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Full Article

Molecular survey of Canine Microfilariae Species in East-Azerbaijan province of Iran

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ABSTRACT

Filariasis in dogs is caused by several species of filariids. Because of importance of this infection in veterinary medicine and public health, it is necessary to carry out an epidemiological and cross sectional studies in various geographical areas and use of well-adapted diagnosis methods. In this study 205 capillary and whole blood samples were collected from doges in various counties of East-Azerbaijan province. Samples after preparation were examined by Knott`s test and light microscope for presence of microfiler. In molecular identification, Pan-filarial and species-specific PCR perimers was used to differentiate among *Dirofilaria immitis, Dirofilaria repens, Acanthocheilonema reconditum, Acanthocheilonema dracunculoides, Brugia malayi* and *Brugia pahangi*. Descriptive statistics were used for data analysis. Total infection prevalence with the microscopic evaluation was 77 (37.5 %) and in PCR test was 94 (45.8 %). The most common species of canine filarial parasite identified in this study was *D. immitis* 54 (57.4 %) followed by Acanthocheilonema species 40 (42.6 %). The molecular evidence on the sequence of the ITS-2 region provided strong evidence that the canine microfilariae discovered in this study belong to a novel species of Acanthocheilonema. Information about infection prevalence helps us to improve disease management practices in the studied area, apply new hygiene policy and reduce extra costs of therapeutic agents and PCR is a quick and accurate molecular genetics method for detection of filarial species.

Keywords: Molecular Characterization, Canine Microfilariae, Iran

INTRODUCTION

Most famous filarial nematodes described in canine are: *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum, A. dracunculoides, Brugia malayi and Brugia pahangi* (Order: Spirurida, Superfamily: Filarioidea, Family: Onchocercidae) (Scaramozzino *et al* 2005, Chansiri *et al* 2002). The interaction between the parasites, hosts, the geographical and environmental conditions plays a central role in the apparent, increase and epidemiology of these infections. The importance of infection with the filarial parasites in canine is heightened by the pathogenic potential of these

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nematodes, the challenges involved in diagnosis and (for some) their zoonotic potential (Traversa *et al* 2010). Because of mentioned importance in veterinary medicine and public health, it is necessary to carry out a diagnosis by efficient and well-adapted methods (Watier-Grillot *et al* 2011). This disease is generally diagnosed by antigen testing for *D. immitis* and/or identification of microfilariae in the blood of infected doges. But some other filariae can produce persistent microfilaremias with negative heart worm antigen tests (Rishniw *et al* 2006). Other identification methods such as Knott`s test and alkaline phosphatase staining are imperfect and rely on specialist training to accurately differentiate the filariae (Fischer *et al* 2002). But species-specific polymerase chain reaction (PCR) using the primers derived from internal transcribed spacer 2 region (ITS2) is a feasible and rapid method for identification (Mar *et al* 2002). The aim of the present study was to determine the molecular prevalence of canine microfilariae spices in some counties of East-Azerbaijan province of Iran.

MATERIALS AND METHODS

Study area. East-Azerbaijan province is located in Northwest of Iran and has an area about 47,830 km² (2.8 % of Iran's area) and is located between 36.45° and 39.26° north latitude and 45.5° and 48.22° east longitude. East Azerbaijan enjoys a cool, dry climate, being in the main a mountainous region. However, the gentle breezes off the Caspian Sea have some influence on the climate of the low-lying areas.

Samples collection. Capillary and whole blood samples were collected from 205 dogs from four different cities, namely Tabriz, Marand, Jolfa and Kaleybar in East-Azerbaijan during 2010-2011.This research was conducted as a cross sectional study. Information (age and sex) of each sample was entered in special forms. All the blood samples were transferred to the parasitology laboratory of Razi research institute immediately and stored at –20 °C prior to examination.

Diagnostic Knott`s test. 1 ml of blood was gently mixed with 9 ml of formaline (2%), to break the erythrocytes and fix the leukocytes and the microfilariae. After 2–3 min, the material was centrifuged for 5 min, at 3000 rpm and the supernatant was discarded. 30 µl of the sediment was smeared on three clean glasses slides, then stained by a methylene blue solution (2%) and examined to determine the presence of microfilariae, by a light microscope (Knott 1939). Slides were photographed using IDS imaging development systems GmbH. Then microfilariae sizes were analyzed with Axiovision LE software.

Molecular identification. Identification was done with 5.8S-ITS2-28S genes amplification with PCR method. Briefly, samples total genomic DNA was extracted according to the AccuPrep kit protocol provided by the manufacturer (BioNeer, Korea). The pointed genes were amplified by using the PCR method with Taq DNA polymerase and Pan-filarial primers (DIDR-F and DIDR-R) for differentiate *D. immitis, D. repens, B. malayi, B. pahangi, A. reconditum* and *A. dracunculoides.*Reaction was carried out in a volume of 20 µl using *ACCUprep* PCR kites with 1 µl DNA and 1 µl each primers and 17 µl DW. The PCR amplification were performed in a termocycler (Eppendorff, Germany) using 31 cycles of 94 °C for 4 minute (min) for a single cycle to primary denaturing, 94 °C for 45 seconds (sec), 65 °C for 30 sec, 72 °C for 45 sec (repeated for 30 cycles) and 72 °C for 10 min for complete extension. Primary positive samples results were confirmed by using the secondary PCR test (Table 1, species specific primers) for identification approves. Unknown PCR results send for sequencing. Sequencing of target gene was carried out in Bioneer (Seoul, Korea). The obtained sequences were assessed, analyzed and manually edited by using of the Choromas lite software package and compared with sequences with in the NCBI database (http://www/ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST).

Statistics analysis. Data were examined using a commercially available statistical package (IBM SPSS version 19 for Windows), and comparisons were made using the descriptive statistics.

RESULTS

Results of the PCR and microscopic examinations of all 205 samples are presented in Table 2. 77 (37.5%) of samples were positive using microscopic screening and 94 (45.8 %) confirmed for filarial species by PCR. The most common species of canine filarial parasite identified in this study was *D. immitis* followed by Acanthocheilonema sp. The prevalence and geographical distribution of this species are summarized in Table 3. Unidentified canine microfilaria species was identified by sequence comparisons of its 534 bp region of the 5.8S-ITS2-28S genes (Acanthocheilonema sp. Razi Marand, GenBank accession number JN819184) with sequences in the GenBank data base revealed that the organism was an Acanthocheilonema sp. with a 99% similarity to the Acanthocheilonema sp. PAMAR-2010 and 86% similarity to the *D. reconditum*.

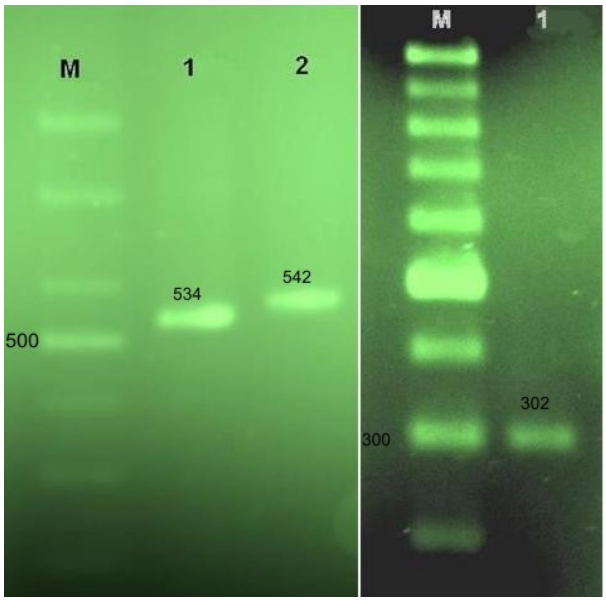

Figure 1. (Left) Differentiation of canine microfilariae spices with Pan-filarial primers (M) marker, 1 (*Acanthocheilonema sp.,* 534bp), 2 (*D. immitis,* 542bp); (Right) Approve of perimery PCR with species-specific primer, (M) marker, 1 (*D. immitis,* 302bp).

DISCUSSION

The prevalence of filarasis in dogs in different regions is variable depending on the environmental and climatic conditions, vector population, diagnostic method, situation of infection (patent or occult), animal travel, international trade and surveillance and control programs (Razi-Jalali *et al* 2010).Different prevalence rates (16-51.1%) reported in previous studies in Iran (Ranjbar-Bahadori *et al* 2009, Razi-Jalali *et al* 2010, Malmasi *et al* 2011, Nematollahi 2010, Ranjbar-Bahadori *et al* 2011, Javidi-Barazandeh 2010) should be related to the mentioned factors. In the present study molecular prevalence rate of infection for all species was 94 (45.8 %). The usefulness of molecular tools for identifying microfilers has been recently demonstrated in epidemiological and clinical studies (Li *et al* 2004, Oh *et al* 2008). A quick and accurate molecular genetics method of detection of filarasis is important for identification because species of microfilers are sometimes difficult to distinguish using morphological criteria (Mar *et al* 2002) and in some samples may we have occult infections. So we used of PCR diagnostic method for species identification. This methodology detected infection agent in 17 samples, which were found to be negative using microscopy (occult infections). In this study the prevalence of infection in various counties was different (39.5% – 48.4%). Also the most common species of canine filarial parasite identified in this study was *D. immitis* (57.4 %) followed by Acanthocheilonema species (42.6 %). Some mutations in ITS2 sequence of isolated Acanthocheilonema sp. Razi marand emphasize the probability of the existence of a new species but genetic distances between this novel species and *A. reconditum* were within the range expected for separate species of the same genera. The first report of this kind species related to Abd-Rani *et al* (2010) from Ladakh, India. At least 2 species of filarial parasite are now known to infect dogs in East-Azerbaijan province of Iran, one of which was reported for the first time in this study, namely a novel species of Acanthocheilonema, which was isolated from various counties of East-Azerbaijan. The study also confirms and extends the known geographical distribution of canine heartworm in East-Azerbaijan province of Iran. So in this study, the influence of the age of dogs has not been considered in

Table 1. Primer sequences used to amplify PCR products from canine blood samples.

	Primer	Primer sequence	Gene Target	Product origin	Product size	References
Pan-filarial	DIDR-F DIDR-R	AGT GCG AAT TGC AGA CGC ATT GAG AGC GGG TAA TCA CGA CTG AGT TGA	5.8S-ITS2-28S	*D. immitis* *D. repens* *B. malayi* *B. pahangi* *A. reconditum* *A. dracunculoides*	542 484 615 664 578 584	Abd Rani et al. 2010
Species-specific	D.imm-F D.imm-R	CAT CAG GTG ATG ATG TGA TGA T TTG ATT GGA TTT TAA CGT ATC ATT T	ITS2	*D. immitis*	302	
	A.rec-F A.rec-R	CAG GTG ATG GTT TGA TGT GC CAC TCG CAC TGC TTC ACT TC	ITS2	*A. reconditum*	348	Rishniw et al. 2006
	D.rep-F D.rep-R	TGT TTC GGC CTA GTG TTT CGA CCA ACG AGA TGT CGT GCT TTC AAC GTG	5SrRNA	*D. repens*	247	
	B.mal-F B.mal-R	GCG CAT AAA TTC ATC AGC AA ATG ACA ACT CAA TAC TCG AC	HR	*B. malayi*	294	Chansiri et al. 2002

Table 2. Prevalence of canine microfilariae in various counties.

Region	Number	Microscopic positive (%)	Primary PCR positive (Pan-filarial primer) (%)
Jolfa	43	15 (34.8)	17 (39.5)
Kaleybar	47	18 (38.2)	22 (46.8)
Marand	51	18 (35.2)	24 (47)
Tabriz	64	26 (40.6)	31 (48.4)
Total	205	77 (37.5)	94 (45.8)

Table 3. Prevalence of positive samples in various counties by PCR.

	Jolfa (%)	Kaleybar (%)	Marand (%)	Tabriz (%)	Overall prevalence (%)
D. immitis[a]	8 (8.5)	12(12.7)	15 (15.9)	19 (20.2)	54 (57.4)
A. reconditum[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
D. repens[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
B. malayi[a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Acanthocheilonema sp.[b]	5 (5.3)	10 (10.6)	9 (9.5)	16 (17)	40 (42.6)

[a] Using Species-specific primers.
[b] Using ITS2 sequencing method.

statistic analysis. Other previous papers are in a discordant way. Experimental infections suggest that higher infection rates in adult individuals are simply related to the longer exposition times (Scaramozzino *et al* 2005, Piergili-Fioretti *et al* 2003, Cringoli *et al* 2001). The data presented in this study indicate a strong need for epidemiological and pathogenetic studies to identify distribution, species of infection, host-parasite relations and infection kinetics. Also these kined studies help us to identify new introduced infections and reduce secondary treatment costs. Rapid and specific diagnosis of the disease is essential for the control of the infection agent and prevention of the disease. For this purpose, PCR is a feasible, rapid, species-specific, meticulous and cheap method.

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References

Abd-Rani, P.A.M., Irwin, P.J., Gatne, M., Coleman, G.T., Mcinnes, L.M., Traub, R.J. (2010). A survey of canine filarial diseases of veterinary and public health significance in India. *Parasites & Vectors* DOI: 10.1186/1756-3305-3-30.

Chansiri, K., Tejangkura, T., Kwaosak, P., Sarataphan, N., Phantana, S., Sukhumsirichart, W. (2002). PCR based method for identification of zoonostic Brugia malayi microfilariae in domestic cats. *Molecular and Cellular Probes*16: 129–135.

Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G. (2001). A prevalence survey and risk analysis of filariosis in dogs from the Mt. Vesuvius area of southern Italy. *Veterinary parasitology* 102(3): 243-252.

Fischer, P., Wibowo, H., Pischke, S., Ruchert, P., Liebau, E., Ismid, I., Supali, T. (2002). PCR based detection and identification of the filarial parasite Buregia timori from Alor Island, Indonesia. *Annals Tropical Medicine Parasitology* 96:809-821.

Knott, J. (1939). A method for making microfilarial surveys on day blood. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 33:191-195.

Li, B. W., Rush, A. C., Tan, J., Weil G.J. (2004). Quantitative analysis of gender-regulated transcripts in filarial nematode *Brugia malayi* by real-time RT-PCR. *Molecular & Biochemical Parasitology* DOI: 10.1016/ *molecular and Biochemical Parasitology journal* 2004.07.002

Malmasi, A., Hosseini, S.H., Aramoon, M., Bahonar, A., Seifi, H.A. (2011). Survey of canine *Dirofilaria immitis* infection in Caspian provinces of Iran. *Iranian Journal of Veterinary Research* 37: 340-344.

Mar, P.H., Yang, I.C., Chang, G.N., Fei, A.C. (2002). Specific polymerase chain reaction for differential diagnosis of *Dirofilaria immitis* and *Dipetalonema reconditum* using primers derived from internal transcribed spacer region 2 (ITS2). *Veterinary Parasitology* 106: 243–252.

Nematollahi, A., Javidi-Barazandeh M.A. (2010). A Survey on Occurrence Dirofilaria immitis in Stray Dogs of Tabriz (Iran). *ACTA VETERINARIA BRNO* 79: 449–451.

Oh, H.W., Jun, H.K., You, M.J., Hayasaki, M., Song, K.H. (2008). Ectopic migration of an adult heartworm in a dog with dirofilariasis. *The Korean Journal of Parasitology* 46:171-173.

Piergili-Fioretti, D., Diaferia, M., Grelloni, V., Maresca, G. (2003). Canine filariosis in Umbria: an update of the occurrence one year after the first observation of autochtonous foci. *Parassitologia* 45: 79-83.

Ranjbar-Bahadori, S.H., Eidi-Delvarzadeh, M., Shemshadi, B. (2009): Dirofilaria immitis infection in stray dogs of Khuzestan, a province in South-Western Iran. *International Journal of Veterinary Research* 3: 133-136.

Ranjbar-Bahadori, S.H., Veshgini, V., Shirani, D., Eslami, A., Mohieddin, H., Shemshadi, B., Masoole, R. (2011). Epidemiological Aspects of Canine Dirofilariasis in the North of Iran. *Iranian Journal of Parasitology* 6: 73-80.

Razi-Jalali, M.H., Alborzi, A.R., Avizeh, R., Mosallanejad B. (2010). A study on *Dirofilaria immitis* in healthy urban dogs from Ahvaz, Iran. *Iranian Journal of Veterinary Research* 33: 357-362

Rishniw, M., Barr, S.C., Simpson, K.W., Frongillo, M.F., Franz, M., Alpizar, J.L.D. (2006). Discrimination between six species of canine microfilariae by a single polymerase chain reaction. *Veterinary Parasitology* 135: 303-314.

Scaramozzino, P., Gabrielli S., Paolo M.D., Sala M., Scholl F., Cancrini G. (2005). Dog filariosis in the Lazio region (Central Italy): first report on the presence of *Dirofilaria repens. Biomedcentral Infectious Diseases* DOI: 10.1186/1471-2334-5-75

Traversa, D., Cesare, A.D., Conboy, G. (2010). Canine and feline cardiopulmonary parasitic nematodes in Europe: emerging and underestimated. *Parasite & Vectors* DOI: 10.1186/1756-3305-3-62

Watier-Grillot, S., Marie, J.L., Cabre, O., Davoust, B. (2011). Survey of Canine Dirofilaria immitis Infection in New Caledonia. *Veterinary Medicine International* DOI: 10.4061/2011/380680