Susceptibility of *Anopheles stephensi* (Diptera: Culicidae) to *Dirofilaria immitis* (Spirurida: Onchocercidae)

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Summary. *Dirofilaria immitis* is endemic in the southern parts of Iran where there is a favourable environment for the development of *Anopheles stephensi*. The aim of the present study was to evaluate the susceptibility of *A. stephensi* as *D. immitis* vector under laboratory conditions. Anticoagulated dog blood containing 1,500 microfilariae ml^{-1} was artificially administered to 140 mosquitoes of the test group (T group), while 94 mosquitoes were left as a control (C group). Blood-fed mosquitoes of the T group were dissected for morphological and molecular analyses at pre-set time points to observe the developmental stage of *D. immitis*. The results showed that the average number of the microfilariae ingested per female in T group was 9.6. At the end of the study, 16 mosquitoes developed infective third-stage larvae of *D. immitis*, and 49 mosquitoes survived at end of incubation period, giving an estimated infection rate of 32.6% and the vector efficiency index of 17%. The infection rate and vector efficiency index suggested that *A. stephensi* could be considered as a potential vector of *D. immitis*.

Key words: dog heartworm, filariasis, microfilariae, molecular analysis, mosquitoes.

The dog heartworm, *Dirofilaria immitis* (Leidy), is a cause of the zoonotic filariasis with a cosmopolitan distribution (Simon *et al.*, 2009). This nematode needs a definite vertebrate host and a culicid, as a vector, to complete its life cycle. It primarily infects canines but may accidentally infect both humans and felines (Simon *et al.*, 2012; Khodabakhsh *et al.*, 2016). Human dirofilariasis, as an emerging zoonotic disease, has been reported increasingly from different parts of the world, including Iran (Negahban *et al.*, 2007; Mirahmadi *et al.*, 2017). Canine dirofilariasis, on the other hand, is endemic in Iran, and its prevalence has been reported to be as high as 33%. However, there are few data related to invertebrate hosts of *D. immitis* in Iran (Sadjjadi *et al.*, 2004; Azari-Hamidian, 2007). The presence of different competent zoophilic vectors in the areas where the prevalence of canine dirofilariasis is high implies that human population is at risk of infection (Simon *et al.*, 2005). Among culicid species, more than 77 species are assumed to be the potential vectors of *D. immitis* (Azari-Hamidian *et al.*, 2009). Few of them such as *Culex pipiens* (Linnaeus), *Aedes vexans* (Meigen), *A. albopictus* (Skuse) and *Anopheles maculipennis* (Meigen) are considered to be efficient vectors for transmitting the infective third-stage larvae (L3) in nature (Aranda *et al.*, 1998; Ferreira *et al.*, 2015). *Culex theileri* (Theobald) is the only reported mosquito in Iran that is able to be infected naturally with infective L3 of *D. immitis* (Azari-Hamidian *et al.*, 2009). The Malpighian tubules of some

mosquitoes are capable of inducing a melanotic response to the invasion of *D. immitis* larvae and providing resistance against this nematode (Bradley & Nayar, 1985). The vector competence is different among mosquito species, strains, or even individuals of the same strain (Serrao *et al.*, 2001). *Anopheles stephensi* is abundant throughout the southern part of Iran (Fig. 1) where the prevalence of dirofilariasis is high (Jafari *et al.*, 1996; Khedri *et al.*, 2014; Salahi-Moghaddam *et al.*, 2017). Previously, it was shown that *A. stephensi* could be infected by feeding on a dog infected with *D. repens* and subsequently, developed the ingested microfilaria (mf) into the infective larvae (Webber & Hawking, 1955). The current study was performed to analyse the susceptibility of the *A. stephensi*, as the dominant strain in Sistan and Baluchestan Province, for transmission of *D. immitis*.

MATERIAL AND METHODS

Mosquitoes' colony. The colony of mosquitoes was originated from eggs collected from Sarbaz city of Sistan and Baluchestan Province, the endemic region of *D. immitis* in the southeast of Iran (Khedri *et al.*, 2014). The larvae were reared to adults in emergent cages (50×50×50 cm) in an insectarium at 26 ± 1°C, with the relative humidity 80%, and 12L: 12D photoperiod. The emerged adults were maintained at the same conditions on 10% sucrose for 5-6 days for mating. The identity of the species of collected mosquitoes was examined at the National Insectarium, Malaria and Vector Research Group (MVRG), Biotechnology Research Centre (BRC), Pasteur Institute of Iran by means of morphological identification keys and a molecular analysis as described (Subbarao *et al.*, 1987; Azari-Hamidian & Harbach, 2009; Chavshin *et al.*, 2014). Briefly, for molecular identification, genomic DNA was extracted from samples using DNAzol® (Invitrogen, Gaithersburg, USA) according to the procedure supplied by the manufacturer. Amplification of the mtDNA-COII partial gene was performed using primer pairs COII-F (5'-ATGGCAACATGAGCAAATT-3') and COII-R (5'-GTATAAAACTATGATTAGC-3').

***Dirofilaria immitis* microfilariae.** The microfilaremic and amicrofilaremic canine blood samples were obtained by one of the authors from his previous study (Zarei *et al.*, 2016). The infected dog was a naturally infected 11-year-old mix breed. The presence of *D. immitis* in the samples was identified and confirmed by Knott's concentration technique (KCT) (Knott, 1939) and PCR (Rishniw *et al.*, 2006), respectively. The concentration of mf was determined by the mean value of ten counts of 20 µl blood smear under magnification 40×.

***Anopheles stephensi* infection.** A colony of 5-8-day-old female mosquitoes was selected and starved for 1 d prior to blood feeding. Two groups of female mosquitoes were fed using microfilariaemic blood (n = 140; test group, T) or amicrofilaremic blood (n = 94; control group, C). The mosquitoes from each group (T and C) were placed separately in cages 50×50×50 cm in size. All mosquitoes in test and control groups were fed using an artificial feeding apparatus (Cosgrove *et al.*, 1994) loaded with positive or negative blood samples containing heparin anticoagulant solution, respectively. The time exposure to blood for each group was one hour. Immediately after blood feeding, the unfed mosquitoes were removed from the cages. All engorged mosquitoes were fed with 10% sucrose solution.

Dissection of mosquitoes colonies. To determine the number of ingested mf, three mosquitos from the T group were killed within 24 h post infection and dissected. For a period of 16 d, the members of the engorged group T were examined at scheduled days (Table 1). The groups of mosquitoes, comprising 1-8 killed or naturally dead mosquitoes, were dissected daily to detect different stages of *D. immitis* larvae. The mosquitoes were killed by putting them in dry ice and dissected in phosphate buffer saline (PBS) solution for detection of *D. immitis* larvae under a stereo-microscope, and the development stages were determined as described previously (Taylor, 1960). After microscopic studies, the slides were rinsed with a few drops of PBS for subsequent molecular analysis. Molecular analysis was applied for each sample to monitor the presence of *D. immitis* infective/non-infective larvae using PCR (Ferreira *et al.*, 2015). For this purpose, DNA extraction was performed separately for both head+thorax and abdomen in order to differentiate between *D. immitis* infective and infected larvae. The amplification of ITS2 region was carried out using DIDR-F and DIDR-R primers.

Susceptibility analysis of *A. stephensi* for transmission of *D. immitis*. The mortality rate of the mosquitoes was calculated daily, within 10 days post infection (dpi) and until 16 dpi as the percentage of naturally dead mosquitoes over the total blood-fed mosquitoes in each cage. The significant differences in mortality rates between the two cages were calculated using Fisher's exact test; *P* values below 0.05 were considered as statistically significant. The infection rate (IR) was defined as the number of blood-fed mosquitoes with infective L3 in their body multiplied by 100 and divided by the number of surviving mosquitoes at the end of

Fig. 1. Map of Iran indicating the distribution of *Anopheles stephensi* and heartworm disease using various markers.

Table 1. *Anopheles stephensi* specimens examined microscopically for microfilaria (mf) and larvae stages of *Dirofilaria immitis* at scheduled days post infection (dpi).

dpi	Total mosquitoes						
	Examined	Killed: total larval stage counted/ mosquito infected; mean			Naturally dead: total larval stage counted/ mosquito infected; mean		
		Mf	L2	L3	Mf	L1	L3
1	3	29/3; 9.6	0	0	–	–	–
2	5	–	–	–	25/5; 5	0	0
3	6	–	–	–	25/2; 12.5	18/3; 6	0
4	7	–	–	–	20/4; 5	17/3; 5.6	0
5	6	–	–	–	0	13/3; 4.3	0
6	6	–	–	–	0	20/4; 5	0
7	8[a]	0	0	0	0	12/4; 3	0
8	4	0	8/3; 2.6	0	–	–	–
9	7[b]	0	0	0	5/3; 1.6	0	0
10	6	0	0	8/2; 4	–	–	–
11	6	0	3/3; 1	0	–	–	–
12	7	0	0	8/3; 2.6	–	–	–
13	8[c]	0	4/1	9/2; 4.5	0	3/1	10/1
14	4[d]	0	0	0	0	0	8/3; 2.6
15	4	–	–	–	0	0	9/3; 3
16	3	–	–	–	0	0	8/2; 4
Total	90	29/3; 9.6	15/7; 2.1	25/7; 3.5	75/14; 5.3	83/18; 4.6	35/9; 3.8

Mosquitoes found dead at the bottom of the cage. Mean indicates the mean number of larvae per infected mosquito.
[a] – three killed and five naturally dead, [b] – four killed and three naturally dead, [c] – five killed and three naturally dead, [d] – one killed and three naturally dead larvae.

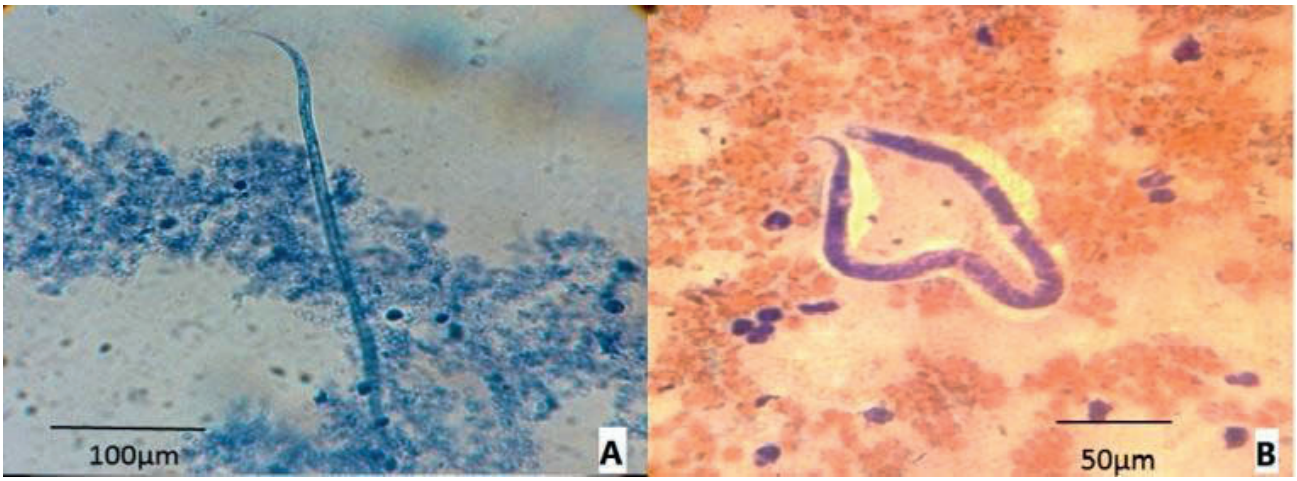

Fig. 2. Morphological identification of the developmental stages of *Dirofilaria immitis* in dog and *Anopheles stephensi*. (A) Microfilariae of *D. immitis* in the dog blood. (B) Microfilariae of *D. immitis* in the abdomen of *A. stephensi*.

incubation period. The vector efficiency index (VEI) was calculated as the average number of L3 developed in the mosquitoes after extrinsic development period, multiplied by 100 and divided by the mean number of ingested mf as described before (Kartman, 1954; Silaghi *et al.*, 2017). The extrinsic development time of *D. immitis* to the L3 stage, based on the *Dirofilaria* development unit, was determined to be 10 d at the temperature of our insectary.

Bioinformatics analysis. Sequencing of the amplified gene of samples was performed at the Bioneer Coporation (Seoul, Korea). The obtained sequences were analysed and manually edited using BioEdit software (version 5.0.6; North Carolina State University). Sequence homology analyses were accomplished using the NCBI databases with BLAST search tool (http://www.ncbi.nlm.nih.gov/).

RESULTS

The morphological and molecular analyses demonstrated that only *A. stephensi* was present. The amplified sequence of *A. stephensi* was deposited in the GenBank with the accession number of KY863454. KCT and morphometric analysis indicated that the suspected dog sample was positive for *D. immitis* infection (Fig. 2A).

The molecular results confirmed the morphological identification of *D. immitis* larvae. The BLAST search identified our sequence as *D. immitis* and showed 99% homology with other sequences of *D. immitis* available in the NCBI database (*e.g.*, AF217800). The amplified sequence was deposited in the GenBank with the accession number of KY863453. After feeding *A. stephensi* with blood meal containing 1,500 mf ml^{-1}, different percentages of the fed female mosquitoes were observed in two groups (64% and 85% in T and C, respectively). The average number of ingested mf per female was 9.6 (ranged from 1 to 18) in T group (n = 90, excluding the three mosquitoes sacrificed for the calculation of the mf intake). The mortality rates of *A. stephensi* in groups T and C at the end of study were 59% (n = 51) and 25% (n = 19), respectively ($P < 0.05$), being higher during the first 10 dpi, *i.e.*, 44% in group T *vs* 13% in group C; $P < 0.05$ (Fig. 3).

Different developmental stages of *D. immitis* (Fig. 2B) in killed and naturally dead mosquitoes are shown in Table 1. The larvae were discriminated based on a morphological identification key as the sausage-like larvae (L1) as well as second (L2) and third (L3) stage larvae. Different parts of *A. stephensi* were screened for *D. immitis* larvae using molecular analyses, which confirmed the morphological identification of *D. immitis* larvae. The L1 began to be identified at 3 d post infection (dpi) and persisted until 13 dpi. L1 was found melanized and dead at 6 dpi but L3 was identified from 10 to 16 dpi. These infective larvae were observed emerging from the proboscis of four mosquitoes. The average number of infective larvae developed in the mosquitoes after extrinsic development period was 1.5. At the end of the study, 16 mosquitoes developed L3, and 49 mosquitoes survived at the end of incubation period, giving an estimated IR of 32.6% and VEI of 17%.

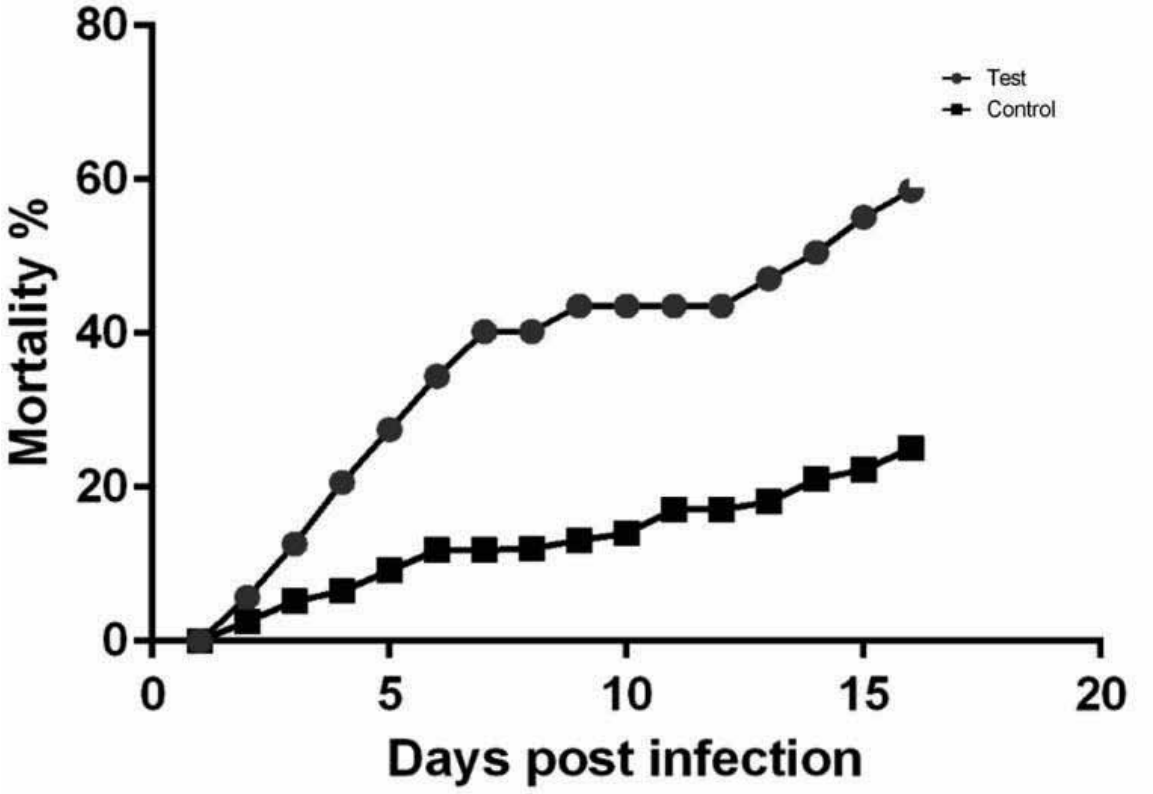

Fig. 3. The mortality rate of test and control groups of the *Anopheles stephensi*.

DISCUSSION

We found that *A. stephensi* mosquitoes isolated from Iran was susceptible to infection with *D. immitis*. To the best of our knowledge, this is the first study to evaluate the susceptibility of wild caught mosquitoes from Iran to *D. immitis* in the laboratory. The mortality rates of *A. stephensi* in T and C groups were 59% and 25%, respectively. The higher rate of mortality in the T group may be due to the development of larvae in the mosquitoes (Montarsi *et al.*, 2015). The rate of mortality in our study is similar to the rates reported for *C. quinquefasciatus* (Say) and *A. aegypti* (Linnaeus) in the previous studies (Serrao *et al.*, 2001; Carvalho *et al.*, 2008). The melanization process only occurred in L1 but not in other stages of *D. immitis* larvae; this result is consistent with the results of other investigations (Nayar & Knight, 1999; Montarsi *et al.*, 2015). The probable role of *A. stephensi*, as a competent vector for *D. immitis*, is also determined using our IR and VEI values (32.6% and 17%, respectively). A mosquito species is assumed to be a competent vector for infection when having the IR value of 10% and VEI of 9% (Kartman, 1953). In this regard, *C. quinquefasciatus*, which was fed with blood containing 1,913 mf ml^{-1} and showing IR of 46% and VEI of 7.8%, was considered as 'refractory' (Carvalho *et al.*, 2008). Recently, *A. koreicus* has been regarded as a competent vector of *D. immitis* that indicates high values of IR and VEI (68.2% and 25.2%, respectively) (Montarsi *et al.*, 2015). Vectorial capacity is a measure not only of vector competence (the ability to become infected and transmit the pathogen) but also of vector density, host preference, feeding frequency, longevity, etc. (Grieve *et al.*, 1983). Despite the probable susceptibility of *A. stephensi*, more investigations on host preference and host-seeking activity are needed to designate this mosquito as a competent vector for canine and human dirofilariasis.

In conclusion, *A. stephensi*, which is a widely distributed species in the Middle East, could be a competent vector of *D. immitis* in that region. The result obtained from the present study demonstrates that the *A. stephensi* represents great transmission potential for *D. immitis* and could be considered as a competent vectors for canine dirofilariasis or probably human dirofilariasis in the southern parts of Iran. Nevertheless, experimental and field evidences are necessary to confirm the role of *A. stephensi* in transmission of human and canine

dirofilariasis. As illustrated in Fig. 1, the distribution of heartworm disease and *A. stephensi* population overlaps only in the southern part of Iran. Therefore, more studies are required to determine the potential vectors of dirofilariasis in other areas of the country.

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R. Solgi, S.M. Sadjjadi, M. Mohebali, N.D. Djadid, A. Raz, S. Zakeri and Z. Zarei. Способность *Anopheles stephensi* (Diptera: Culicidae) переносить *Dirofilaria immitis* (Spirurida: Onchocercidae).

Резюме. Нематоды *Dirofilaria immitis* обычны в южных провинциях Ирана, где складываются походящие условия для развития комаров *Anopheles stephensi*. Проведено исследование способности *A. stephensi* служить вектором-переносчиком для нематод *D. immitis* в лабораторных условиях. Кровь собак с добавлением антикоагулянтов, содержащие 1,500 микрофилярий в 1 мл была использвоана для заражения голодавших самок комаров. Всего 140 комаров было использовано для эксперимента (Т группа) и 94 особи комаров служили контролем (С группа). Комаров из Т группы вскрывали через определенные промежутки времени для морфологического и молекулярного выявления развивающихся стадий *D. immitis*. Результаты показали, что в среднем каждая самка комара заглатывала 9.6 микрофилярий. В конце эксперимента было установлено, что 49 самок пережили условия данного опыта (32.6%) и у 16 самок комаров развились инвазионные личинки 3-й стадии *D. immitis.* Таким образом, индекс эффективности переноса составил 17%. Сделан вывод о способности *A. stephensi* служить вектором для *D. immitis*.