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ORIGINAL PAPER

Molecular identification and phylogenetic analysis of *Dipetalonema evansi* (LEWIS, 1882) in camels (*Camelus dromedarius*) of Iran

Alireza Sazmand[1,4,5] · **Barbara Eigner**[1] · **Mohammad Mirzaei**[2] · **Seyedhossein Hekmatimoghaddam**[3] · **Josef Harl**[1] · **Georg Gerhard Duscher**[1] · **Hans-Peter Fuehrer**[1] · **Anja Joachim**[1]

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Abstract Despite the economic importance of camels, the parasites that affect them have not received adequate attention so far and molecular studies are scarce compared to other livestock. In this study, we characterized peripheral blood microfilariae in 200 healthy one-humped camels (*Camelus dromedarius*) from south-east Iran by microscopy and molecular tools to receive a more detailed insight into prevalence and species that affect them. Moreover, adult specimens of the filarial nematode *Dipetalonema evansi* were collected from the carcass of an infected animal. Microscopic examination was performed on Giemsa-stained blood smears, and blood was also spotted on Whatman FTA® cards for DNA analysis. Genomic DNA was extracted, and PCR was carried out for the detection of filaroid helminths, followed by sequence analysis of positive samples. Four samples were positive for microfilariae by microscopy, while 16 animals (8 %) were positive by PCR. Sequence analysis revealed *D. evansi* in all cases. Phylogenetic analysis of a cytochrome C oxidase subunit I (COI) sequence of filaroid nematodes showed that most species in a single genus cluster in the same clade; however, *D. evansi* and *D. gracile* are not monophyletic and branch rather at the base of the tree. Further studies on the life cycle of *D. evansi*, specifically the identification of intermediate host(s), have become feasible with the provision of the first specific COI sequences in this study.

Keywords Camels · *Dipetalonema evansi* · Filariosis · Hemoparasites · PCR · COI

✉ Anja Joachim
Anja.Joachim@vetmeduni.ac.at

[1] Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Vienna, Austria

[2] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Postal Code 7616914111 Kerman, Iran

[3] Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Postal Code 8916188635 Yazd, Iran

[4] Present address: Department of Agriculture, Payame Noor University, P.O. Box 193953697, Tehran, Iran

[5] Iranian Research Center for Zoonotic Diseases, School of Health, Shahid Sadoughi University of Medical Sciences, Postal Code 8915173160 Yazd, Iran

Introduction

Camels are multi-purpose animals in arid and semi-arid regions of the world, with a global population of over 27 million animals. According to official estimates, 160,000 camels live in Iran, and almost all of them are one-humped dromedaries (*Camelus dromedarius*) (Ministry of Agriculture Jihad 2014). Camels are resistant to harsh environmental conditions and were formerly thought to be resistant to a wide range of pathogens, but new studies have confirmed that they are susceptible to various pathogens (El Harrak et al. 2011), including filarial nematodes. *Dipetalonema evansi* (LEWIS, 1882) and *Onchocerca fasciata* (RAILLIET & HENRY, 1910) are filaroid nematodes specific to camels, and infection of camels with *Onchocerca armillata* (RAILLIET & HENRY, 1909) and *Onchocerca guttorosa* (NEUMANN, 1910) of bovine origin have also been reported (Nagaty 1947; Schillhorn-Van-Veen et al. 1976; Dakkak and Ouhelli 1987; Hussein et al. 1988). Although onchocerciasis is a common finding in skin biopsies of camels, microfilariae are rarely detected in peripheral blood, and it is postulated that these animals are naturally

tolerant to these infections and show no clinical signs (Wernery et al. 2014). *D. evansi* is the only filaroid helminth thought to induce clinical illness. The life cycle has not been described in detail, but mosquitoes of the genus *Aedes*, especially *Aedes* (*Ochlerotatus*) *detritus* and *Aedes caspius*, are thought to act as intermediate hosts for this nematode (Kataĭtseva 1968; El Bihari 1985; Lehane 2005). Adult worms are usually intertwined in affected organs and accumulate in large numbers that can become a golf ball-sized mass. Males measure 8–11 cm and females 14.5–18.5 cm in length, and microfilariae are 200–315 μm long (Nagaty 1947). The adult worm of *D. evansi* is seen mostly in testicles, epididymis, spermatic cord, lungs, and heart. Its sheathed microfilariae may be found in the peripheral blood. Mild infections are usually asymptomatic; however, acute infections may cause respiratory symptoms, emaciation, apathy, pale mucous membranes, orchitis, aneurysm of the spermatic cord, arteriosclerosis, heart failure, and nervous impairments (Oryan et al. 2008). High burdens of mature parasites can cause pulmonary distress and even death in exhausted animals (El Bihari 1985). The microfilaremia of *D. evansi* has a peculiar biphasic periodicity which peaks around daybreak (05:00) and early evening (20:00) (Elamin et al. 1993). Administration of ivermectin is the recommended therapy, and infected camels become amicrofilaremic 2–5 days post-treatment (Chhabra and Gupta 2006). Despite several prevalence reports from different camel rearing areas around the world (Sazmand et al. 2013), molecular characterization of filaroid helminths in these animals is lacking.

Filariae of domestic and farm animals often infect humans, and reports of clinical cases due to various species of *Dipetalonema*, *Onchocerca*, *Brugia*, *Dirofilaria*, and *Loaina* spp. are increasing. These zoonotic infections are usually cryptic and asymptomatic or diagnosed incidentally (Orihel and Eberhard 1998; Grácio et al. 2015). For this reason, the filarial parasites have been extensively studied. Nonetheless, phylogenetic relationships and bionomics of some known species such as *D. evansi* remain dubious (Krueger et al. 2007).

With the exception of trypanosomosis, hemoparasitic diseases of camels have not been investigated in great detail and most of the knowledge in the field of cameline parasitology is based on studies using light microscopy and/or serology.

In the field of camel filarial worms, a few studies have been performed on histochemical distribution of several hydrolytic enzymes and dehydrogenases in adult *O. fasciata* (Omar and Raoof 1994; Omar et al. 1996). Fine structure of female *O. fasciata* has been also described using transmission electron microscope examination (Determann et al. 1997).

This study aimed to investigate the infection rate of blood microfilariae in camels of Iran in more depth by PCR and sequence analysis to determine parasites with high sensitivity and specificity.

Materials and methods

Study area and sampling

The sampling was carried out from June to July 2014 and included 200 clinically healthy one-humped dromedaries (*C. dromedarius*) of both genders (36 females and 164 males) aged between 1 and 9 years. All camels were kept by local farmers in central and south-eastern Iran (Fig. 1). The two provinces of Kerman and Sistan-va-Baloochestan which were chosen for sampling host were almost half of the camels in Iran. The samples were derived in equal numbers from both provinces, each from three different localities that had the highest numbers of animals (Table 1). Although it is advisable to take samples from superficial blood vessels and in the time of nocturnal activity of microfilariae but in the study area situation, this consideration was not applied and blood samples were taken from jugular vein between 06:00 and 10:00 except samples from Kahnoodj ($n=20$) which were taken between 16:00 and 17:00. Drops (approximately 100 μl) of EDTA blood were spotted on Whatman FTA® Elute sample collection cards (GE Healthcare, Little Chalfont, UK) and air dried. The cards were shipped to the Institute of Parasitology, University of Veterinary Medicine Vienna, Austria, for further examination.

Microscopic examinations

From each sample, thin blood smears were prepared and stained with Giemsa for light microscopic examination of hemoparasites.

DNA isolation and PCR assay

Disks of spotted blood (approximately 5 mm^2) were cut out from the FTA® cards with sterile blades, and genomic DNA was extracted with the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. PCRs targeting a section of the mitochondrial cytochrome C oxidase subunit I (COI) were performed for the detection of filaroid helminths in the camels' blood. Because of the lack of sequences for *D. evansi* in GenBank®, DNA was isolated from three adult worms collected from male genitalia of a naturally infected camel in Iran and morphologically identified as *D. evansi* according to Nagaty (1947). DNA was extracted from tissue samples with the QIAamp® DNA Blood Mini Kit and subjected to PCRs targeting sections of COI and the 16S rRNA (16S) genes. All PCRs were performed using the GoTaq G2® Polymerase (Promega, Wisconsin, USA) using an Eppendorf MasterCycler Pro® (Eppendorf, Hamburg, Germany). Primer sequences and PCR conditions are listed in Table 2. The amplified products were detected by electrophoresis on 1.8 % agarose gels

Fig. 1 Map of Iran showing sampling sites in Kerman (Shahr-e-Babak, Kerman, and Kahnoodj cities) and Sistan-va-Baloochestan provinces (Zabol, Zahedan, and Mirdjaveh cities). The map was drawn by using Arcinfo (ESRI®Arcmap™ 10.0, Redlands, CA, USA) and DIVA-GIS (http://www.diva-gis.org/Data)

stained with Midori-Green Advance® (Biozym, Hessisch Oldendorf, Germany).

Sequencing

Purification and sequencing of PCR products (both directions) were performed by LGC (Berlin, Germany). Sequence reads were analyzed with BioEdit® Sequence Alignment Editor (Hall 1999) and curated manually by removing all primer sequences.

Phylogenetic analysis

In order to investigate the phylogenetic relationships between *D. evansi* and other filaroid nematodes, we calculated a maximum likelihood (ML) tree with the COI sequences obtained in the present study and sequences from GenBank®. A BLAST search was performed at the NCBI database (http://www.ncbi.nlm.nih.gov/) with a COI sequence of *D. evansi* (accession number KR184821), resulting in a total of 573 COI sequences of filaroid nematodes. For the phylogenetic tree calculation, only those 133 sequences which showed at least 600-bp overlap with the query sequence were included, and the ends of the sequences were manually trimmed so that the final alignment showed 580 bp in length. Identical sequences were collapsed using DAMBE v.5.2.78 (http://dambe.bio.uottawa.ca/dambe.asp; Xia 2013), resulting in a total of 105 unique haplotypes. A model test was performed with MEGA v.5.1 (Tamura et al. 2011). The model GTR + G + I was suggested to describe the substitution pattern best according to the AICc values (Akaike information criterion corrected) [the discrete gamma distribution (+G) is used to model evolutionary rate differences among sites and the rate variation model (+I) allows for some sites to be evolutionarily invariable]. A ML tree was calculated with MEGA, applying the model GTR + G + I and subtree-pruning-regrafting (SPR) as heuristic method. A ML bootstrap analysis was calculated with identical settings and 1000 replicates.

Statistical analyses

Pearson's chi-squared and Kruskal-Wallis tests were used for the determination of correlations between infections and gender or age of the camels. Differences were considered

Table 1 Distribution of camel samples according to sampling sites and gender of the animals

Province	Sampling site	Number of collected samples (gender)
Kerman	Shahr-e-Babak	20 (17 males and 3 females)
	Kerman	60 (50 males and 10 females)
	Kahnoodj	20 (19 males and 1 female)
Sistan-va-Baloochestan	Zabol	30 (16 males and 14 females)
	Zahedan	10 (2 males and 8 females)
	Mirdjaveh	60 (all males)
Total		200 (164 males and 36 females)

Table 2 PCR conditions for detection of parasites in camel blood

Target organism	Target	Sequence (5′→3′)	No. of cycles	Annealing temperature (°C)	Primer concentration (pmol)	Product size (bp)	Reference
Blood microfilariae	COI	H14FilaCOIF GCC TAT TTT GAT TGG TGG TTT TGG H14FilaCOIR AGC AAT AAT CAT AGT AGC AGC ACT AA	35	53	10	724	Hodžić et al. 2015
Dipetalonema evansi worm	COI	H14FilaCOIF GCC TAT TTT GAT TGG TGG TTT TGG H14FilaCOIR AGC AAT AAT CAT AGT AGC AGC ACT AA	35	53	10	724	Hodžić et al. 2015
Dipetalonema evansi worm	16S rRNA	O16F GCG TGA TGG CAT AAA AGT AGC O16R CAA CCC TGT TAA CTC CGG AG	30	55	20	272	Koehsler et al. 2007

significant at $p < 0.05$. The analyses were performed with IBM SPSS Statistics v.20.0 software.

Ethical statement

Samples from Kerman province were obtained from slaughtered camels, and samples of Sistan-va-Baloochestan province were taken from live animals with official permission and under supervision of the Provincial Veterinary Organization in accordance with the veterinary laws of I.R. Iran.

Results

In light microscopy, *D. evansi* was detected in four infected camels (Fig. 2).

Using PCR, 16 out of 200 (8 %; 95 % CI = 4–12 %) samples were positive for filaroid helminths, including those positive by microscopy. Statistical analysis showed that the infection rate significantly decreased with increasing age of the animals ($p = 0.041$); 15/164 camels aged up to 4 years (9.2 %) were positive, while only 1/36 animals aged 5–9 years (2.8 %) harbored microfilariae. However, gender of the host animals was not significantly correlated with infection; 13/164 males (7.9 %) vs. 3/36 females (8.3 %) were infected. All positive samples were from Kerman province.

The filaroid COI sequences (adults and larval stages) were identical ($n = 12$) or differed only in single substitutions from each other ($n = 6$), suggesting that the larvae and the adult specimens all belonged to the same species. The sequences deposited in GenBank® (http://www.ncbi.nlm.nih.gov/) under the accession numbers KR184801 to KR184818 (*D. evansi* COI) and KR184821-KR184823 (*D. evansi* 16S).

The ML tree calculated with a COI sequence of *D. evansi* and sequences from GenBank® was displayed as a radial tree (Fig. 3). Mostly, species of a single genus come out monophyletic in the same clade (e.g., *Onchocerca*, *Cercopithifilaria*, and *Setaria*), but *D. evansi* and *D. gracile* (accession number AJ544877; Casiraghi et al. 2004) are not monophyletic and branch of rather basal in the tree. A more robust phylogenetic analysis requires analysis of more conserved genes because evolutionary distances are too large between higher taxa of filaroid nematodes.

Discussion

In the present study, blood stages of filaroid helminths of healthy Iranian dromedary camels were examined by microscopy and molecular techniques.

D. evansi microfilariae were present in the peripheral blood of 8 % of tested camels. Previous studies on blood

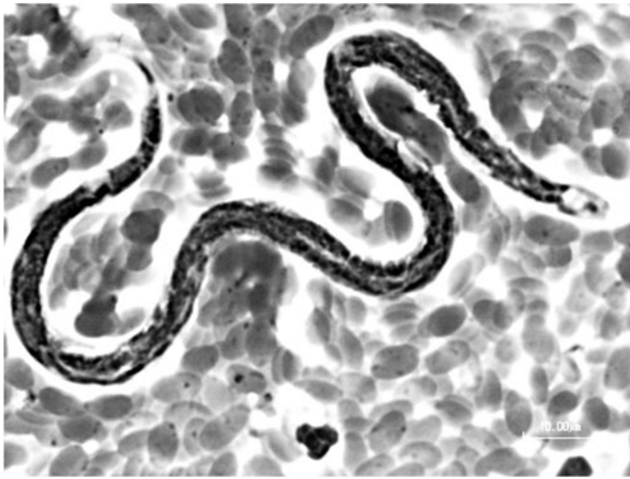

Fig. 2 Microphotograph of a *Dipetalonema* microfilaria in camel blood

microfilariosis in different parts of Iran have reported varying infection rates of 0.88–47 % (Mowlavi et al. 1997; Oryan et al. 2008; Borji et al. 2009; Sazmand et al. 2013; Karimi et al. 2015) and recovery of adult worms from up to 50 % of affected organs of slaughtered camels (Mowlavi et al. 1997). Modified Knott's technique for concentration of microfilariae is the method of choice for microscopic diagnosis of the larvae however, since the aim of current project was molecular identification of the parasites, and stained blood smears were prepared also for detection of *Trypanosoma* spp., piroplasms and *Anaplasma* spp. (will be published elsewhere); the stained slides were examined for *D. evansi* as well. Molecular detection of filaroid helminths using filter papers was more sensitive than light microscopy as PCR detected 16 microfilaremic camels, while microscopy was able to detect only four.

Despite the clinical importance and distribution of this nematode in camel-rearing areas of the world, no published reference sequences were previously available for this species. In agreement with previous reports on decreasing susceptibility to infection with advanced age (Chhabra and Gupta 2006), in this study, a significantly higher infection rate for *D. evansi* was found in younger camels.

Studies on the molecular phylogeny of *Dirofilariinae* and *Onchocercinae* indicate that these subfamilies appear as polyphyletic groups (Lefoulon et al. 2015). In our study, also *D. evansi* and *D. gracile* were found not to be monophyletic.

Members of the genus *Aedes* which are believed to be possible intermediate hosts are commonly found in Iran (Azari-Hamidian 2007). However, so far, no data is available on infections of possible vectors (and their vector competence) of *D. evansi* in Iran and neighboring countries, Pakistan and Afghanistan, where dromedaries are commonly free-roaming. The availability of specific DNA sequences for *D. evansi* now enable large-scale studies on its possible vectors in endemic areas by molecular typing.

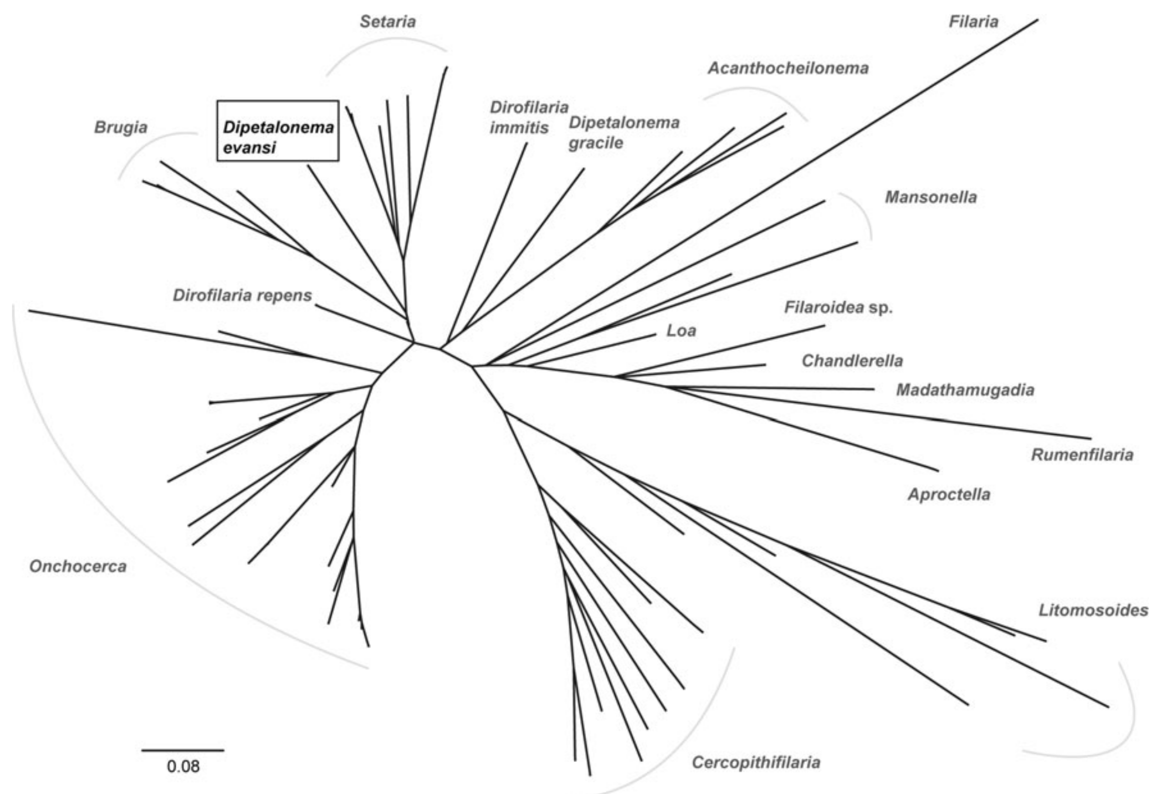

Fig. 3 Unrooted radial maximum likelihood tree with COI sequences of *Dipetalonema evansi* (in frame) and other filarioid nematodes obtained from GenBank®. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Conclusion

Molecular identification of *D. evansi* adds to the current knowledge on vector-borne diseases of camels. The comparison of COI nucleotide sequences places *D. evansi* among filaroid helminths as expected. Since there is no confirmed information about intermediate host(s) of camel filariosis based on molecular methods, identification of its vectors for studying the nematode's life cycle is suggested.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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