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Short communication

# Molecular features of hookworm larvae (*Necator* spp.) raised by coproculture from Ugandan chimpanzees and Gabonese gorillas and humans

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ABSTRACT

Species composition of *Necator* hookworms was surveyed in (i) Ugandan chimpanzees living around farms and villages at Bulindi, (ii) Gabonese gorillas under habituation in Moukalaba-Doudou National Park (MDNP), and (iii) Gabonese villagers living adjacent to MDNP. Internal transcribed spacers (ITS) of rDNA and partial cytochrome *c* oxidase subunit 1 (*Cox*1) gene of mtDNA were analyzed from larvae obtained by coproculture. Three ITS types (I, II and III) and three *Cox*1 haplotype groups (A, B and C) were demonstrated. ITS type I and *Cox*1 haplotype group A, representing *Necator americanus*, were demonstrated in the hookworm larvae from Gabonese gorillas and humans, but not from Ugandan chimpanzees. Type II and haplotype groups B and C, presumably representing *N. gorillae*, were found in larvae from Ugandan chimpanzees and Gabonese gorillas and humans. These features were overall similar with those found previously in the Central African Republic. Meanwhile, type III was proven in a larva from a Gabonese gorilla as the first demonstration from a non-human primate. *Cox*1 haplotypes obtained from Ugandan chimpanzees formed a subgroup within group B, presumably reflecting dispersal and diversification processes of the apes.



Hookworms are major blood-sucking nematode parasites of humans and other mammals causing anemia that is sometimes fatal [1]. It is estimated that 740 million people are infected globally, including a high prevalence in sub-Saharan Africa [1]. *Necator americanus* is the predominant species of hookworm infecting humans today [1]. Besides humans, *Necator* hookworms are also known to infect some non-human primates including great apes, and some non-primate mammals [2]. All great ape species and subspecies are classified as Endangered or Critically Endangered [3]. However, they increasingly inhabit disturbed environments in close proximity to humans [4]. The infective larvae of *Necator* infect by skin penetration when a host walks on contaminated soil or through grasses to which the larvae are clinging [1]. Thus, enhanced conditions for zoonotic transmission of *Necator* have potential consequences for both great ape conservation and public health.

In 2014, it was proven that *N. americanus* and a second species, referred to as '*Necator* sp.', are shared by humans and western lowland gorillas (*Gorilla gorilla gorilla*) in tropical rainforest in the Dzanga-Sangha Protected Areas (DSPA), Central African Republic (CAR) [2]. Recently, a morphological study of hookworms expelled from researchers, who had participated in gorilla surveys in DSPA, demonstrated *Necator gorillae* besides *N. americanus* [5], though molecular comparisons of adult worms are still underway. It was strongly suggested that the great apes could be a reservoir of *Necator* hookworms for humans and vice versa in DSPA [2]. Presumably, a similar situation may occur in other African sites, where humans and great apes share habitats. Although undetermined *Necator* species have been demonstrated by DNA analysis from chimpanzees at Kibale National Park, Uganda [6,7], and bonobos in Bolobo Territory, Democratic Republic of Congo [8], transmission to humans has not yet been proven. In this study, we

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aimed to identify the species composition of *Necator* hookworms among East African chimpanzees (*Pan troglodytes schweinfurthii*) in Bulindi, Uganda, and western lowland gorillas in Moukalaba-Doudou National Park (MDNP), Gabon. Nineteen human inhabitants living adjacent to MDNP were also examined; however, it was not possible to sample humans from Bulindi in the present study.

Bulindi (1°28′N, 31°28′E) is situated in a region of notably close spatial overlap and contact between wild chimpanzees and local humans [9,10]. Here, chimpanzees inhabit small, degraded forest fragments amid farmland and villages [11,12]. The study group comprised 19 individuals during the sampling period. They are sympatric with four other diurnal non-human primates: olive baboons (*Papio anubis*), tantalus monkeys (*Chlorocebus tantalus*), black and white colobus monkeys (*Colobus guereza*) and blue monkeys (*Cercopithecus mitis*).

MDNP (2°26′S, 10°25′E) is composed of a mosaic of forest, savanna, and swamp. It is inhabited by western lowland gorillas and Central African chimpanzees (*P. t. troglodytes*). A habituation program for one group of gorillas, numbering 20 individuals during the sampling period, has been carried out since 2005 [13]. The Moukalaba River provides a natural barrier between local villages and the home ranges of great apes. Besides great apes, nine species of primates, i.e., mandrill (*Mandrillus sphinx*), grey-cheeked mangabey (*Lophocebus albigena*), northern talapoin (*Miopithecus ogouensis*), red-capped mangabey (*Cercocebus torquatus*), crowned monkey (*Cercopithecus pogonias*), putty-nosed monkey (*Cercopithecus nictitans*), moustached monkey (*Cercopithecus cephus*), elegant needle-clawed galago (*Euoticus elegantulus*) and lesser galagos (*Galago* spp.), inhabit the park [14].

Fecal samples from great apes were collected non-invasively as reported previously for Bulindi [15] and MDNP [16]. Fecal samples from farmers of the western Bantu language group living near MDNP were also obtained [16]. They had no direct contact with gorillas, but often entered the forest [17]. All feces collected were subjected to the modified Harada-Mori filter paper culture [18]. After 7–14 days, the bottom water was checked with a magnifying glass, and larva-positive water was transferred to a 5 mL serum tube using a disposable plastic pipette, fixed and stored in >99% ethanol in Uganda [15] and 80% ethanol in Gabon [16].

Methods of DNA extraction, amplification and sequencing followed those employed previously [2,15]. Internal transcribed spacers (ITS) of rDNA and partial cytochrome *c* oxidase subunit 1 (*Cox*1) gene of mtDNA were sequenced. Types of ITS and *Cox*1 sequences were determined according to those reported previously [2]. Phylogenetic analysis was made for *Cox*1 nucleotide and amino acid sequences by neighbor-joining (NJ) and maximum-likelihood (ML) methods, using MEGA5 (v. 5.03) [19] as employed previously [2,15]. The nucleotide sequences determined were registered in DDBJ/EMBL-Bank/GenBank with accession numbers LC088261 to LC088302 (rDNA and ITS) and LC088303 to LC088321 (*Cox*1).

Twenty-five (65.8%) of 38 cultures and 4 (22.2%) of 18 cultures made from feces of chimpanzees in Bulindi and gorillas in MDNP, respectively, were positive for *Necator* larvae. Eleven (57.9%) of 19 cultures made from feces of Gabonese villagers were hookworm-positive [16], but only four *Necator*-positive cultures each with a few larvae were available for the present study. Thirty-four larvae from Ugandan chimpanzees and 23 larvae from Gabonese gorillas were selected randomly and subjected to DNA sequencing (Table 1). Only three larvae from two Gabonese villagers responded to DNA amplification (Table 1).

Sequencing was often difficult, especially in the middle area of ITS1 of type II (=*Necator* sp.) where many repetitions of TG appear, giving incomplete sequences. Full lengths of both ITS1 and ITS2 were successfully sequenced in only four and one larvae from Ugandan chimpanzees and Gabonese gorillas, respectively. However, typing of the incomplete sequences was possible based on characteristic indels (Table 1, Supplemental Fig. 1). Only type II sequences were found from Ugandan chimpanzees, whereas all three types were demonstrated from Gabonese gorillas (Table 1). This is the first record of type III (also assigned to *Necator* sp. [2]) from a non-human primate. Types I (=*N. americanus*) and II sequences were found in the larvae from Gabonese humans (Table 1). The type I sequence obtained from one Gabonese gorilla was identical with those reported previously from DSPA humans and gorillas (Supplemental Figs. 1, 2). The type II sequences obtained were mostly identical with that reported from DSPA [2], but some had a few substitutions (Supplemental Fig. 1). The type III sequence differed slightly from those reported in DSPA by having one to three substitutions (Supplemental Fig. 1).

The larvae from Gabonese gorillas reacted effectively to the primers tested for *Cox*1 amplification, and gave sequences with 603 bp or more (Table 1). Meanwhile, most larvae from Bulindi chimpanzees did not respond to the primers, and only two short sequences with 245 bp and 589 bp, respectively, were obtained (Table 1). NJ analyses on the two sets of *Cox*1 sequences each with 254 and 589 bp, respectively, resulted in phylogenetic trees of largely similar topology, giving three clear haplotype groups (Fig. 1). Isolates from Gabonese gorillas were scattered in the three groups. The isolates from Ugandan chimpanzees were in group B, but diverged at a more basal position than those of Gabonese and DSPA isolates. Presumably, chimpanzee-parasitic *Necator* sp. in East Africa forms a subgroup, reflecting dispersal and diversification processes of the apes. NJ and ML analyses on amino acid converted sequences from the longer sequences did not give trees with branch bootstrap support within *Necator* spp. above 52%. Among 11 larvae in which both ITS and *Cox*1 were successfully sequenced, only three combinations, I-A, II-B and II-C, were demonstrated (Table 2).

Local villagers in Bulindi sometimes defecate outdoors at the edges of agricultural fields and inside the forest [20]. Because the villagers are strongly presumed to harbor *N. americanus*, the chimpanzees could be exposed to the hookworm larvae, especially when foraging or travelling on agricultural land [9,11]. Hence, absence of *N. americanus* in Bulindi samples was unexpected. Interestingly, *N. americanus* was also not found from three chimpanzees examined in DSPA [2]. This may imply that chimpanzees are less susceptible to *N. americanus* in natural environments.

*Necator* sp. (presumably *N. gorillae* [5]) has zoonotic potential because it was demonstrated from humans, gorillas and chimpanzees in the present study, and in the same host species in the previous study in DSPA [2]. In Bulindi, chimpanzees defecate in agricultural fields and near human homes when travelling between forest patches and foraging on agricultural crops [20]. Bulindi villagers most likely also harbor this hookworm species. However, extensive parasitological surveys of local people are necessary to demonstrate this zoonotic infection.

**Table 1**
Number of *Necator* larvae analyzed and their ITS and *Cox*1 types.

| Locality | Host | No. fecal cultures utilized | No. larvae analyzed | ITS1 | ITS2 | Both ITS1 & ITS2 | ITS type | | | *Cox*1 | *Cox*1 haplo type group | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | I | II | III | | A | B | C |
| Bulindi, Uganda | Chimpan-zee | 12 | 34 | 30 (7)[a] | 30 (13) | 26 (4) | | 34 | | 2 | | 2 | |
| Moukalaba-Doudou, Gabon | Gorilla | 5 | 23 | 14 (3) | 3 (3) | 3 (1) | 3 | 10 | 1 | 17 | 5 | 6 | 6 |
| | Human | 2 | 3 | 3 | | | 2 | 1 | | | | | |

[a] Whole length sequenced.