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ABSTRACT Cutaneous leishmaniasis is one of the main public health problems in Afghanistan, particularly in Herat. To identify Leishmania spp., molecular techniques were applied to samples from 64 cutaneous leishmaniasis patients referred to Herat regional hospital during 2013. Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis of the ribosomal RNA gene internal transcribed spacer-1 (ITS1) was used. Most of the patients demonstrated dry type single lesions on the head. The results of direct microscopy detection using Giemsa-stained skin scrapings were compared with that of ITS PCR–RFLP for the diagnosis of cutaneous leishmaniasis. Light microscopy examination showed 37/64 positive cases (58%). PCR revealed 50 positive cases (78%), from which ITS PCR–RFLP identified 48 cases (96%) as L. tropica and 2 cases (4%) as L. major. Cutaneous leishmaniasis in Herat appears to be endemic and of the clinically dry type, caused mainly by L. tropica and occasionally by L. major.

Détection moléculaire des espèces de Leishmania isolées à partir de

lésions cutanées chez des patients orientés vers l'hôpital régional d'Herat (Afghanistan)

RÉSUMÉ La leishmaniose cutanée est l'un des principaux problèmes de santé publique en Afghanistan, et particulièrement à Herat. Pour identifier les espèces de Leishmania, des techniques moléculaires ont été appliquées sur des échantillons prélevés chez 64 patients atteints de leishmaniose cutanée orientés vers l'hôpital régional d'Herat en 2013. L'analyse du polymorphisme de la longueur des fragments de restriction par amplification en chaîne par polymérase de l'espaceur interne transcrit 1 au sein du gène d'ARN ribosomique (ITS PCR – RFLP) a été utilisée. La plupart des patients présentaient des lésions uniques sèches localisées sur la tête. Les résultats du dépistage microscopique direct à partir de grattages cutanés colorés au Giemsa ont été comparés à ceux de l'analyse par ITS PCR-RFLP pour le diagnostic de la leishmaniose cutanée. L'examen microscopique optique a dépisté 37/64 cas positifs (58 %). L'amplification en chaîne par polymérase a permis de dépister 50 cas positifs (78 %) ; parmi ces cas, l'analyse par ITS PCR-RFLP a identifié 48 cas (96 %) de L. tropica et 2 cas (4 %) de L. major. La leishmaniose cutanée à Herat semble endémique et de type sec au plan clinique, principalement causée par L. tropica et occasionnellement par L. major.

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Introduction

Leishmaniasis threatens 350 million people in 98 countries, with a global estimated incidence of 2 million cases per annum. There are 3 major manifestations of this parasitic disease: cutaneous, mucocutaneous and visceral (1,2). In Afghanistan, cutaneous leishmaniasis is one of the main public health problems. While the estimated annual incidence of cutaneous leishmaniasis in Afghanistan ranges from 113 100 to 226 200 cases, the number of reported cases of cutaneous leishmaniasis are only 22 620 annually (3).

Each Leishmania sp. has a unique epidemiological profile and therefore identification of Leishmania spp. in different regions is indispensable. Conventional methods such as

microscopy and culture of amastigotes are not utilized for Leishmania spp. identification as they have an unsatisfactory level of sensitivity. Instead, DNA-based methods, such as the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of the internal transcribed spacer-1 (ITS1) region of the ribosomal RNA gene (rRNA), are increasingly used for identification worldwide (4–6). DNA cards such as FTA® and KBC® are immediate, affordable, safe and portable tools for collection, banking and transportation of leishmaniasis samples at room temperature prior to DNA extraction, in order to carry out ecological and epidemiological studies in Afghanistan (7–9).

There is a scarcity of comprehensive data about the epidemiology of this neglected disease in Afghanistan, although there has been recent important work by Reithinger et al. (10–16). This follows earlier studies of disease foci by Eliseev and Kellina in 1962 (17) and on the epidemiology of cutaneous leishmaniasis throughout the country by Nadim et al. in the 1970s (17–20). Later, Reyburn et al. carried out a cross-sectional study of a prolonged epidemic of anthroponotic cutaneous leishmaniasis during 1997–98 in Kabul (21). More recently, Faulde et al. made a molecular study of cutaneous leishmaniasis in northern parts of Afghanistan (22). Rowland et al. reported an outbreak of cutaneous leishmaniasis in an Afghan refugee camp in north-west Pakistan (23). Plourde et al. studied genetic polymorphisms and drug susceptibility of 4 isolates of Leishmania tropica acquired from Canadian soldiers returning from Afghanistan (24).

Cutaneous leishmaniasis is endemic in Herat in the west of Afghanistan and represents one of the independent foci of the disease (3). While Ahrari and Yaminhad made a 9-month demographic analysis of cutaneous leishmaniasis patients referred to Herat regional hospital (25), there is a lack of molecular data about the epidemiology of cutaneous leishmaniasis in Herat. The main aim of the present study therefore was to use molecular methods to identify Leishmania spp. isolated from cutaneous samples of patients referred to Herat regional hospital.

Methods

Study design and setting

This descriptive study was performed in Herat, a city situated in the west of Afghanistan at an altitude of 930 m above sea level. According to previous studies carried out in Herat, most cutaneous leishmaniasis cases are known to occur in early spring (March and April) and early fall (September and October) (25). So the study was designed to cover these periods during 2013.

Sampling

Samples were taken from 64 patients (41 males and 23 females) with clinically diagnosed

cutaneous leishmaniasis who were referred to the dermatology section of the Herat regional hospital for confirmation of diagnosis (by microscopic analysis) and treatment (with pentavalent antimony).

Data collection

A questionnaire was completed which included questions about patients' demographic information and the characteristics of the lesions, and then photos of the lesions were taken and kept for future reference. Treatment was free of charge for the patients so we categorized patients' income as low if they said they would not come to hospital if treatment was not free, as intermediate if they might come to hospital if treatment was not free, and high if they would come to hospital even if treatment was not free.

Patients had not received any treatment before sample collection. Slash skin smears from the margin of lesions were also taken on DNA banking cards (for molecular analysis) and slides (for microscopic analysis).

Microscopic analysis

The slides were stained by Giemsa staining, then scanned for existing amastigotes using ×100 oil-immersion light microscopy.

Molecular analysis (PCR–RFLP)

Disks (2 mm in diameter) were punched out from each DNA and washed 3 times with KBC DNA banking card purification buffer and twice with distilled water. The disks were air dried and put directly through PCR processing.

PCR was performed on the ITS1 region of the rRNA gene as described previously by Schönian et al. (26). Initial denaturation at 95 °C for 5 min was followed by 35 cycles (95 °C for 30 s,

55 °C for 30 s and 72 °C for 45s) and final extension for 6 min at 72 °C. Amplicons were detected in a 1% agarose gel holding 0.2 mg/mL ethidium bromide. Hae III was the restriction enzyme used. The PCR-RFLP products were detected in 4% agarose gel.

The PCR product of each sample and primers were sequenced by the ABI3730XL sequence analyser (Macrogen, Korea). After editing and aligning of sequences using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), the sequences were compared with reference sequences from GenBank. For L. major and L. tropica ITS1 sequences, the phylogenetic tree was created with the neighbour-joining algorithm using Molecular Evolutionary Genetics Analysis (MEGA) software, version 6.0, including sequences of prototypical L. major and L. tropica isolates from GenBank.

Geographical mapping

The geographical locations of cases according to species of Leishmania were located using the Google Earth software. The topography of Herat was isolated from the whole topography of Afghanistan using ArcGIS geographic information system software and saved in separate files. The marginal lines contained within geomaps of Herat were indexed in the same folder. The geographical locations of cases of L major and L. tropica were established on the final map using longitude and latitude techniques.

Results

Demographic characteristics

Out of 64 patients who were sampled for this study 41 were male (64%) and 23 female (36%). Although the patients' ages ranged from 1 to 85 years, the majority of patients (67%) were aged 1–20 years. According to patients' occupation high proportion were students (45%), and the rest were manual workers (17%), housewives (14%), children below school age (14%) or in other employment (9.5%). The patients were classified into 3 income categories, based on their ability to afford their drugs: low (56%), intermediate (33%) or high (11%) (Table 1).

Clinical profile

Of the total patients 61% had a single lesion, 19% had 2 lesions and 20% had more than 2 lesions. The size of lesions varied from 0.5 to 10 cm. A majority of cutaneous leishmaniasis lesions were on the head (41%) or hand (36%), and the rest were on the foot (8%) or a combination of 2 or more sites (15%). The majority of patients (88%) presented with dry type lesions (Table 1).

Identification and mapping of Leishmania spp.

Among the 64 slides examined for amastigotes using light microscopy, 37 (58%) were found positive and 27 (42%) negative.

The PCR technique identified 50 positive cases out of these 64 cutaneous leishmaniasis cases (78%). Amplified fragments of the ITS1 region of the rRNA gene on gel electrophoresis are shown in Figure 1, lane 4. Of these PCR-positive cases, RFLP identified 48 cases (96%) as L. tropica and 2 cases (4%) as L. major (Figures 1 and 2).

The phylogenetic relationships of Leishmania genotypes from Herat province were compared with other species in GenBank using the neighbour-joining algorithm (Figure 3). The sequences obtained were annotated in GenBank by accession numbers from KJ420582 to KJ420587.

The geographical distribution of cutaneous leishmaniasis cases in Herat, by species, is presented in Figure 4 . The great majority of cases clustered in Injil district of Herat, with 2 cases in Zindajan and 1 case in Adraskan .

Discussion

There are many studies concerning the identification of Leishmania spp. throughout the world, especially in the Middle East and Central Asia (3). The current study was a preliminary molecular report on cutaneous leishmaniasis in Herat province. All patients were local residents of Herat. Our results revealed that anthroponotic cutaneous leishmaniasis due to L. tropica was the dominant form of leishmaniasis in Herat (48/50 PCR-positive cases), although we cannot ignore the presence of zoonotic cutaneous leishmaniasis due to L. major (2/50 cases).

Research in the Islamic Republic of Iran, which shares a border with Afghanistan, indicated that L. major was the dominant species of Leishmania (27–31), except in Mashhad province, which is located near Herat in Afghanistan, where the larger portion of Leishmania cases belongs to L. tropica (27). A study in Turkey showed that L. tropica was the only existing species of Leishmania in Sanliurfa province (32). A recent study also revealed that L. tropica was the causative agent of leishmaniasis in humans in most parts of Turkey (33). A study on leishmaniasis in Yemen revealed that L. tropica was the paramount species of Leishmania, although L. infantum and L. donovani were also identified (34). Most studies in Pakistan indicated that L. major was the major species of Leishmania in lowland areas, while L. tropica dominated in highland areas located near Afghanistan (22).

Many studies have indicated the existence of L. major either in Central Asia or northern parts of

Afghanistan. Faulde et al. in 2007 reported that zoonotic cutaneous leishmaniasis caused by L. major was endemic in Balkh province, north of Afghanistan (35). They found that of 3958 cases, 3782 (95.5%) were zoonotic and thus concluded that L. major was the principal species of Leishmania in Balkh. In another study, the same authors found an aggressive strain of L. major in Uzbekistan (36). Moreover, according to Larréché et al. some virulent strains of L. major occur in various areas of Central Asia, especially in Turkmenistan (37).

We found that 64% of patients were male, 67% were aged under 20 years and 56% were of low income. The most common pattern of lesions was 1 lesion (61%), of dry type (88%) and situated on the head (41%). Another study conducted by Ahrari and Yamin also in Herat regional hospital produced findings roughly in line with our data (25); 48% of patients were male, a majority were under 20 years old and 83% were of middle or lower socioeconomic class. They also found that 43% of lesions were located on the head and 60% of the patients had a single lesion. As in our study, most of their patients were resident in Injil district (25). It should be noted, however, that Injil is the nearest district to Herat hospital and it is likely that districts further from the hospital are equally affected by leishmaniasis but that patients could not afford to come to the hospital and therefore were not included in the study.

Myint et al. in their research in Pakistan reported that among 69 cutaneous leishmaniasis cases, 45 (65%) were males. Furthermore, 44 (64%) of the patients were under 20 years old, of whom 42 (61%) suffered from wet lesions and 46% had single lesions (22). Their findings about the age and sex distribution of patients roughly correspond to our data. However, we found that 88% of patients had dry lesions and 61% had only a single lesion. Rassi et al. in a study in Qom, Islamic Republic of Iran, found that 46.7% of patients were males and 75.0% were aged 15 years and above. Their results about lesions showed that 64.4% of patients suffered from 1 lesion and 48.0% of the lesions occurred on the hands (31). Kato et al. in Venezuela found that 55% of the patients were under 20 years old, of whom 61% were males and 39% females (8). Their results were relatively in line with our data. Khatri et al. did similar research in Yemen. His patients comprised 66% males and 34% females and they showed that 65% of the patients possessed single lesions and a majority of the lesion were of the dry type, 1 to

In conclusion, our results reveal that L. tropica was more prevalent than L. major in Herat province of Afghanistan. Hence, the major strategies for prevention of cutaneous leishmaniasis should be directed towards treatment of infected individuals, due to the anthroponotic nature of transmission of L. tropica. However, we should not overlook the presence of L. major in Herat province which points to zoonotic reservoirs of the disease. The existence of both L. tropica and L. major in Afghanistan calls for serious prevention strategies, treatment and vaccination. The lack of modern, sensitive equipment for diagnosis and of effective health care facilities is problematic for physicians and patients.

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