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PCR Based Identification Of Five Indian Phlebotomine Species From New Foci

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Abstract

Phlebotomine sandflies are key vectors in the transmission of leishmaniasis, and their accurate identification is essential for effective vector surveillance. Traditional morphological methods often fail to differentiate closely related species, especially in newly emerging foci where species diversity is high. The present study employs PCR-based molecular techniques to identify five Indian phlebotomine species collected from new endemic areas. Species-specific primers targeting mitochondrial and ribosomal gene regions were used to amplify diagnostic fragments. Distinct PCR profiles successfully differentiated *Phlebotomus argentipes*, *P. papatasi*, *P. sergenti*, *P. major* and *P. salehi*. The findings demonstrate that PCR assays are reliable, rapid and highly sensitive tools for species identification, contributing significantly to early vector detection, mapping of new foci and improved leishmaniasis control strategies in India.

Keywords- Phlebotomine sandflies, PCR-based identification, *Phlebotomus* species, Molecular diagnostics, New endemic foci, Vector surveillance, Leishmaniasis, Species-specific primers

Introduction

Leishmaniasis is a heterogeneous group of diseases with a history of more than 4500 years [1]. The causative agent of this malady is the protozoan species *Leishmania*. This disease results in heightened provocation because of the life cycle of pathogens, including vectors from the class Nematoceran, usually female sandflies of the Phlebotominae family, and various mammalian hosts [2]. Approximately 20 *Leishmania* species and 90 sandfly species have been reported with three main forms of the disease in humans: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) or kala-azar, and mucocutaneous leishmaniasis (MCL). As per WHO, CL is the most prevalent, VL is the most severe form, and MCL is the most disabling form [3].

Most cases of leishmaniasis have been reported from various parts of the world, including Mexico, Central America, parts of South America, Southern Europe, Central and Southern Asia, the Middle East, and Africa, with annual increments of approximately 700,000-100,000 [3]. A major part of this invasion-linked malady has been documented in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil [4].

In 2020, India was declared endemic with 18% Kala-Azar (VL) cases. The 54 districts of four states-Bihar, Jharakhand, Uttar Pradesh, and West Bengal were majorly endemic areas. VL has also been found to cover other parts of the country, such as Delhi, Punjab, Gujarat, Sikkim, Assam, and Madhya Pradesh, for sporadic cases. India has shown 98% decline in cases from 1992 (77,102 cases) to 2021 (1,275 cases), but the rise of new foci in the Himalayan and sub-Himalayan regions is becoming a point of concern [5-6].

According to Sharma et al. 2003, leishmaniasis was absent in Himachal before 1988, but 38 cases of CL were reported during the 1988-2001 period because of migrations from endemic parts of country [7]. In 2005, Sharma et al. confirmed 285 cases of LCL until January 2005 in their study highlighting regions of Pooh subdivision of Kinnaur district to Kumarsain subdivision of Shimla district with adjoining Nirmand subdivision, Kul1u district comprising 86 villages [8], illustrating 161 new cases of VL [9]. Again, Sharma

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et.al in another study in 2009 confirmed 100% VL and 31.8% LCL human patients via rapid rK39 immunochromatographic dipstick test [10]. Kumari & Garg, 2018 reported 337 cases of CL mainly from Shimla, Kullu, and Kinnaur districts within a span of one year only , i.e., 2016-2017 [11]. As presented in **Fig. 1.**

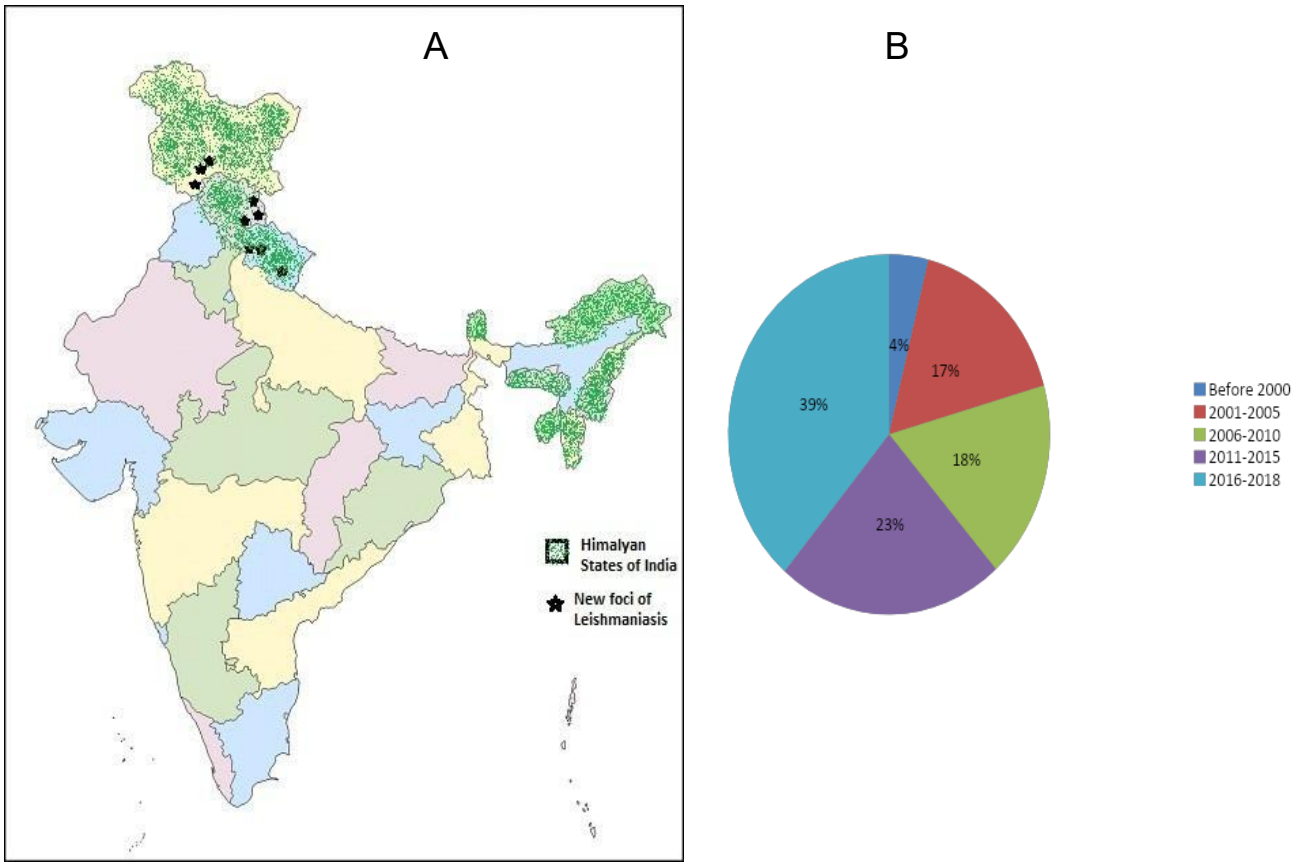

Fig. 1: A) New foci of Leishmaniasis among Himalayan states of India . B) Leishmaniasis cases reported from new foci of the Himalaya, India: which is observed to be increasing from last two decades.

This neglected tropical disease has spread from their endemic areas to different regions of Himachal [12-13]. Researchers have even illustrated the recent adaptation of sandfly vector species to high altitudes, due to global rise in temperature and anthropogenic activities [13]. Therefore, our study was aimed to investigate Leishmania vectors and their modes of invasions from endemic areas to new geographical locations in India.

Methodology

Collection of Specimens:

Between 2013 to 2017, sand flies were collected from various districts with reported cases of Leishmaniasis in the Himalayan regions of Jammu and Kashmir, Himachal Pradesh, and Uttarakhand. Methods such as mouth aspirators, CDC light traps, and handhold alcohol-dipped brushes were used to collect specimens indoors and outdoors [8, 14-16] .

Morphological & Anatomical identification:

The collected sandfly specimens were sorted, dissected, and taxonomically identified using Lewis identification key [17]. Peculiar taxonomic characteristics were considered while morphologically identifying

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the fly, including the shape of the cibarium and the number of cibarial teeth, pharyngeal armature, shape and segmentation in the spermatheca, and spermathecal ducts.

Molecular identification:

Molecular markers based on mitochondrial DNA sequence were used to investigate the diversity and genetic inter relationship in between diverse populations of sandfly **[15]**. The insect DNA barcode locus, a PCR based amplified segment of mitochondrial cytochrome oxidase I gene (mt-COI), was used as a molecular marker to determine the genetic relationship among diverse strains of Leishmania **[16]**. A total of 762 specimens of sand fly (*Phlebotomus*) were analyzed. For each specimen of sand fly collected, genomic DNA was isolated using E.Z.N.A ® Insect DNA isolation kit (Omega Bio-Tek) in accordance with the manufacturer's instructions. Extracted DNA samples were stored at -20 ºC until further use. The quality of the extracted DNA samples was assessed via Nanodrop (Thermo Scientific Nanodrop 2000).

Mt-COI barcoding was achieved by targeting PCR based amplification of specific fragments of mt-DNA. The amplification primers included a forward primer (5'-AGGCTCATTCAGTCGCTTTC-3') and a reverse primer (5'-AAGCTTATGACTCAACAC TT-3') **(Reference)**. The PCR based amplification was performed in 25 µL of total volume, containing 1 µL of DNA template, 2.5 µL of 10x PCR buffer (Mg^{2+} free), 4 µL of dNTP mix (2.5 mM of each dNTP), 0.2 µL of TaKaRa LA Taq® DNA Polymerase, 0.75 µL of each primer (10 mM), and 16 µL of ddH_2O. Thermocycler based amplification of the mixture was carried out in accordance to the following steps: ▪ Initial denaturation for 1 min at 94 ºC. ▪ Primer Annealing at 45 ºC for 90s. ▪ Extension at 72 ºC for 1 min. The process was repeated for 40 cycles with increasing annealing temperature of 0.5 ºC for every cycle, in order to maintain the fidelity of the amplification cycle. The final extension step was performed at 72 ºC for 5 min **[16]**.

PCR based amplification product (5 µL) were analyzed by agarose gel electrophoresis (1x TAE), along with a DNA ladder. Electrophoresis was performed at 150 V for 35 min, and the amplified PCR products were visualized using EtBr under UV light. The separated DNA band was eluted out and transferred to a nitrocellulose sheet of paper via southern blotting [18]. The experiments were performed in triplets to minimize the chances of any non specific amplification of mt-COI barcoding DNA sequences, by inappropriate binding of primers.

Phylogenetic analysis:

Phylogenetic analysis was performed to infer the evolutionary history using maximum-likelihood method in MEGA software (Molecular Evolutionary Genetics Analysis, version 11.0.13-1). Mitochondrial COI sequences of five sandfly species: *Phlebotomus papatasi* (accession no. KY701781.1), *Sergentomyia eadithae* (accession no. KY883628.1), *Sergentomyia minuta* (accession no. KY883630.1), *Sergentomyia clydei* (accession no. KY883631.1), and *Phlebotomus argentipes* (accession no. KY883622.1) were identified by Nucleotide BLAST on NCBI and forwarded for phylogenetic analysis. The sequences were trimmed to use only 585 positions due to missing positions.

Result

A total of 762 specimens were collected during the study. Based on morphological identification 198 were confirmed to be females and 564 were males. Of all female flies collected during the study, 33 were recognized as engorged females, whereas the rest were unfed.

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Refer to Table 1.

Table 1: Collection details				
S. No.	**Place of Collection**	**Total engorged female specimens**	**Total unfed female specimens**	**Total male Specimens**
1.	Uttarakhand	03	02	05
2.	Jammu & Kashmir	20	106	456
3.	Himachal Pradesh	10	57	103
TOTAL		**33**	**165**	**564**

Morphological and molecular identification approaches identified five species in the study, i.e., *Phlebotomus argentipes* Annandale & Brunetti*, P. papatasi* Scopoli, *Sergentomyia punjabensis* Sinton*, S. babu* Annandale*, and S. clydei Sinton, and* estimated their numbers as 61, 67, 277, 352, and 5, respectively. Of these identified specimens, P. papatasi Scopoli, P. argentipes Annandale & Brunetti, and S. babu Annandale have been reported as provoking species by many researchers.[19][20][21] Refer **to Fig. 2A** Phylogenetic tree in which these five species are aligned to confirm the homology of their mitochondrial COI. **Refer to fig.2B.**

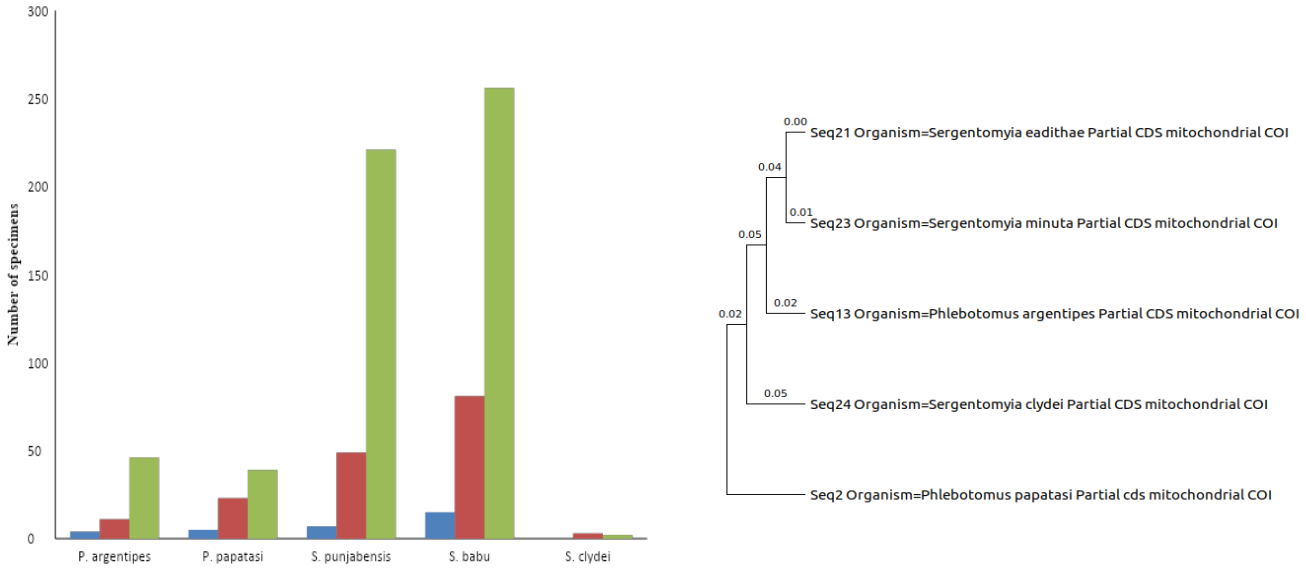

Fig. 2 A) Details of Taxonomically Identified specimens: where range of *S. punjabensis* and *S. babu* has been found more. **B) Phylogenetic tree of mitochondrial COI:**islolated from new foci in Himalyan regions of India. Tree includes species: *Phlebotomus papatasi* (accession no. KY701781.1), *Sergentomyia eadithae* (accession no. KY883628.1), *Sergentomyia minuta* (accession no. KY883630.1), *Sergentomyia clydei* (accession no. KY883631.1), and *Phlebotomus argentipes* (accession no. KY883622.1). Number along branches indicates bootstrap values.

Discussion

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Six species of Nematoceran, sandfly, were found in the new focus, which were observed earlier in the endemic states of India. These were the low-altitude states of Bihar, West Bengal, Jharkhand, and Uttar Pradesh.[22] It is observed that the initial invasion of Leishmaniasis in India occurs through trade routes. The dispersion of these vectors has been suggested as the effect of various factors such as deforestation, urbanisation, agricultural development, migration of non-immune people to endemic areas, road construction or man-made activities in which humans intrude into the habitat of the vector and new settlements in zoonotic foci are responsible for the spread of the disease to new focal zones [23, 24, &25].

Ghosh et al suggested that soil properties, i.e., moisture, pH, temperature, and soil organic carbon content, also have an impact on the distribution of sandflies. Generally, high clay content in the soil and an average pH of 9.0 are significant features of the areas where leishmaniasis is prevalent in India.[26] Kesari et al vitalised the above fact by observing that the soil pH and moisture content of endemic areas are comparatively higher than those of non-endemic areas [27]. Picado et al and Singh et al reported that temperature, rainfall, and humidity also affect the seasonality and distribution of vector species. [28][29]

In continuation of the aforementioned factors, changes in climatic conditions also have a significant impact on the adaptation of the vectors in the new foci. According to Sarkar et al., the systematic temperature increment i. e. 0.01-0.04ºC per year, according to the ICIMOD scenarios will allow tropical diseases to expand their range to higher elevations at which they did not occur before, and this could also be a cause of the upward spread of Leishmaniasis.[30][31] Savoia also stated the need to control pollution so that its negative impact on global temperature and soil pH can be minimised. [32].

According to a report by the World Health Organization (1990), the emergence of sporadic cases of Cutaneous Leishmaniasis in Himachal Pradesh was due to anthropogenic activities, such as the construction of roads, horticulture development, and establishment of new residential colonies, leading to clearing of forests and intrusion into the sylvania cycle of the vector species [5].

This research develops a clear vision of the severity of the disease in Himalayan regions where Leishmaniasis cases were negligible before two decades. Identification of the vector species and knowledge of the mode of their transmission to new focal zones will help in the development of preventive and control measures for the disease

Mt-COI based analysis was preferred over nuclear DNA sequences, due to its multiple copy number per cell, and a faster rate of evolution in animals. Furthermore the mt-COI do not participate in crossing over, as well as lack introns, so remains conserved from one generation to the other [16]. Thus primars can be effectively designed to work with a diverse range of Leishmania species and comparative phylogenetic analysis ensured [16]. The method is of immense importance in case of non-model organisms where limited investigation has been made.

Conclusion

Both vectors and pathogens require adaptations in new foci, which can be acquired by adaptations at the molecular level. Therefore, we adopted a morpho-molecular approach for species identification. Two species of *Phlebotomus* and three species of *Sergentomyia* i. e. *Phlebotomus argentipes* Annandale & Brunetti, *P. papatasi* Scopoli and *Sergentomyia punjabensis* Sinton, *S. babu* Annandale and *S. clydei* Sinton were identified during the research using morpho-molecular assays. The current status of invasion of Leishmaniasis vectors to the new focal zones is interpreted and their mode of transmission is also discussed in detail to develop an understanding of the grievousness of the disease in the regions and the need to develop control strategies for the disease, particularly in the Himalayan regions.

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