-1 sequences.

# A Consensu<br>1. HT\_002<br>2. HT\_003<br>3. HT\_010<br>4. Sample!<br>5. Sample!<br>7. Sample!<br>9. Sample!<br>9. Sample!<br>10. Sample!

B



**Appendix Figure 8.** Sequence alignments of ITS-2 amplicon sequence variants from patient fecal samples collected in Côte d'Ivoire with whole-genome sequences from 8 adult worms obtained through an expulsion study. A) Multiple sequence alignments of the 424-bp ribosomal internal transcribed spacer (ITS) 2 sequences generated from the *Trichuris* population in Côte d'Ivoire by using amplicon sequencing of fecal samples and ITS-2 sequences extracted after whole-genome sequencing of adult worms. Dissimilarities are highlighted by black lines or different colors. B) Distance matrix displaying the percentage identity among the ITS-2 sequences.