Acta Tropica 172 (2017) 147–155

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Epidemic outbreak of anthroponotic cutaneous leishmaniasis in Kohat District, Khyber Pakhtunkhwa, Pakistan

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ARTICLE INFO

Keywords:
Epidemic
Leishmaniasis
PCR
Promastigote
Kohat

ABSTRACT

An epidemiological and molecular study was carried out for the first time in Kohat District of Khyber Pakhtunkhwa (KP) province, Pakistan from April 2015 to May 2016 to determine the prevalence of Cutaneous Leishmaniasis (CL) in local population and Internally Displaced People (IDPs). In 13 different villages, a total of 1359 (out of 26,250 individuals belonging to local population) and 140 (out of 3615 IDPs residing in these villages) cases were recorded and 300 samples were collected. The total prevalence of CL in local population was 5.17% with active lesions and scar prevalence of 3.91% and 1.26% respectively. Similarly a prevalence of 3.86% for CL was recorded in IDPs. Highest number of IDPs having CL active lesions and scars were recorded in villages Sherkot, Surgul, and Jarma and their presence was positively correlated with CL in local population. Age wise prevalence was highest in young children of age group 1–15 years. The microscopic examination showed 64.33% (193/300) positive samples while kinteoplastic PCR showed 84.66% (254/300) positive. For the first time in KP province, 2/784 sandflies trapped from the study villages was found positive for *Leishmania* by PCR. Restriction Fragment Length Polymorphism of patients and sandflies samples revealed *L. tropica* as the prevalent *Leishmania* species in this district. The results of sequencing and RFLP identified *L. tropica* in *Phlebotomus sergenti*. This is the first ever report of molecular identification of *L. tropica* from sandflies of genus *P. sergenti* in Khyber Pakhtunkhwa province. This data can be helpful for health authorities in finding out new CL foci and to plan effective strategies for the provision of health facilities to poor people of this area.

1. Introduction

Cutaneous Leishmaniasis (CL) is most widespread form of leishmaniasis caused by *L. tropica* and *L. major* that invade the host macrophagic cells causing skin lesion on exposed parts of the body such as the face, arm and legs (Azizi et al., 2006). The disease is endemic in Asia, Europe, and South America and WHO has announced it as one of the most serious disease of the world due to ever increasing number of new cases (more than million) each year (WHO, 2001a,b). The ten countries including Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru together account for 70–75% of the global estimated CL incidence (Akhoundi et al., 2016). The disease is transmitted by the blood sucking sandflies (Phlebotomines) whose 700 species has been identified all over world, out of which 37 species have been identified in Pakistan (Jamjoom et al., 2002). Species belonging to genera *Phelobotomus* are involved in transmission of parasites in old world while in new world transmission is through members of genus *Lutzomyia* respectively. These flies have wide range of habitat from tropical rain forest to desert and also have wide range of host including human, livestock, dogs, chickens, vertebrates as well as some mammals (Desjeux, 2004; Durrani et al., 2011; Kassi et al., 2008; Lewin, 2000; Noyes et al., 1998; Shaw, 1994).

Anthroponotic cutaneous leishmaniasis (ACL) caused by *L. tropica* is reported from all over Pakistan with majority of cases in Khyber Pakhtunkhwa (KP) province (Bhutto et al., 2003; Jaffernay and Nighat, 2001; Kakarsulemankhel, 2011; Rahman and Bari, 2003). It is reported as an endemic in many districts of KP province (Amtul and Shaheen, 2001; Durrani et al., 2011; Noyes et al., 1998) as outbreak

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http://dx.doi.org/10.1016/j.actatropica.2017.04.035
Received 14 February 2017; Received in revised form 23 April 2017; Accepted 23 April 2017
Available online 02 May 2017
0001-706X/

from many settled districts (Kohat, Hangu, Nowshehra, Peshawar, Cherat, D.I. Khan, Bannu, Karak, Peshawar, Dir, Dargai, Shangla) and Federally Adminitered Tribal Areas (FATA) (Teerah, Khyber agency, Parachinar, Kurram agency, Orakzai agency, North Waziristan and South Waziristan agency) had been reported (ul Bari et al., 2016). The vectors like *Phlebotomus papatasi* and *P. sergenti* are widespread in the areas of Bannu, Dera Ismail Khan, Tank, Kohat, Lahore, Miranshah and Nowshera (Kakarsulemankhel, 2011; Sinton, 1924). Moreover, this province hosts more than 2 million Afghan Refugees which were a continuous source of bringing the infection to this part of Pakistan since 1980 (Rowland et al., 1999). Unfortunately, there are few studies available regarding prevalence of CL in Afghan refugees camps (Brooker et al., 2004; Kolaczinski et al., 2004). Terrorism and counter-insurgency military operations in FATA lead to large scale migration of internally displaced people (IDPs) in Pakistan's KP province. Within the Federally FATA Khyber and Kurram agencies are currently the worst-affected areas. More than 415,000 people were newly displaced in 2012 (WHO, 2013). There were 1.1 million IDPs registered as displaced in the KP, and many more are unregistered in the region and elsewhere (WHO, 2013).

Regarding Leishmaniasis, Kohat is one of the most important southern districts of KP province as it hosted about 0.2 million IDPs (WHO, 2013). However, no precise report about leishmaniasis in local as well as IDPs, is yet available (Kakarsulemankhel, 2004a). Therefore objective of the current study was to analyze the prevalence of CL, identification of causative species and vector (sandfly) in local as well as IDPs habituating rural areas of Kohat district of KP, Pakistan.

2. Materials and methods

2.1. Geographical and political profile of Kohat district

Kohat is a medium sized district of KP province located at a distance of about 47 km from Peshawar, the capital of KP. It is located at 33°35′13N 71°26′29E with an altitude of 489 m (1607 feet) with total area is 2973 km^2. According to data from Pakistan's last census in 1998, the district's population stands around 562,640 with an annual growth rate of 3.25% (present projected population 7, 82,070). About 80% peoples are habituated in rural areas. The topography of the district is dominated by the mountains and hills. The climate of Kohat district is usually dry with extreme hot in summer (around 40° C) and extreme cold and dry winters (about 6° C). The average annual rainfall is about 400 mm. This district has boundaries with Orakazai Agency, district Hangu, Karak, Nowshera and Punjab. The district also hosts many afghan refugees and IDPs residing in camps and villages (WHO, 2013).

2.2. Study area

A prospective study was conducted from April 2015 to May 2016 at different villages Barh, Jabbi, Gul Hassan Banda, Surgul, Tolanj, Chorlaki, Sherkot, Sudal, Sheen Dhand, Mir Ahmad Khel, Ghamkol, Jarma and Jabar in district Kohat (Fig. 1). These 13 villages were selected according to the data of the Basic Health Unit (BHU) located near village who indicate persons exhibiting cutaneous lesions. All the information from patient including name, sex, age, address, occupation, nature of lesion (active/scar/dry/wet), number of lesions, location of lesion, travel history to endemic area, type of house (mud made/brick made), presence of animals and treatment profile was also recorded on a questionnaire.

2.3. Sampling

A total of 29,865 individuals including local population 26,250 and IDPs (n = 3615) residing in the study villages were surveyed. House to house survey was carried out by a team comprising of research students of Kohat University, Principal investigator of research project and a medical dispenser/technologist a representative of BHU located near each study village. Information was taken from head of each house from randomly selected houses in east west and north south directions. The sample size was determined after the formation of clusters. The sample size of each cluster was determined by assuming that in each clusters the prevalence of cutaneous leishmaniasis is the same (3%) with the marginal error of 2% and 95% confidence interval. All individuals of a selected endemic village were eligible, however, for clinical sample collection, only those individuals with suspected cutaneous leishmaniasis active lesions were considered. Informed written consents were used before sampling patients.

A total of 300 (264 local people and 36 IDPs) smears were prepared from active skin lesions of willing patients (Fig. 2). All the patients in study were referred to nearest government hospital for free treatment. The samples were transported in ice and kept at −20 °C until further use for DNA extraction.

2.4. Collection of sandflies

For studying sandflies, reference points were set within a 1.5 km radius from houses of infected patients. Sandflies were collected by sticky papers (castor oil used) and some CDC light traps (Model 512, John Hock and Co., USA) set outside and inside of houses. The CDC traps were set before sunset and left overnight till dawn. One trap was placed outdoor while another trap was placed inside the houses of CL patients. These sandflies were stored in 70–80% ethanol till further use for morphological identification and *Leishmania* testing by PCR (Parvizi et al., 2005). All sandflies were identified based on external and internal morphological characters of the head and abdominal Terminalia (Nadim and Javadian, 1976; Parvizi et al., 2012), which were slide-mounted in Berlese's fluid (Lewis, 1982), following dissection with sterilized forceps and micro-needles (Testa et al., 2002). Female sandflies were used for parasite detection by kDNA PCR.

2.5. Collection of rodents

The rodents were captured by locally made steel traps baited with bread, peanut butter, vegetables and fruits placed in the fields, bushes and rock holes and crevices near the houses of CL patients, humanely euthanized and inspected for any gross lesions of CL on skin and different body parts (Mirzaei et al., 2011). The spleen, liver and skin were collected and stored at −20 °C until further use (Ayaz et al., 2011).

The samples (human, sandflies and rodents) were examined both microscopically (giemsa staining) and molecularly (PCR-RFLP) at Department of Microbiology Kohat University of Science and Technology (Pakistan) and ANSES, Animal Health Laboratory Maisons Alfort, France.

2.6. Microscopy

Slides were prepared, fixed in methanol, stained with Giemsa stain and examined under the microscope at 10×, 40× and 100× magnification for *Leishmania* amastigotes.

2.7. Extraction and amplification of DNA

DNA extraction from all types of samples (human, rodents and sandfly) was carried out as reported previously (Motazedian et al., 2002). Briefly, 200 µl of sample was mixed with equal volume of lysis buffer (50 mm Tris-HCl pH 7.6, 1 mm EDTA pH 8.0, 1% Tween 20, 15 µl Proteinase K solution 19 mg/ml) and incubated for 1 h at 56 °C. The lysate was then extracted twice with phenol. DNA was also extracted using DNA extraction kit (Nucleospin II, Machery Nagel, Germany). Two type of primers were used in study. kDNA Oligonucleotide primers (Rodgers et al., 1990). Forward primers Rv1, F 5'-CTTT-