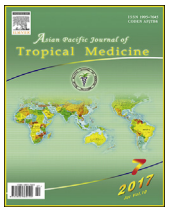
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm



Case report http://dx.doi.org/10.1016/j.apjtm.2017.07.015

First report on molecular characterization of *Leishmania* species from cutaneous leishmaniasis patients in southern Khyber Pakhtunkhwa province of Pakistan

Mubbashir Hussain[1✉], Shahzad Munir[2✉], Sultan Ayaz[3], Bahar Ullah Khattak[1], Taj Ali Khan[1], Niaz Muhammad[1], Muhammad Anees[1], Hazir Rahman[1], Muhammad Qasim[1], Muhammad Ameen Jamal[4], Irfan Ahmed[5], Kashif Rahim[6], Humaira Mazhar[1], Noha Watanay[7], Mohamed Kasbari[8]

[1]*Vector Borne Diseases Laboratory, Department of Microbiology, Kohat University of Science and Technology, Kohat, KP 26000, Pakistan*

[2]*Department of Plant Pathology, Faculty of Plant Protection, Yunnan Agricultural University, Kunming 650201, China*

[3]*College of Animal Husbandry and Veterinary Sciences, Abdul Wali Khan University Mardan, Pakistan*

[4]*College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China*

[5]*Yunnan Provincial Key Laboratory of Animal Nutrition and Feed, Yunnan Agricultural University, Kunming 650201, China*

[6]*Beijing Key Laboratory of Genetic Engineering Drug and Biotechnology, Institute of Biochemistry and Biotechnology, College of Life Sciences, Beijing Normal University, Beijing 100875, China*

[7]*US Naval Medical Research Unit-3, (NAMRU 3), Cairo, Egypt*

[8]*French Agency for Health and Safety Animal Health Laboratory, Maisons-Alfort, France*

ARTICLE INFO

Article history:
Received 22 Jan 2017
Received in revised form 28 Apr 2017
Accepted 20 Jun 2017
Available online 27 Jul 2017

Keywords:
Cutaneous leishmaniasis
ITS-PCR
RFLP
Leishmania tropica
Khyber Pakhtunkhwa

ABSTRACT

Objective: To report presence of *Leishmania major* in Khyber Pakhtunkhwa of Pakistan, where cutaneous leishmaniasis (CL) is endemic and was thought to be caused by *Leishmania tropica* only.

Methods: Biopsy samples from 432 CL suspected patients were collected from 3 southern districts of Khyber Pakhtunkhwa during years 2011–2016. Microscopy on Giemsa stained slides were done followed by amplification of the ribosomal internal transcribed spacer 1 gene.

Results: *Leishmania* amastigotes were detected by microscopy in 308 of 432 samples (71.3%) while 374 out of 432 samples (86.6%) were positive by ribosomal internal transcribed spacer 1 PCR. Subsequent restriction fragment length polymorphism confirmed *L. tropica* in 351 and *L. major* in 6 biopsy samples.

Conclusions: This study is the first molecular characterization of *Leishmania* species in southern Khyber Pakhtunkhwa. It confirmed the previous assumptions that anthroponotic CL is the major CL form present in Khyber Pakhtunkhwa province. Furthermore, this is the first report of *L. major* from a classical anthroponotic CL endemic focus identified in rural areas of Kohat district in southern Khyber Pakhtunkhwa.

✉First and corresponding author: Mubbashir Hussain, Department of Microbiology, Kohat University of Science and Technology, Kohat, KP, 26000, Pakistan. Tel: +92 3334380950; Shahzad Munir, Faculty of Plant Protection, Yunnan Agricultural University, Kunming 650201, Yunnan, China. Tel: +86 15559883719.
E-mails: mubashirbangash@gmail.com, mubbashir@kust.edu.pk, shazid_10@yahoo.com
Peer review under responsibility of Hainan Medical University.

1. Introduction

Leishmaniasis is categorized as a class I disease by the World Health Organization [1], and the three important clinical forms are visceral, cutaneous and mucocutaneous [2,3]. In Pakistan, cutaneous leishmaniasis (CL) is estimated to affect 15 000–20 000 people annually [4]. CL cases are widely distributed in all provinces of Pakistan and are being reported from Sindh, Balochistan, Khyber Pakhtunkhwa (KP) and Punjab [5–7]. The KP province is located in the north-west of Pakistan and shares about 2300 km long border (Durand line) with Afghanistan. Kabul the capital of Afghanistan which is located at distance of 290 km from Peshawar (KP) is thought to have the highest incidence of CL in the world, with an estimated 67 500–200 000 cases annually among population of 3.7 million individuals [8]. After Afghan–Russia war, millions of Afghan refugees migrated to Pakistan. In 1990, the official figures of Afghan refugees population was 3.27 million but the actual number was more than 5 million Afghan refugees with majority of them residing in different camps and others illegally in different areas of Balochistan province and KP province [9]. In 1997, a major outbreak of anthroponotic CL was reported from Timargara Afghan refugee camp located in Dir District north western Pakistan [10]. During a survey of Afghan Refugee camps and some local Pakistani population in KP in 1998, the average annual anthroponotic CL infection rate was estimated to be 4.6 cases per 1 000 persons every year [11]. Although parasite identification was not carried out in that study and it is assumed that *Leishmania tropica* (*L. tropica*) is the etiologic agent seems probable because of its characteristic dry form lesions and also because of endemicity of anthroponotic CL in South Asia, Central Asia [10,11] and Palestine [12].

However, recent molecular studies have determined that dry and wet lesions do not automatically indicate that infections are caused by *L. tropica* and *Leishmania major* (*L. major*), respectively, demonstrating that the manifestations of the skin lesions do not indicate the etiological *Leishmania* species [6]. CL has a high prevalence in the KP province and federal administered tribal areas at Pak-Afghan border because of cross border movements and presence of dry, hot and humid climate with abundance of sand fly vector [7,8]. Kohat, Karak and Hangu are Southern districts of KP. Kohat and Hangu are important because they host more than 2 million of internally displaced people that have migrated from CL hyper endemic zone (Pak–Afghan border) where military operations against terrorist camps are in progress [13]. CL outbreaks are frequently reported from different parts of Southern KP, particularly Karak [14,15].

Surprisingly, considering the importance of KP province as a focus of CL, little is known about the identity of *Leishmania* species responsible for CL in this area. The differentiation of *Leishmania* species is important for eco-epidemiology, clinical diagnosis, and management of patients. Previously, no report was available regarding presence of *L. major* in KP province where anthroponotic CL caused by *L. tropica* was thought to be the only major form of CL. The objective of this study was molecular identification of *Leishmania* species from biopsy samples of CL patients from Southern KP.

2. Materials and methods

2.1. Study area

KP province is located in the north-west of Pakistan (27° 2′N to 31° 42′ N and 50° 42′ E to 55° 36′E). The topography of the Kohat, Hangu and Karak districts are well dominated by series of small mountains and hills. The present study was conducted at Department of Microbiology, Kohat University of Science and Technology, Kohat in collaboration with ANSES, Animal Health Laboratory, Maisons-Alfort, France.

2.2. Sample collection

A study was carried out from April 2011 to March 2016 to record the cases of CL in Kohat, Hangu and Karak districts. The study was ethically approved by Research Ethical Committee of Kohat University of Science and Technology, Kohat and Higher Education Commission, Pakistan. Informed consents on consent form were obtained before sample collection from suspected CL patients. Data regarding age, sex, characteristic of lesions, traveling history and other related information were recorded on questionnaires from 432 patients visiting hospitals of the three districts. Two biopsy smears were obtained from each of suspected CL patients. One of these smears was used for microscopy while the second part was used for DNA extraction. The smears were prepared as described by Evans in 1989 [16] on a microscopic slide, air dried, fixed with absolute methanol, stained by Giemsa 10% and examined microscopically.

2.3. DNA extraction, internal transcribed spacer 1-PCR analysis and RFLP

DNA was extracted from 432 biopsy smears using Tissue DNA extraction kit (Nucleospin II, Machery Nagel, Germany) as per manufacturer's protocol and were analyzed by internal transcribed spacer 1 (*ITS1*) PCR as described by Schonian *et al* [17]. The positive *ITS1* amplicons (374) were analyzed by RFLP using *Hae* III enzyme (Biolabs, UK). Further, 30 positive samples were confirmed by *ITS1* gene sequencing (Eurofins, MWG, Operon, Germany). The sequences were cleaned and aligned, and phylogenic analysis was conducted using Clustal W software.

3. Results

Leishmania amastigotes were detected by microscopy in 308 of 432 samples (71.3%) while 374 out of 432 samples (86.6%) were positive by *ITS1* PCR that produced approximately 330–350 bp products for all reference strains of *L. tropica*, *Leishmania infantum* and *L. major* and clinical samples. RFLP analysis using *Hae* III showed restriction profile (20, 57, 60 and 200 bp) similar to *L. tropica* for 351 out of 374 samples while 6 samples produced *L. major* specific restriction pattern (2 bands of 130 and 220 bp). The sequence analysis of 30 samples confirmed RFLP results and revealed *L. tropica* as the major prevailing specie in southern districts of KP province whereas *L. major* was also described for the first time. Phylogenetic tree

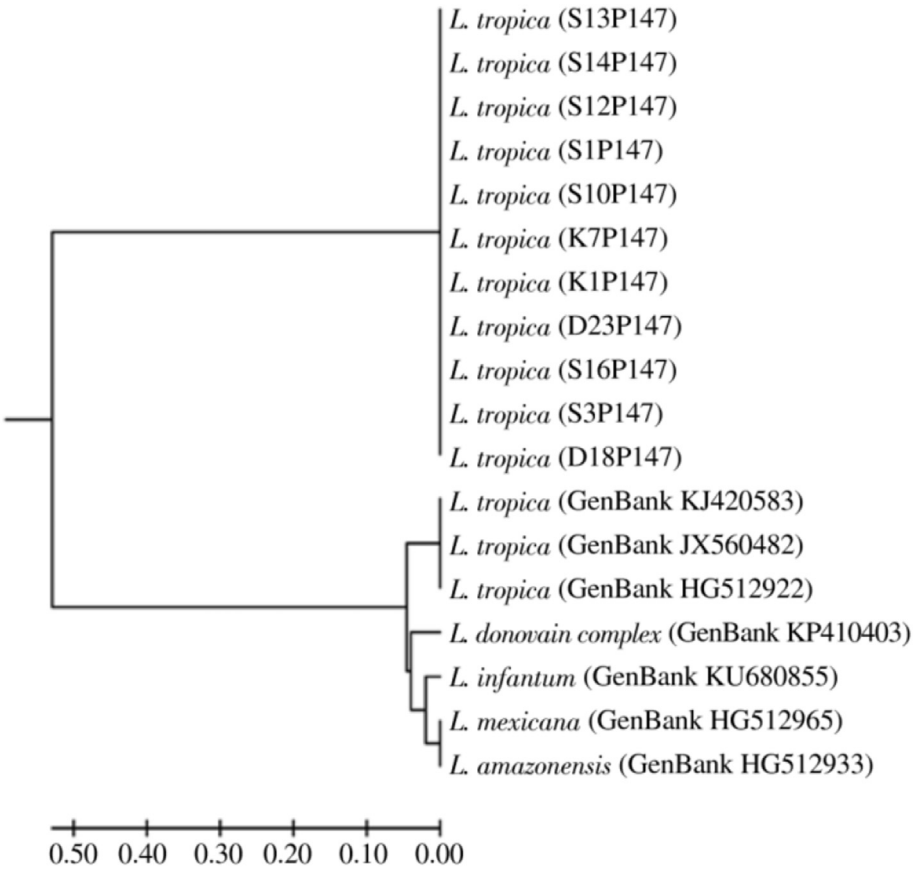


Figure 1. Phylogram (*ITS*1 gene) built using clustal W and Mega 7 software.
S13P147, S14P147, S12P147, S10P147, K7P147, K1P147, D23P147, S16P147, S3P147 and D18P147 represent Pakistani strains of *L. tropica* isolated from study districts of khyber pakhunkhwa province of Pakistan.

showed that the *Leishmania* strains from Pakistan are close (99%–100% similarity) to the already deposited sequences of reference *L. tropica* strains in GenBank database (Accession numbers KJ420483, JX560482, HG512922, KP410403, KU680855, HG512965, HG512933). All of the Pakistani strains sequenced were different variants of *L. tropica* (Figure 1).

4. Discussion

The endemic profile of CL in KP particularly Southern districts could be attributed to migration of large number of internally displaced people from CL hyper endemic tribal zone (federal administered tribal areas) [7,8,13]. Previously, *L. tropica* has been molecularly characterized only from Dir district (northern KP) whereas in southern KP, prevalent *Leishmania* species were unknown [11]. In this study, the *ITS1*-PCR-RFLP analysis and sequencing identified *L. tropica* as prevailing species in Southern KP. It confirmed previously through epidemiological studies that were not based on molecular analysis but on typical dry form of active CL lesion [11,18]. Various epidemiological studies also described the presence of *L. tropica* in all provinces of Pakistan [5,19]. It was therefore concluded that *L. tropica* is the prevailing specie in KP, which confirms previously epidemiological studies that were not based on molecular analysis [11]. In the present study *L. major* was identified in five samples from Hangu and one from Karak. Two of the patients diagnosed with *L. major* infection had traveling history to Sindh province and one of them had traveled to Afghanistan. The remaining three (from Kohat) claimed that they had never traveled anywhere in the past 2 years. This is the first ever report of autochthonous *L. major* infection from any part of KP province. Rodents are usually reservoirs of *L. tropica* so further studies by our research group on screening of rodents in this area are in progress. In Pakistan, zoonotic CL caused by *L. major* is mainly found in Sindh province and Southern Balochistan bordering Iran while in KP it was always considered to be anthroponotic [20]. Afghanistan is considered to be one of world's largest endemic anthroponotic CL foci but zoonotic CL (*L. major*) is endemic in Mazar-e-Sharif Province of Afghanistan [21]. *Phlebotomus paptasi* and *Phlebotomus sergenti* that are considered as vectors of anthroponotic CL and zoonotic CL respectively are widely distributed in KP province [22]. The zoonotic CL focus (three autochthonous *L. major* cases without traveling history) in Hangu district has dry habitat but extensive cultivation of seasonal crops is practiced, using irrigation systems of canals and springs that favors dense populations of vectors and rodents. The high densities of the natural zoonotic CL animal reservoirs and its associated sand-fly vector are the key elements in establishing new disease foci. But to date animal reservoirs are still unknown in any part of Pakistan [18,20].

Establishment of new zoonotic CL foci could be strongly related to human activities, such as climate changes, mass migration of people during war (internally displaced people in present case) or natural disasters, development of agricultural and industrial projects. In general, the boundaries between the different nosogeographical forms of leishmaniasis overlap and have ability to progress [23]. The present findings about *L. major*

may predict in future the possibility of encountering new endemic zoonotic CL foci in other southern districts of KP. Since intraspecific polymorphisms were found among Pakistani *L. major* populations [5] as well as among *L. major* populations in Central Asia, the Middle East, and Africa [24] further studies like isoenzyme electrophoresis, multi-locus sequencing technique and microsatellite analysis are inevitable to reveal the origin of *L. major* strains identified in present study. Such data represent a first step for future incrimination of vectors, animal reservoirs, disclosing the eco-epidemiologic picture of this disease in KP province.

This is the first report of molecular detection of *L. major* and *L. tropica* co-existence from southern KP province. However this work has shown that *L. tropica* is probably prevalent specie in this region. The results indicated that *L. major* has recently become established in this classical focus of *L. tropica*. Although three cases of *L. major* claimed not to have traveled out of the vicinity of Kohat district, local sand fly vectors must be caught, identified and, if possible, shown to harbor *L. major* infections. Furthermore, role of animal reservoir host should also be explored to endorse whether the cases caused by *L. major* were autochthonous or imported.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgments

We are grateful to Higher Education Commission Government of Pakistan for providing fund Grant No: 1384 to Kohat university of Science and technology Kohat, Pakistan. We are also grateful to French Embassy, Islamabad for funding under their split PhD fellowship programs, a 6 months PhD fellowship to Dr. Mubbashir Hussain at ANSES, Animal Health Laboratory, Maisons-Alfort, France. We are thankful to Dr. Christophe Ravel CNRL, Montpellier, France for provision of reference *Leishmania* strains to be used as control during our molecular studies. We express our deep gratitude to US Naval Medical Research Unit 3 (NAMRU3), Cairo, Egypt for establishing State of Art Vector Borne Diseases Management Center at Department of Microbiology, Kohat Univeristy. We also acknowledge all CL patients, doctors and lab staff District Head Quarter Hospitals, Kohat, Karak and Hangu for helping in this study.

References

[1] Rezvan H, Moafi M. An overview on *Leishmania* vaccines: a narrative review article. *Vet Res Forum* 2015; **6**(1): 1-7.
[2] Correa E, Quinones W, Robledo S, Carrillo L, Archbold R, Torres F, et al. Leishmanicidal and trypanocidal activity of *Sapindus saponaria*. *Bol Latinoam Caribe Plantas Med Aromát* 2014; **13**(4): 311-323.
[3] Souto DEP, Silva JV, Martins HR, Reis AB, Luz RCS, Kubota LT, et al. Development of a label-free immunosensor based on surface plasmon resonance technique for the detection of anti-*Leishmania infantum* antibodies in canine serum. *Biosens Bioelectron* 2013; **46**: 22-29.
[4] Alvar J, Velez ID, Bern C, Herrero M, Desjeux P. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 2012; **5**: e35671.
[5] Marco JD, Bhutto AM, Soomro FR, Baloch JH, Barraso PA, Kato H, et al. Multi-locus enzyme electrophoresis and cytochrome B gene sequencing based identification of *Leishmania* isolates from different foci of cutaneous leishmaniasis in Pakistan. *Am J Trop Med Hyg* 2006; **75**: 261-266.
[6] Myint CK, Asato Y, Yamamoto Y, Kato H, Bhutto AM, Soomro FR, et al. Polymorphisms of cytochrome b gene in *Leishmania* parasites and their relation to types of cutaneous leishmaniasis lesions in Pakistan. *J Dermatol* 2008; **35**: 76-85.
[7] Hussain M, Munir S, Jamal MA, Ayaz S, Akhoundi M, Mohamed K. Epidemic outbreak of anthroponotic cutaneous leishmaniasis in Kohat District, Khyber Pakhtunkhwa, Pakistan. *Acta Trop* 2017; **172**: 147-155.
[8] Reithinger R, Aadil K, Kolaczinsky J, Mohsen M, Hami S. Social impact of leishmaniasis. *Afghan Emerg Infect Dis* 2005; **11**: 634-636.
[9] Wallace MR, Hale BR, Utz GC, Olson PE, Earhart KC, Thornton SA, et al. Endemic infectious diseases of Afghanistan. *Clin Infect Dis* 2002; **34**: S171-S207.
[10] Rowland M, Munir A, Durrani N, Noyes H, Reyburn H. An outbreak of cutaneous leishmaniasis in an Afghan refugee settlement in north-west Pakistan. *Trans R Soc Trop Med Hyg* 1999; **93**(2): 133-136.
[11] Brooker S, Mohammed N, Adil K, Agha S, Reithinger R, Rowland M, et al. Leishmaniasis in refugee and local Pakistani populations. *Emerg Infect Dis* 2004; **10**: 1681-1684.
[12] Jawabreh AA, Dumaidi K, Ereqat S, Jawabreh HA, Nasereddin A, Azmi K, et al. Molecular epidemiology of human cutaneous leishmaniasis in Jericho and its vicinity in Palestine from 1994 to 2015. *Infect Genet Evol* 2017; **50**: 95-101.
[13] OCHA. Intercluster Mission to Kohat and Hangu. [Online] Available from: http://oneresponse.info/Countries/Pakistan [Accessed on 22nd January, 2017]
[14] Bari A, Hasshim R, Mahmood K, Muhammad I, Shahbaz M, Tariq KM. Clinico-epidemiological pattern of cutaneous leishmaniasis in armed forces personnel fighting war against terrorism in Khyber Pakhtunkhwa Province and Fata regions. *J Pak Assoc Dermatol* 2011; **21**: 10-15.
[15] Ayaz S, Khan S, Khan S, Shams S, Saqalain M, Ahmad J, et al. Cutaneous leishmaniasis in Karak, Pakistan: report of an outbreak and comparison of diagnostic techniques. *Afr J Biotechnol* 2011; **10**(48): 9908-9910.
[16] Evans D. *Handbook on isolation, characterization and cryopreservation of Leishmania*. Geneva: World Health Organization; 1989.
[17] Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, et al. Diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 2003; **47**(1): 349-358.
[18] Kolaczinski J, Brooker S, Reyburn H, Rowland M. Epidemiology of anthroponotic cutaneous leishmaniasis in Afghan refugee camps in northwest Pakistan. *Trans R Soc Trop Med Hyg* 2004; **98**(6): 373-378.
[19] Noyes HA, Reyburn H, Bailey JW, Smith D. A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J Clin Microbiol* 1998; **36**: 2877-2881.
[20] Bhutto AM, Soomro RA, Nonaka S, Hashiguchi Y. Detection of new endemic areas of cutaneous leishmaniasis in Pakistan: a six year study. *Int J Dermatol* 2003; **42**: 543-545.
[21] Faulde M, Heyl G, Amirih M. Zoonotic cutaneous leishmaniasis outbreak in Mazar-e Sharif, Afghanistan. *Emerg Infect Dis* 2006; **12**: 1623-1624.
[22] Lewis DJ. The Phlebotomine sand flies of West Pakistan (Diptera; Psychodidae). *Bull Br Mus (Nat Hist) Entomol* 1967; **19**: 14-46.
[23] Ben Salah A. Zoonotic cutaneous leishmaniasis in central Tunisia: spatio-temporal dynamics. *Int J Epidemiol* 2007; **36**: 991-1000.
[24] Parvizi P, Mauricio I, Aransay AM, Miles MA, Ready PD. First detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* from Isfahan province, Iran: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. *Acta Trop* 2004; **93**: 75-83.