Annals of Tropical Medicine & Parasitology, Vol. 99, No. 5, 511–517 (2005)

Differentiating *Dracunculus medinensis* from *D. insignis*, by the sequence analysis of the 18S rRNA gene

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Received 15 February 2005, Revised 9 May 2005, Accepted 10 May 2005

This study, undertaken as a component of the global Dracunculiasis Eradication Program (DEP), was designed to provide molecular tools to distinguish *Dracunculus medinensis*, the nematode causing human dracunculiasis, from other tissue-dwelling nematodes, including other *Dracunculus* species that infect humans and other animals. DNA was extracted from *D. medinensis* and from a closely related species that infects North American carnivores, *D. insignis*, so that the genes coding for the small-subunit ribosomal RNA (18S rRNA) of the parasites could be amplified, sequenced and compared. Sequences were obtained for 20 specimens of *D. medinensis* (from humans in Pakistan, Yemen and six African countries endemic for dracunculiasis) and three of *D. insignis* (from raccoons trapped in the state of Georgia in the southern U.S.A.). All of the *D. medinensis* 18S-rRNA sequences were found to be 1819 bases long and identical. The three *D. insignis* 18S-rRNA sequences were also found to be identical to each other but were 1821 bases long and differed from the *D. medinensis* 18S- rRNA sequence at eight positions (representing a difference of 0.44%). The 18S-rRNA coding region of a Guinea worm extracted from a dog in Ghana was indistinguishable from that of the *D. medinensis* isolates from human cases. These results provide the basis for the molecular differentiation of *D. medinensis* that will permit the DEP to determine, rapidly and accurately, whether a worm recovered from an area considered dracunculiasis-free is a specimen of *D. medinensis* or not.

One of the critical premises for considering human dracunculiasis or 'Guinea worm' disease eradicable is that there is no animal reservoir from which the causative parasite, *Dracunculus medinensis*, can be transmitted to humans. The scientific literature, however, contains numerous reports of natural infections with *D. medinensis*, or a morphologically similar species, in dogs, cats, horses, cows and other (non-human) mammals from many parts of the world. Cases of such 'animal dracunculiasis' (caused by unknown species of *Dracunculus*) have recently been reported, formally or informally, in countries where human dracunculiasis is or was endemic — in dogs in Pakistan (Muhammad *et al.*, 2005), Mauritania (S. Mohamed, unpubl. obs.) and Ghana (P. Apoya, unpubl. obs.) and in a donkey in Mali (I. Degoga, unpubl. obs.).

Currently, the taxonomy and species classification of the dracunculid parasites that infect humans and/or other mammals is based on the type of host and the morphological features of the parasite. Unfortunately, as most of the key morphological features that allow classification to species level are only present on the male worms (which are rarely available for study), the

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DOI: 10.1179/136485905X51355

female worms can often be identified only to genus level by morphology. Claims of non-human infection with *D. medinensis* that are based solely on the morphological features of female worms — especially those from areas that have no, or no recent, history of the endemic transmission of *D. medinensis* — therefore need to be interpreted with great caution.

It would be extremely useful and convenient if female specimens of *Dracunculus* could be identified to species level by molecular characterization. Even a small portion of female worm could be investigated by the PCR-based amplification of a particular region of the parasite's DNA and subsequent sequence analysis. The results of such analysis could help to elucidate the epidemiology, clinical significance and genetic diversity of *Dracunculus*.

One aim of the present study was to determine if there was intra-specific variability in the nuclear small-subunit ribosomal RNA (18S-rRNA) genes of *D. medinensis* isolates from humans. The corresponding gene of *D. insignis*, a well recognized and available species that infects wild mammals in the U.S.A. and Canada, was also sequenced so that it could be compared with that of *D. medinensis*. Since Blaxter *et al.* (1998) found the highly conserved regions in the ribosomal repeat to be useful for the molecular taxonomy of the Nematoda, the 18S-rRNA sequences were then used to determine the phylogenetic relationship of *D. medinensis* and *D. insignis*.

MATERIALS AND METHODS

Parasites

Dracunculus medinensis

The purpose of the study was explained to the national co-ordinators of the Guinea-worm eradication programmes (GWEP) in Côte d'Ivoire, Ghana, Nigeria, Pakistan, Sudan, Togo, Uganda and Yemen. In these countries, those with patent *Dracunculus* infections have their Guinea worms manually extracted, following traditional methods and the norms established by each national GWEP and Ministry of Health. The support of the co-ordinators, for the collection and shipment of *D. medinensis* specimens, was sought. Each co-ordinator was provided with forceps, vials of 70% ethanol for the preservation of the extracted worms, and mailing tubes for shipping the specimens back to the research laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, U.S.A.

In addition to the specimens collected and preserved in 70% ethanol by the national co-ordinators, some pre-emergent worms were extracted from patients in Ghana, frozen, and shipped to the CDC, by other workers employed in the Ghanaian GWEP. A curious 'red' Guinea worm collected from a patient in Togo and a worm collected from a dog in Ghana (both preserved in 70% ethanol) were also studied.

Dracunculus insignis

Raccoons (*Procyon lotor*), opossums (*Didelphis virginiana*), bobcats (*Lynx rufus*), red foxes (*Vulpes vulpes*), grey foxes (*Urocyon cinereoargeuteus*) and coyotes (*Canis latrans*) that had been trapped as part of an ongoing control programme in a rural area near Albany, in the state of Georgia in the southern U.S.A., were examined for infection with *D. insignis*. The mammals were killed humanely and examined either immediately, on site, or after storage in a refrigerator for approximately 24 h. The skin was removed from the limbs of the animals to facilitate visual inspection for worms. A single gravid female *D. insignis* was recovered from each of three raccoons. These three worms were placed, alive, in Hank's balanced salt solution (HBSS) and then carried, on ice packs, to the research laboratory.

DNA Extraction and Preparation

The specimens reaching the research laboratory varied in quality: the *D. insignis* from raccoons arrived as intact worms but most

of the *Dracunculus* specimens from humans consisted of the posterior segments of broken or ruptured female *D. medinensis* (with large numbers of larvae forming sediments at the bottom of many of the collection vials). After the careful decanting off of any ethanol or saline, each sample was washed three times with 0.01 M phosphate-buffered saline (pH 7.2) containing 1 mM EDTA. The sample was then soaked overnight (12–18 h), at 37°C, in 1 ml of the CLS-TC buffer from the FastDNA® kit (Q-BIOgene, Irvine, CA), to facilitate the rehydration of any DNA. Approximately 20 mg of larvae or 2–3 cm of adult worm were used for each DNA extraction. Before processing, each 2- to 3-cm-long piece of adult worm was placed in a Petri dish with 1 ml CLS-TC buffer and cut into small pieces with a disposable scalpel blade. Each sample of larvae or chopped adult worm was then homogenized, with an electric homogenizer, in a 2.0-ml microcentrifuge tube. The FastDNA kit and the FastPrep® cell disruptor (Q-BIOgene) were then used, according to their manufacturer's recommendations, to extract the DNA. The cell disruptor was run, for 20 s at setting 5.0, either once on each sample or, if intact tissue was still visible, twice. Some samples of extracted DNA were found to contain PCR inhibitors and these samples were further purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendations.

PCR-based Amplification of the Region Coding for the *Dracunculus* 18S rRNA Gene

Extracted DNA was amplified using the custom-made forward primer NEMFG1 (5′-TCT CCG ATT GAT TCT GTC GGC GAT TAT ATG-3′), which matches bases 1–30 of the 18S-rRNA sequence of *Gnathostoma binucleatum* (GenBank accession number Z96946), and the standard, eukaryotic-18S-rRNA reverse primer CRYPTOR (5′-GCT TGA TCC TTC TGC AGG TTC ACC TAC-3′).

Each 50-µl PCR reaction mixture consisted of 1× buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$, at pH 9.0), 200 µmol of each deoxynucleotide triphosphate, 25 pmol of each primer, 2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), and 0.1 or 1 µl of DNA. An automated thermocycler (GeneAmp PCR System 9700; Applied Biosystems) was set to give an initial polymerase-activation step of 15 min at 95°C, followed by 45 cycles, each of 30 s at 94°C, 30 s at 60°C and 90 s at 72°C, and then a final extension step for 10 min at 72°C.

Verification of PCR Products

The PCR products were resolved by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized on an ultra-violet trans-illuminator (Fotodyne, Hartland, WI). A 100-bp ladder (Invitrogen, Carlsbad, CA) was used as the size standard.

DNA Sequencing

Amplification products were purified using the StrataPrep® PCR purification kit (Stratagene, La Jolla, CA) according to the instructions from the manufacturer, and eluted in 50 µl ultra-violet-irradiated water. The sequencing reactions used to identify the *Dracunculus* species represented by each sample were performed, on both strands of the purified products, using the NEMFG1 and CRYPTOR primers as well as the internal primers NEM1F (5′-CTG CCT TAT CAA CTT TCG ATG-3′), NEM1R (5′-CAT CGA AAG TTG ATA AGG CAG-3′), NEM2F (5′-GCG GTT AAA AAG CTC GTA GTT GG-3′), NEM2R (5′-CCA ACT ACG AGC TTT TTA ACC GC-3′), NEM3F (5′-GCG GCT TAA TTT GAC TCA ACA C-3′), NEM3R (5′-GTG TTG AGT CAA ATT AAG CCG C-3′), NEM4F (5′-CCG GGA CTG AGC CGT TTC GAG-3′) and

NEM4R (5′-CTC GAA ACG GCT CAG TCC CGG-3′).

The products of each reaction were purified with Centri·Sep™ (Princeton Separations, Adelphia, NJ), to remove the dye terminators, then dried in a SpeedVac DNA 120 unit (Thermo Electron, Philadelphia, PA), and re-suspended in 20 µl formamide. Sequencing was carried out in a Model 3100 genetic analyser (Applied Biosystems). The sequences determined were then assembled and analysed using the SeqManII program from the Lasergene® software package (DNAStar, Madison, WI).

Phylogenetic Analysis

Phylogenetic analysis was performed, with version 5.2 of the TREE-PUZZLE program (Strimmer and Haeseler, 1996), version 4.0b10 of the PAUP program (Swofford, 1996) and programs from version 3.63 of the PHYLIP software package (Felsenstein, 1989), after aligning the sequences using version 1.83 of the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic trees were then created using version 1.6.6 of the TreeView program (Page, 1996).

RESULTS

The complete coding sequences for the *D. medinensis* and *D. insignis* 18S-rRNA genes were amplified using a custom-made PCR primer for the 5′ end (NEMFG1), and the standard primer for eukaryotic 18S rRNA (CRYPTOR) for the 3′ end. Several other forward PCR primers that were designed on the basis of the known sequences of the 18S rRNA genes of *Brugia* and *Caenorhabditis* failed to amplify this region from *Dracunculus* DNA (data not shown).

Complete 18S-rRNA sequence data were obtained for 18 human isolates of *D. medinensis* collected by the national co-ordinators of GWEP (one each from Côte d'Ivoire, Pakistan and Togo, two each from Nigeria, Sudan, Uganda and Yemen, and seven from Ghana). The 18S-rRNA gene in each of the three raccoon isolates of *D. insignis* (all originating from Georgia, U.S.A.) was also successfully amplified and sequenced. The lengths of the 18S-rRNA sequences were 1819 nucleotides for *D. medinensis* and 1821 nucleotides for *D. insignis*. Although no variability was detected among isolates of the same species, there were differences between *D. medinensis* and *D. insignis* at eight sequence positions (giving a similarity score of 99.5%). Two of the eight inter-specific differences were insertions/deletions and the rest were substitutions. All six substitutions were transitions (five T/C and one G/A). The 18S-rRNA sequences were submitted to GenBank (under accession numbers AY947720 for *D. medinensis* and AY947719 for *D. insignis*).

The initial Clustal alignment of the 15 sequences used for the phylogenetic analysis had 1936 columns. After flush-trimming the ends and removal of all columns containing gaps and unresolved characters, the length of the alignment was reduced to 1514 columns. The results of the phylogenetic analysis revealed that *D. insignis* and *D. medinensis* form a well-resolved clade with two other species from the superfamily Dracunculoidea: *Margolisianum bulbosum* and *Philonema* sp. Although the results obtained with all the phylogenetic methods employed were similar, the topology of the tree obtained with TREE-PUZZLE and quartet puzzling (see Figure) was closest to the results presented by Blaxter *et al.* (1998).

The sequences for the 18S-rRNA gene of the canine isolate from Ghana and of the 'red' worm of human origin from Togo were identical to that of all the other specimens from humans. (All the alignments and trees may be requested from the authors.)

DISCUSSION

As the campaign to eradicate dracunculiasis progresses and transmission of *D. medinensis*

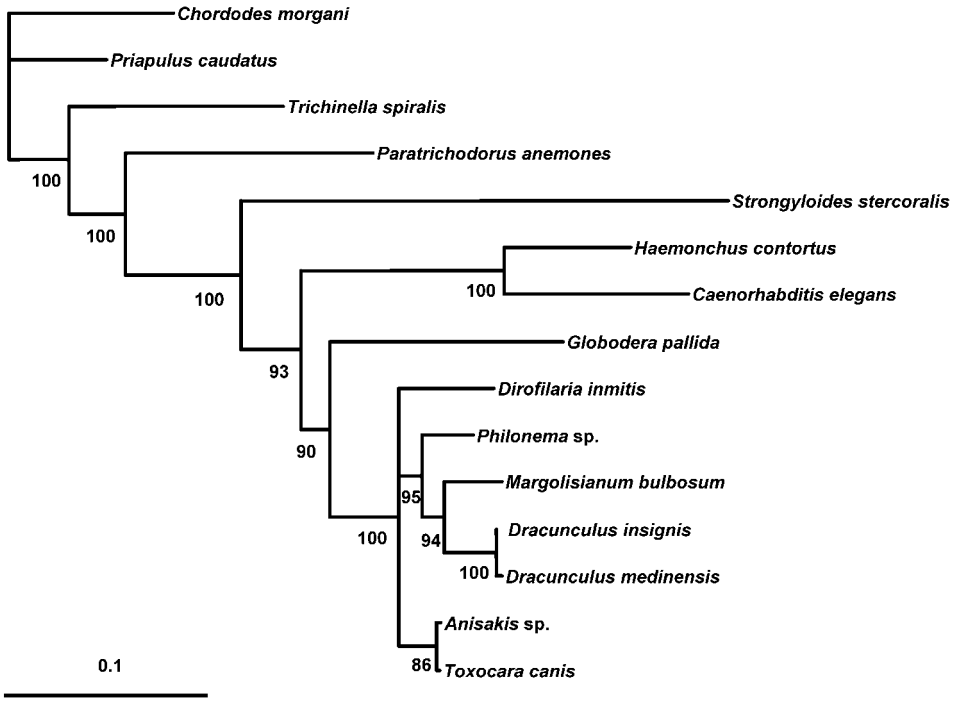

FIG. Phylogenetic tree based on the small-subunit-ribosomal-RNA (18S-rRNA) sequences of *Dracunculus medinensis* and *D. insignis* (as determined in the present study) and those of selected Nematoda (that were already available in the GenBank database). Quartet-puzzling maximum-likelihood results (Strimmer and Haeseler, 1996) are shown, with *Priapulus caudatus* (phylum Priapulida) and *Chordodes morgani* (phylum Nematomorpha) used as outgroups. Numbers to the left of the nodes indicate the quartet-puzzling support for each internal branch. The scale bar indicates an evolutionary distance of 0.1 nucleotide/position in the sequence. Vertical distances are for clarity only. The nematodes included in the analysis were (with the GenBank accession codes for the sequences investigated): *Anisakis* sp. (U94365), *Caenorhabditis elegans* (X03680), *Chordodes morgani* (AF036639), *Dirofilaria immitis* (AF036638), *Dracunculus insignis* (AY947719), *Dracunculus medinensis* (AY947720), *Globodera pallida* (AF036592), *Haemonchus contortus* (L04153), *Margolisianum bulbosum* (AB185161), *Paratrichodorus anemones* (AF036600), *Philonema* sp. (U81574), *Priapulus caudatus* (X87984), *Strongyloides stercoralis* (M84229), *Toxocara canis* (U94382), and *Trichinella spiralis* (U60231).

is stopped in more and more areas, the possibility of an unexpected human infection with *Dracunculus* occurring in an area considered dracunculiasis-free increases. The response to each of these unexpected cases will depend partly on the travel history of the patient during the preceding year and partly on the confirmation that the parasite involved is *D. medinensis* and not a zoonotic infection with another species of nematode. The discovery of a viable adult *D. medinensis* in an area categorised as dracunculiasis-free would be cause for considerable concern to the DEP — particularly if the infection could only have been acquired in such an area — and would necessitate extensive interventions to contain and eventually stop any transmission. If, on the other hand, a suspected case of dracunculiasis was found to be infected only with a species of *Dracunculus* other than *D. medinensis*, there would be no cause for alarm and no public-health interventions would be necessary.

On only three occasions during the last 78 years have cases of human dracunculiasis been reported from countries that have never been known to have the endemic disease: Korea (Hashikura, 1926, 1927), Japan (Kobayashi *et al.*, 1986) and China (Wang *et al.*, 1995). In all three instances

the parasite involved was described as *D. medinensis*, even though the subjects found infected had no history of travel to a country with endemic human dracunculiasis and no further transmission of the infection to other people was observed. These puzzling infections were probably not *D. medinensis* but with a species of *Dracunculus* that, though not usually a parasite of humans, had been picked up by humans when they ate a paratenic host of the parasite. Such a mechanism of transmission has been postulated for the dracunculid parasites of raccoons, mustelids and reptiles, and has been confirmed experimentally (Crichton and Beverly-Burton, 1977; unpubl. obs.). All the odd cases of apparent human dracunculiasis reported in Korea, Japan and China had eaten uncooked freshwater fish prior to their infection. The case from Japan, for example, had ingested loaches (Cobitidae), which are effective predators of copepods (Kobayashi *et al.*, 1986). People in Asia may accidentally acquire *Gnathostoma*, a parasite of carnivores that also uses copepods as its intermediate hosts, by eating various raw fish (Beaver *et al.*, 1984).

Canine infections with *D. medinensis* have been reported in Uzbekistan (Litvinov and Litvinov, 1981; Litvinov and Lysenko, 1982), and the Indian state of Tamil Nadu (Joseph and Kandasamy, 1980; Lalitha and Anandan, 1980) — two regions where human dracunculiasis was formerly endemic. There is, however, no evidence to indicate that the transmission of *D. medinensis* to humans has ever been reintroduced in any formerly endemic country (e.g. Uzbekistan, Iran, India, Pakistan, Yemen and Senegal) as the result of canine or other, non-human infections. Humans are the principle host of this infection. That the 18S-rRNA coding region of the canine isolate investigated in the present study was found to be identical to that of all the human isolates indicates that *D. medinensis* can, on occasion, infect animals other than man. There have been previous, albeit unconfirmed reports of dogs naturally infected with *D. medinensis* (Joseph and Kandasamy, 1980; Lalitha and Anandan, 1980; Litvinov and Litvinov, 1981; Litvinov and Lysenko, 1982). In endemic communities, the incidental infection of dogs, and perhaps other non-human mammals, with *D. medinensis* appears likely. Not surprisingly, dogs have been the most common experimental hosts of *D. medinensis*, although the parasite has also been maintained in rhesus monkeys (Muller, 1971).

It is now possible not only to differentiate *D. medinensis* from *D. insignis* by the parasites' 18S-rRNA sequences but also to verify whether or not dracunculid worms of non-human origin are *D. medinensis*. Even in the study of the dracunculid parasites that are never found in humans or other mammals, the molecular approach used in the present study may still be useful, negating the need for more complex and more labour-intensive morphology based on light or electron microscopy (Moravec and Little, 2004). A nematode from the Ugandan DEP, recently received at the CDC, could not be identified as *D. medinensis* by light microscopy, and sequence analysis of the extracted and amplified DNA confirmed that the specimen was not *Dracunculus* (unpubl. obs.).

Although all the isolates identified as *D. medinensis* in the present study — including the 'red' worm from a patient and the canine isolate — had the same 18S-rRNA sequence, they probably varied in another region of their genome. Future research will include attempts to explore such intraspecific variation, probably, initially, by investigating the intergenic transcribed sequences (ITS) in the rRNA cluster.

The results of the phylogenetic analysis of the 18S-rRNA coding region (see Figure) demonstrated that *D. medinensis* and *D. insignis* form a sister group to *M. bulbosum*, and these three species form a sister group to *Philonema* sp. Both *M. bulbosum* and *Philonema* are fish parasites in the family Philonematidae, superfamily

Dracunculoidea. As seen previously (Anderson, 1988; Blaxter *et al.*, 1998), the Ascaridida and Spirurida did not resolve well. It was not possible to identify which group of the spirurids is most closely related to the dracunculids. The phylogenetic relationship of the dracunculids within the Spirurida therefore remains to be elucidated.

ACKNOWLEDGEMENTS. The financial support of the CDC, the WHO Collaborating Center for Research, Training and Eradication of Dracunculiasis, and the Global 2000 Program of the Carter Center is gratefully acknowledged. The authors wish to thank E. Nace, K. Won, H. Bishop and G. Punkosdy for their invaluable assistance in collecting the *D. insignis* for this study.

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