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Detection of natural infection of *Leishmania donovani* (Kinetoplastida: Trypanosomatidae) in *Phlebotomus argentipes* (Diptera: Psychodidae) from a forest ecosystem in the Western Ghats, India, endemic for cutaneous leishmaniasis

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ABSTRACT

A new focus of transmission of *Leishmania donovani* causing cutaneous manifestations (CL) was reported by us earlier, in the Western Ghats region of Thiruvananthapuram district, Kerala, India. 12,253 sand fly specimens, comprising of three species belonging to the genus *Phlebotomus* (24.7%) and 16 species belonging to the genus *Sergentomyia* (57.3%) were collected from the region during 2012–2014. Among *Phlebotomus* species, *Phlebotomus argentipes* was found predominant (77.3%), followed by *Phlebotomus colabaensis* (21.7%) and *Phlebotomus stantoni* (1.6%). From these collections, 793 *P. argentipes* (88 pools), 123 *P. colabaensis* (31 pools) and three *P. stantoni* (three pools) female specimens were processed for detection of natural infection with *L. donovani* parasites using a multiple genetic marker (kinetoplast DNA; 3′UTR of HSP70 gene & HSP70 gene) approach. Five pools of *P. argentipes* specimens (Unfed (one), Fulfed (one) and Gravid (two)) among these, were found positive for *L. donovani* infection. HSP70 gene sequences of the parasites in the vector species was found genetically identical with the human isolates reported earlier, evincing the role of *P. argentipes* in the transmission of CL in this region. This is the first finding of natural infection of *P. argentipes* with *L. donovani* (causing CL) from India.

1. Introduction

Leishmaniases are caused by the parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) and transmitted by female Phlebotomine sand fly species (Diptera: Psychodidae). About 800 species of Phlebotomine sand flies are prevalent throughout the world and of which 98 have been implicated to be the vectors of more than 20 different species of *Leishmania* parasites (Alvar et al., 2012; Maroli et al., 2013). Leishmaniases have been reported from 98 countries, both in the tropical and subtropical regions. At present, more than 350 million people live at risk of infection of leishmaniases and an estimated 12 million cases exist worldwide. About 1.5–2 million new cases are estimated to occur annually, of which 1–1.5 million cases are cutaneous leishmaniasis (CL) and 0.5 million cases are visceral leishmaniasis (VL) (WHO, 2010).

While *Leishmania major* and *Leishmania tropica* are the parasite species involved in CL in old world countries, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, *Leishmania peruviana*, *Leishmania mexicana*, *Leishmania amazonensis* and *Leishmania venezuelensis* are found responsible for this manifestation in the new world countries (Alrajhi, 2003). In the Sahara and Saudi Arabia, *Phlebotomus bergeroti* has been suspected to be the vector of *L. major* (Nadim et al., 1979). *Phlebotomus duboscqi* common in houses, was found infected with *L. major* in Niger and Senegal (Dedet et al., 1980). *Phlebotomus papatasi*, a domestic species and an aggressive man-biter was reported to transmit *L. major* in Central Asia. *Phlebotomus sergenti* was reported to be specific towards *L. tropica infection* in India and Afghanistan, while *Phlebotomus longipes* and *Phlebotomus pedifer* to *L. aethiopica* in Ethiopia.

In India, CL was confined to hot, dry north-western region, particularly in the western Thar Desert of Rajasthan (Kumar et al., 2007). Recently, CL cases were recorded in Himachal Pradesh (Sharma et al., 2005, 2009). In southern India, until 1988 no case of CL was recorded. Two cases of CL were accounted for the first time during 1988 in Thiruvananthapuram District, Kerala, reportedly imported from Saudi Arabia (Lohidakshan et al., 1988) and the first indigenous case was reported from Malappuram District,

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Table 1
Number of sand flies obtained from resting collections made from the Kani tribal dwellings.

Tribal settlements	Total. No. of suspected CL cases	*P. argentipes*		*P. colabaensis*		*P. sintoni*		*Sergentomyia* spp.	
		Male	Female	Male	Female	Male	Female	Male	Female
Ariyavila	1	37	113	9	32	1	7	66	312
Chonambara	0	6	17	6	21	0	0	109	516
Kamalagam	0	12	36	13	47	0	0	182	863
Kombidi	1	35	107	6	21	0	0	304	1436
Mela Amala	12	115	192	33	117	2	11	89	421
Keezhe Amala	11	203	844	61	214	4	19	245	1204
Podium	2	12	36	2	7	0	0	342	1621
Ayiram Kala	0	71	143	4	14	0	0	69	313
Cherumangal	0	18	54	6	22	0	5	186	902
Kunnadi	0	73	219	1	3	0	0	21	21

Table 2
Sand fly samples screened for natural infection with *Leishmania* parasites, if any, using molecular methods.

Area	Species	UF	FF	SG & G	Total specimens processed	Pools tested	No. of pools +ve
Keezheamala	*P.argentipes*	348	135	306	789	86	5
	P.colabaensis	50	26	33	109	28	nil
	P.stantoni	0	1	2	3	3	nil
Meleamala	*P.argentipes*	3	0	1	4	2	nil
	P.colabaensis	7	3	4	14	3	nil
Grand total	*P.argentipes*	351	135	307	793	88	5
	P.colabaensis	57	29	340	123	31	nil
	P.stantoni	0	1	2	3	3	nil

after two years (Muhammed et al., 1990). Very recently, leishmaniasis cases with cutaneous manifestation were reported from the Kani tribes, settled on the southernmost part of the Western Ghats, in Thiruvananthapuram District, Kerala, India (Kumar et al., 2015), where, sand flies were also abundant (Srinivasan and Subramanian, 2015). This study was carried out to find out the vector species involved in transmission of CL infection in the settlement of Kani tribes.

2. Materials and methods

2.1. Study area

Description of the Kani tribe settlements was already described (Srinivasan et al., 2015). Kani tribes, one of the ancient ethnic groups of India and the hereditary proprietors of the land are scattered in many settlements on the southernmost part of the Western Ghats. The settlements ($n = 28$) are spreading over at different altitude ranging between 267 and 2,425 ft. Two settlements namely, Melaamala and Keezheamala are located on the western side of the Neyyar dam reservoir. Transport, communication, electricity and protected drinking water facilities to these settlements are inadequate. The local government has made provisions to cater the basic health needs by setting up a Primary Health Centre and to provide education to tribal children by opening schools in the foothill area. The tribal population collect non-timber forest products, such as honey, bee-wax, medicinal plants, gums etc. and sell in local market, located at the foothill and get things needed for their livelihood. Majority of the dwellings of the tribes are made of natural materials like dry leaves, wooden logs, grasses, bamboos and mud (72.6%) and the remaining with cement and bricks (23.8%).

2.2. Sand fly collection and morphological identification

Sand fly collections were made in 10 tribal settlements (08°37′49.7″N; 77°11′29.7″E & 08°36′51.2″N; 77°09′54.9″E) with altitude, ranging from 267 to 2425 ft during June 2012–May 2014, using hand-held aspirator. Since, the sand fly population was abundant and the number of CL suspected/cases were found to be relatively larger in two of the 10 tribal settlements, namely, Keezheamala (08°33′10.0″N, 77°12′07.2″E, altitude 733 ft) and Meleamala (08°33′52.8″N, 77°11′29.7″E, altitude 1221 ft), sand fly females collected from these settlements were used to detect natural infection.

Each female sand fly was dissected and head and last three segments of abdomen were mounted on glass slides. The specimens were identified to species, following the standard keys (Lewis, 1978). Individual code number was assigned to each specimen. The remaining portions of each dissected specimen, namely, thorax and major portion of abdomen were preserved in 1.5 ml Eppendorf safe-lock micro-centrifuge tubes containing 70% alcohol (50 μl) and used for PCR assay.

2.3. Extraction of total DNA

Sadlova et al. (2013) reported that *Sergentomyia* sp. are not competent vectors for *Leishmania donovani* and other *Leishmania* species pathogenic to humans. *Leishmania* parasites ingested by these sandflies, along with blood meal, failed to colonize the thoracic midgut and were defecated. Hence, only the females of *Phlebotomus* genus ($n = 919$) (*Phlebotomus argentipes*, *Phlebotomus colabaensis* and *Phlebotomus stantoni*) were used for detection of natural infection (Table 1). The female specimens were grouped according to gender and abdominal conditions, namely, un-fed (UF) full-fed (FF), semi-gravid (SG) and gravid females (G) based on Sella's stage (WHO, 1975). The specimens were pooled according to the area of collection. The pool size ranged from 1–10 specimens. Altogether, 793 *P. argentipes* female specimens in 88 pools, 123 *P. colabaensis* in 38 pools and 3 specimens of *P. stantoni* in 3 pools were processed (Table 2).

The specimens thus pooled were transferred again to 1.5 ml eppendorff tubes containing the lysis solution and homogenized, with Kontes pellet pestles using its motor for roughly about 5–10 minutes or until a uniform homogenate was obtained. The DNA extraction was carried out using, SIGMA DNA extraction Kit (Sigma–Aldrich, USA), following the manufacturer's protocol. The extracted total DNA was eluted from the silica columns to about