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Molecular detection of *Murshidia linstowi* in a free-ranging dead elephant calf

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Abstract: Gastrointestinal helminths are ubiquitous in both domestic and wild animals. Infections are often sub-clinical except in circumstances of destabilization of host-parasite equilibrium by innate or environmental factors. The present case deals with microscopic and molecular diagnosis of *Murshidia linstowi* recovered from an elephant. A post-mortem examination of a free-ranging juvenile male elephant calf that had died of electrocution in Athagarh Wildlife Division revealed the presence of slender, whitish nematodes in the stomach. No gross lesions were noticed either in the site of predilection or any other internal organs. The average length of the parasites was 3.8cm. These parasites were collected for further gross as well as microscopic examination following routine parasitological techniques. Temporary mounts prepared after cleaning the nematodes in lactophenol were observed under a microscope. Morphological features such as a well-developed mouth collar, large and globular buccal capsule with fine tubercles, cone shaped oesophageal funnel, short bursa having indistinctly divided lobes and closely apposed ventral rays and stout spicules with club shaped tips bent dorsally corroborated with that of *M.linstowi* (male). Amplification of the rDNA from the internal transcribed spacer (ITS) region using universal nematode primers NC2 and NC5 revealed a product size of 870bp. The PCR product was subjected to sequencing followed by NCBI-BLAST which revealed 98% homology with *M. linstowi*. A phylogenetic study showed a maximum similarity with *M.linstowi* recovered from elephants in Kenya. This particular nematode species belonging to the family Strongylidae and sub-family Cyathostominae appears to be the first documented report in India.

Keywords: Gastrointestinal helminths, infection, nematode.

Helminths are ubiquitous across vertebrate taxa. They pose a threat to the welfare, management and conservation of captive as well as free-ranging elephants. Strongyles of the genus Murshidia reside in the alimentary canal of Indian and African elephants. *Murshidia* spp. affecting elephants include *M. linstowi* (Heinrich 2016; McLean et al. 2012), *M.* murshida (Ajitkumar et al. 2009; Chandra et al. 2018; Edwards et al. 1978; Muraleedharan 2016), *M.* falcifera (Ajitkumar et al. 2009; Chandra et al. 2018; Edwards et al. 1978; Matsuo and Supramah 1997),*M. longicaudata* (Heinrich 2016; McLean et al. 2012), *M. indica* (Ajitkumar et al. 2009; Muraleedharan 2016) and *M. Africana* (McLean et al. 2012). Murshidiasis in elephants has been reported from across the globe like Sri Lanka, Nigeria, Kenya, Burma, Indonesia and India. The present case report deals with molecular identification of *Murshidia linstowi* recovered from a free-ranging elephant calf that died of electrocution.

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History

A free-ranging juvenile, male elephant calf about 4½ years that had died of electrocution in Athagarh Wildlife Division (20.32^{0}N & 85.41^{0}E) was referred for investigation. The body condition of the elephant was almost normal. During post-mortem examination, two types of helminths (trematode and nematode) were recovered from its stomach. No gross lesions were noticed either in the site of predilection of the parasites or any other internal organs.

Materials and Methods

These parasites thus collected (Image1) were subjected to gross and microscopic examination following routine parasitological techniques and identified based on their features (Singh 2003).

Molecular diagnosis

The internal transcribed spacer (ITS) region within the rDNA provides a reliable marker to differentiate between several strongyles. Genomic DNA of the parasite was extracted using commercially available DNA mini kit (QIAGEN, Germany). According to the manufacturer's instructions, 25mg of the parasite was taken for the said purpose. The universal nematode primers NC2 (5'- TTAGTTTCTTTTCCTCCGCT-3') and NC5 (5'- GTAGGTGAACCTGCGGAAGGATCATT-3') were used for amplification (McLean et al. 2012). PCR was carried out in a 24µl reaction mixture containing2µl (640ng/µl)of genomic DNA, 2.4µl 10X PCR buffer, 2.4µl of 25mM $MgCl_2$, 0.16µl DNA polymerase, 1.2µl of each primer(10mM) and 2.4µl of dNTP mixture (2pmol). Amplification was preceded by a 10 minute polymerase activation step at 95ºC followed by 40 cycles of 45 sec each at 95ºC, 55ºC and 72ºC. A 5-min extension step at 72ºC concluded the reaction. The amplification products were subjected to electrophoresis on 1.5% agarose gel. The parasite sample was run in duplicates along with nuclease free water as negative control. The purified PCR products were subjected to sequencing for further identification. The similarity of the sequence with Genbank database submissions was carried out by using BLAST (http://blast.ncbi.nlm.nih.gov) (Altschul et al. 1990). The sequence was submitted to Genbank for generation of accession number. Additional 21 gene sequences were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). All the sequences were aligned and compared using ClustalW (http://www.ebi.ac.uk), with gaps and missing data eliminated from the dataset ("complete deletion option").There were a total of 707 positions in the final dataset. Molecular phylogenetic analysis was performed using MEGA 6.05. The best fit model for nucleotide substitution was selected from 24 models using MEGA 6.05 (Tamura et al. 2013) based on the minimum Bayesian Information Criterion (BIC) value (Nei and Kumar 2000; Schwarz 1798). The best fit nucleotide substitution model was used for testing the phylogenetic hypothesis using maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The branch support for the correct location of branches was assessed through 1,000 bootstrap replicates.

Image 1. Helminths recovered from stomach of elephant.

Results and Discussion

Microsopic examination of the anterior end of the slender whitish nematode measuring about 3.8cm revealed the presence of a well-developed mouth collar, large and globular buccal capsule having fine tubercles and cone shaped oesophageal funnel (Image2). The posterior end consisted of a short bursa having indistinctly divided lobes and closely apposed ventral rays. Spicules were stout, straight with club shaped tips bent dorsally (Image3). Such morphological features corroborated with those of the male *M. linstowi* (Singh 2003). Molecular analysis showed a product size of 870bp (Image4). The sequencing results were compared with reference sequences of NCBI database using BLAST and 98% similarity was found with *M. linstowi* recovered from elephants in Kenya. The sequence was submitted to GenBank, with the accession number MK968095. Nucleotide substitution model with invariant sites (T92+I, BIC=3284.19, InL= -1282.61, I = 0.69) was chosen

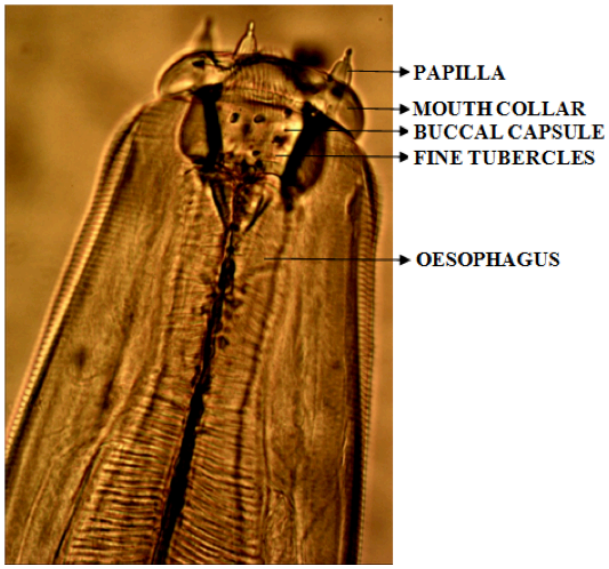

Image 2. Anterior end of *Murshidia linstowi* (male). © Sonali Sahoo

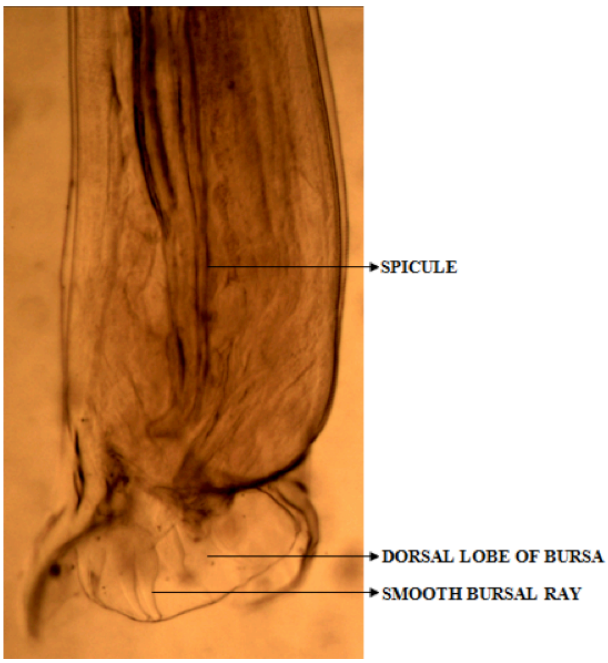

Image 3. Posterior end of *Murshidia linstowi* (male). © Sonali Sahoo

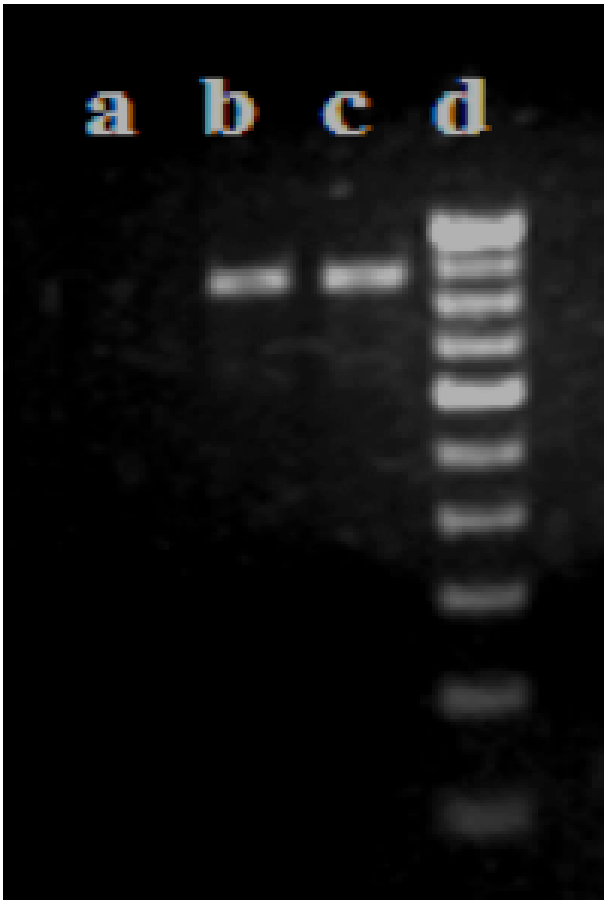

Image 4. Lane a—negative control | Lanes b & c—samples in duplicate (870bp) |Lane d—100bp DNA ladder. © Sonali Sahoo

as the best nucleotide substitution model (Tamura 1992). Nucleotide sequence of the sample and 21 reference sequences were used for the construction of a maximum likelihood phylogenetic tree (Figure 1). The bootstrap values shown in the nodes of the branches within the different clusters of *Murshidia* are relatively high. Therefore, the sample is likely to be *M. linstowi.*

Based on gross and microscopic examinations, the trematode was identified as *Pseudodiscus hawkesii* (Singh 2003). *P.hawkesi* measuring approximately 3.6–11mm in length and 2–6mm in breadth possessed the salient features like ventral mouth opening with oral suckers, well developed esophageal muscular bulb, lobed testes, sub-median ovary and coiled uterus.

Like other members of the subfamily Cyathostominae, *M.linstowi* probably has a direct life cycle. Eggs passed in the faeces hatch on the ground to release the first stage larva which subsequently develops into the third stage. These strongyles are inadvertently ingested by their hosts as infective third-stage larvae on vegetation (Newton-Fisher et al. 2006). Helminthic infections in many wild animals are often sub-clinical except in circumstances where the host-parasite equilibrium is being destabilized by stressors like concurrent infections, pregnancy, lactation and changes in climatic conditions. Clinical signs such as reduction in feed intake, edematous swelling on dependent parts of body, debility and reduction in body weight have been recorded in elephants suffering from murshidiasis (Tripathy et al. 1991). However, in the present case, no such clinical signs were evident. A single

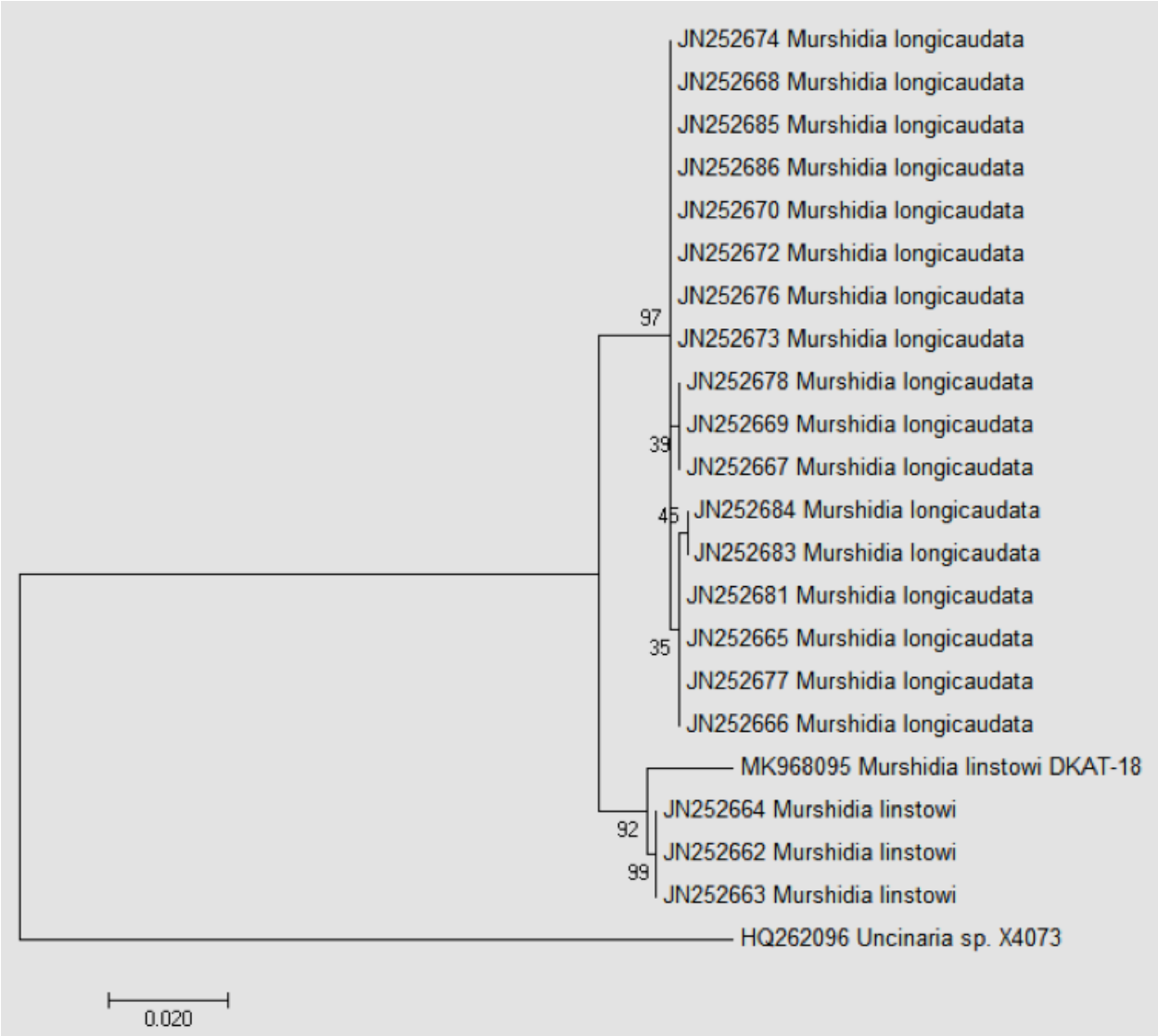

Figure 1. Evolutionary analysis of *M. linstowi* (DKAT-18) using 16s rDNA sequencing.

dose of fenbendazole at the rate of 5mg/kg body weight has been found to be successful against murshidiasis in elephants (Nei and Kumar 2000).

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