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Research paper

Immunodetection and molecular determination of visceral and cutaneous *Leishmania* infection using patients' urine

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ABSTRACT

The diagnosis of leishmaniasis relies mainly on the use of invasive processes, to collect the biological material for detecting *Leishmania* parasites. Body fluids, which can be collected by non-invasive process, would greatly facilitate the leishmaniasis diagnosis. In the present study, we investigated the potency of urine immunoblotting to diagnose cutaneous and visceral leishmaniasis and we compared with routine molecular methods. A total of 80 samples, including 40 sera and their 40 corresponding urine samples were collected from 37 suspected patients with cutaneous and visceral leishmaniasis, and 3 healthy individuals (as control), in Ilam and Ardabil provinces of Iran. All sera and urine samples were analyzed, using immunoblotting. The confirmation of leishmaniasis infection was performed, using conventional and quantitative PCRs as well as by sequencing the amplicons. Among 37 suspected patients, 23 patients presented cutaneous lesions (CL) and 14 exhibited clinical symptoms reminiscent of visceral leishmaniasis (*L. infantum*). Among cutaneous patients, 15 were positive for zoonotic cutaneous leishmaniasis (*L. major*), and eight for anthroponotic cutaneous leishmaniasis (*L. tropica*). Molecular quantification of *Leishmania* parasites was performed on sera, urines and cutaneous biopsies of CL and VL patients, demonstrating that parasite load is lower in urines, compared to sera or biopsy. DNA can be detected in 20 out of 23 (86.9%) CL urine samples and in 13 out of 14 (92.8%) VL urine samples. Immunodetection analysis demonstrates that 22 out of 23 (95.6%) sera from CL patients and all patients suspected with VL are positive. For urine samples, 18 out of 23 (78.2%) urine of CL patients and 13 out of 14 (92.8%) urine of VL patients were positive, using Western blot. Therefore, immunodetection and molecular analysis using urine samples can be used as a diagnostic tool for surveying cutaneous and visceral leishmaniasis.

1. Introduction

Leishmaniases are vector-borne diseases, caused by obligate protistan parasites from the genus *Leishmania* (Trypanosomatida: Trypanosomatidae). They are endemic in large areas of the tropics, subtropics and the Mediterranean basin, spanning > 98 countries and territories. There are almost 350 million people at risk and 12 million cases, with an estimated worldwide annual incidence of 0.7–1.2 million cases of cutaneous leishmaniasis (CL) and 0.2–0.4 million cases of visceral leishmaniasis (VL) (Alvar et al., 2012). *Leishmania* parasites are transmitted to vertebrates by the bite of infected female phlebotomine sandflies, and are frequently hosted by canids, rodents, marsupials, mongooses, bats and hyraxes (Akhoundi et al., 2016, 2017).

In Iran, both VL and CL are identified important public health problems. The causative agents of VL in Iran are *L. donovani* and *L. infantum*. The latter is sporadically reported throughout the country in four main endemic foci, located in the north-western (Ardabil and Azerbaijan-e- Sharqi) and southern Iran (Fars and Bushehr) (Akhoundi et al., 2013). Zoonotic Cutaneous Leishmaniasis (ZCL) due to *L. major,* is a zoonotic disease with rodents as reservoirs. It occurs mainly in rural

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areas of 15 out of the 31 provinces of Iran (Akhoundi et al., 2013). The most prevalent endemic foci of ZCL are located in Turkmen Sahara and Lotf Abad, in north-east of Iran, Abardezh Varamin, Esfahan and Yazd, in center of Iran, Fars and Sistan-Baluchestan in south and south-east and Ilam and Khuzestan in south-west of Iran (Nadim and Seyedi-Rashti, 1971; Mohebali et al., 2004). Anthroponotic Cutaneous Leishmaniasis (ACL) due to *L. tropica,* mainly anthroponotic in nature, occurs in Tehran, Khorasan-e-Razavi in the north-east and Fars and Kerman provinces in the south of Iran (Nadim and Seyedi-Rashti, 1971; Mohebali et al., 2004).

The diagnosis of leishmaniasis is puzzled, by the diversity of the clinical pathology profiles of the disease; varies from simple cutaneous lesions to visceral forms. Variability in the clinical presentation is also reported, within each clinical form (VL, CL). Parasitological confirmation *via* culture or molecular techniques is often complex, invasive, and requires a sophisticated laboratory infrastructure. Molecular methods, including PCR-based approaches are highly sensitive and specific (Akhoundi et al., 2017), and they required sophisticated laboratory infrastructures. In addition, a substantial proportion of healthy individuals has parasite DNA in the blood, detected by PCR (Bhattarai et al., 2009). Serological tests like indirect fluorescent antibody test (IFAT), Western blot, or direct agglutination test (DAT), are used for leishmaniasis diagnosis (Mikaeili et al., 2007; Sarkari et al., 2008; Castellano et al., 2010). But these immunodetection methods present drawbacks because they detect antibodies, still present after a cure, as well as past or present asymptomatic infection.

Currently, all rapid diagnostic tests (RDT) rely on the principle of the antibody detection (see Boelaert et al., 2014). RDT were specifically developed for the field, used in VL-endemic areas. They also can be used, if sufficiently accurate, for the early diagnosis of VL, at peripheral and central levels of the health system (Boelaert et al., 2014). The most sensitive and specific RDT for VL is the rK39 immunochromatographic test (ICT) with 91.9% sensitivity and 92.4% specificity (Boelaert et al., 2014). In the recent years, rK-39 ICT has been carried out using patient urine samples and showed high sensitivity (ranging from 95 to 97.7%) and specificity (93.3 to 100%) (Musawwir Khan et al., 2010; Singh et al., 2013; Ghosh et al., 2016). In addition, qPCR analysis of the VL urine showed good prospect for *Leishmania* DNA detection (Pessoa-E-Silva et al., 2016).

In the majority of countries endemic for leishmaniasis, more than one human pathogenic *Leishmania* species are reported. The higher diversity of *Leishmania* including eight different pathogenic species is reported in Brazil (Akhoundi et al., 2017). Nevertheless, mentioned methods cannot discriminate between the infecting *Leishmania* species.

Because urine samples are easy to collect and process, therefore we decided to test the efficiency of Western blot, using patients' urine, (i) to diagnose *Leishmania* infection and (ii) to evaluate the sensitivity of such approaches for serological and molecular diagnosis.

2. Materials and methods

Clinic and samples

A prospective study was conducted between March 2015 and June 2017 on the patients referring to Dehloran, Mehran and Meshkin shar health centers, in the north-west and south-west of Iran. Serum, biopsy and urine samples were collected from individuals, with symptoms reminiscent of leishmaniasis (CL and VL). A total of 37 patients were selected: 23 with cutaneous lesions (8 ACL and 15 ZCL) and 14 suspected having VL. For CL, personal information, lesion duration, type of lesion, lesion location, prescribed medications and travel history in endemic regions, were recorded for each person.

Biopsies of cutaneous lesions were collected and the smears of cutaneous lesions were prepared according to the protocol developed by Evans (Evans, 1989). Briefly the samples were smeared on a microscopic slide, air dried, fixed with absolute methanol, stained by 10% Giemsa and examined under a light microscope with high magnification (1000×). In addition, the skin lesions were cleaned with 70% ethanol, a thin needle and syringe, containing 0.2 ml sterile PBS was inserted intradermally into the margin of the cutaneous lesion and then the lesion fluid was aspirated slowly and transferred into a new sterile 1.5 ml microtube for molecular investigation. In addition, 10 ml of serum and urine were collected from each CL patient, for serological and molecular analyses.

The suspected VL patients presented the symptoms reminiscent of visceral leishmaniasis infection such as the fever, weakness, loss of appetite, weight loss, anemia, abdominal distension, due to hepatomegaly and splenomegaly or both, and lymph node enlargement. Ten ml of serum and 10 ml of urine were collected from each suspected patient. In addition, serum and urine samples were collected from three healthy donors as control. None of the patients were under anti-leishmaniasis therapy at the time of evaluation.

Blood (10 ml) collected individually was centrifuged at 2000 rpm (4 °C) for 10 min in order to remove the clots. The serum was then carefully transferred into a clean polypropylene tube, using a Pasteur pipette and then kept at 4 °C for further serological and molecular analyses.

Half of each urine sample was frozen at −20 °C for further serological analysis. The second part of the urine was centrifuged at 14000 rpm in sterile tubes for 8 min; the supernatant was discarded and the pellet was washed twice in sterile distilled water and centrifuged again. The final pellet was re-suspended in 100 µl sterile distilled water for molecular analysis.

2.1. Western blot

All sera and urines collected from both groups of CL and VL patients, as well as from healthy individuals were analyzed by Western blot, using in-house Western blot kit (LDBIO diagnostics, France) (Mary et al., 1992). The assays were carried out, according to the manufacturer's recommendations with some modifications. Briefly, strips were put in a WB tray and, 1.2 ml of sample buffer was added. One minute later, 25 µl of serum or urine of each patient was added to each channel and gently shaken, for 90 min. Then, serum or urine was removed by Pasteur pipette and the strips were washed three times with 2 to 3 ml of wash buffer (10× buffer), with 5 min intermediate incubation. Then 1.2 ml of a mix, including 600 µl goat anti-human IgG, conjugated to horseradish peroxydase and 600 µl in-house anti-human IgG goat alkaline phosphatase conjugate (1:1000) (Dayangac et al., 2004), was added, and the strips were incubated for 60 min. Then, the strips were washed three times with “wash buffer”, and subsequently 1.2 ml of NBT/BCIP substrate was added and incubated for 60 min.

Two matrixes for each of serum and urine immunoblot analyses were constructed based on manually identifying band on the strips (Supplementary Tables 1 & 2). In order to investigate the relations between protein bands specific for each *Leishmania* species, Principal Component Analysis (PCA) were carried out on each matrix (Dray and Dufour, 2007, R Core Team, 2014).

2.1.1. DNA extraction and amplification

DNA extraction from serum, urine and biopsy samples was carried out using QIAamp® DNA Mini Kit (Valencia, CA, USA), according to the manufacturer's protocol. The DNA concentration was measured by a NanoDrop spectrophotometer (BioTek Synergy 2, USA). In order to identify *Leishmania* species, the standard PCR, targeting *Leishmania* kDNA minicircle was carried out, using previously described specific internal primers of 13Z (5′- ACTGGGGGTTGGTGTAAAATAG-3′) and LiR (5′-TCGCAGAACGCCCCT-3′) (Noyes et al., 1998). Each reaction included 25 µl master mixture, containing 1× PCR, 1.5 mM MgCl2, 0.2 mM dNTP, 2.5 U Taq (Qbiogene, Irvine, CA) and 1 µM of each primer. A total of 35 cycles was performed by a PCR-Thermal-Cycler (Senso Quest, Germany), under the following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 94 °C for 30s, 55 °C