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MULTILOCUS ENZYME ELECTROPHORESIS AND CYTOCHROME *B* GENE SEQUENCING–BASED IDENTIFICATION OF *LEISHMANIA* ISOLATES FROM DIFFERENT FOCI OF CUTANEOUS LEISHMANIASIS IN PAKISTAN

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Abstract. Seventeen *Leishmania* stocks isolated from cutaneous lesions of Pakistani patients were studied by multilocus enzyme electrophoresis and by polymerase chain reaction amplification and sequencing of the cytochrome *b* (*Cyt* b) gene. Eleven stocks that expressed nine zymodemes were assigned to *L. (Leishmania) major*. All of them were isolated from patients in the lowlands of Larkana district and Sibi city in Sindh and Balochistan provinces, respectively. The remaining six, distributed in two zymodemes (five and one), isolated from the highland of Quetta city, Balochistan, were identified as *L. (L.) tropica*. The same result at species level was obtained by the *Cyt* b sequencing for all the stocks examined. No clear-cut association between the clinical features (wet or dry type lesions) and the *Leishmania* species involved was found. *Leishmania (L.) major* was highly polymorphic compared with *L. (L.) tropica*. This difference may be explained by the fact that humans may act as a sole reservoir of *L. (L.) tropica* in anthroponotic cycles; however, many wild mammals can be reservoirs of *L. (L.) major* in zoonotic cycles.

INTRODUCTION

The leishmaniases are a group of diseases that threaten 350 million people in 88 countries with an incidence estimated at between 1.5 and 2 million new cases per year. Nevertheless, this estimated global burden of disease might be inaccurate, because of passive case detection data from many endemic areas.[1] The diseases are caused by parasitic protozoan flagellates of the genus *Leishmania*, which includes ~30 different taxa. The intrinsic diversity and the immune response of the host are responsible for the variability of clinical manifestation of the diseases,[2] which can be pleomorphic, and one species can be responsible for more than one clinical form. Therefore, the differentiation of these parasites and their accurate identification, which are relevant to eco-epidemiology, clinical diagnosis, and management of patients, must be based on molecular approaches, because parasitologic, clinical, and epidemiologic features are, by themselves, insufficient for this task.[3] Among the molecular techniques that have been proposed for the characterization of *Leishmania,* multi-locus enzyme electrophoresis (MLEE) is the gold standard method, whereas DNA-based techniques are being used increasingly.[2] One of these techniques, the polymerase chain reaction (PCR) amplification and sequencing of cytochrome *b* (*Cyt* b) gene was recently established,[4] has been very promising for species identification over wild isolates.[5]

Pakistan, a tropical and subtropical country located in the northwest of South Asia, is highly endemic for the leishmaniases. The Old World visceral leishmaniasis (VL), which is considered deadly if it is not treated, mainly occurs in the northern region of the country, in areas such as Baltistan, Chilas, and Azad Jammu and Kashmir (AJK).[6] It has also been reported sporadically from the vicinity of the Northwest Frontier Province (NWFP), Balochistan, and Punjab provinces, although no cases of VL have yet been documented from Sindh province.[7] In the Himalayan region, *L. (L.) infantum* has been incriminated as the causal agent of VL using MLEE, with dogs as a reservoir.[6,8]

Two types of cutaneous leishmaniasis (CL), Old World anthroponotic (ACL) and zoonotic (ZCL) forms, are also prevalent in Pakistan. Locally, the lesions are often called tropical or oriental sores, Baghdad or Delhi boils, and Kandahar or Lahore sores.[9]

The ACL that usually affects urban or city dwellers and is clinically characterized as "dry-type lesions" has been reported from Multan city located in the southern part of Punjab province, Quetta, and other cities of Balochistan province, and also from AJK and Timargara Afghan refugee camps in the NWFP. In addition, some districts in Sindh province could be considered endemic for ACL.[10–13] Recently, it has been suggested that ACL transmission is autochthonous in a given endemic area of Pakistan, characterized by household clustering of the cases and a higher risk among children.[13,14] In most of the studies done in Pakistan to date, the etiologic *Leishmania* identification was made, mainly based on clinical features, epidemiology, and vector sand fly fauna. Only in limited leishmaniasis-endemic areas has *L. (L.) tropica* been incriminated as the causal agent of ACL by using molecular methods. The species has been isolated from patients and characterized by MLEE in Multan city,[15] AJK, Rawalpindi, Besham (NWFP), and Afghan refugee camps of Islamabad.[16] *Leishmania (L.) tropica* was also identified by a nested PCR-based schizodeme method in clinical samples from Timargara refugee camps in the NWFP.[10,17]

In Pakistan, ZCL mainly occurs in rural and semi-urban areas of Balochistan and neighboring Punjab and Sindh provinces; clinically, the disease form has been associated with "moist or wet-type lesions,"[12,13,18] and unusual clinical forms

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TABLE 1
Characterisitics of Pakistani *Leishmania* stocks and clinico-epidemiologic data of the patients

Zymodeme	Species*	Designation	Lesion features	Lesion location	Evolution (days)	Sex	Age (years)	Geographical origin
KMS 10	*L. (L.) mj*	MHOM/PK/03/SK2	Single, dry ulcerative	Shin	90	M	38	Larkana, Sono Khan
KMS 10	*L. (L.) mj*	MHOM/PK/03/SK7	Single, wet ulcerative	Shin	30	M	2	Larkana, Sono Khan
KMS 10	*L. (L.) mj*	MHOM/PK/03/TH1	Single, dry active	Arm	30	F	45	Larkana, Tharri Hajra
KMS 11	*L. (L.) mj*	MHOM/PK/03/WR3	Multiple (4), wet ulcerative	Ankle, leg	60	M	25	Larkana, Warah
KMS 12	*L. (L.) mj*	MHOM/PK/03/SK14	Multiple (4), dry active	Forearm, hand, wrist	30	F	10	Larkana, Sono Khan
KMS 13	*L. (L.) mj*	MHOM/PK/03/LR1	Multiple (15), wet ulcerative	Arm, back, chest, forearm	60	M	15	Larkana, Lalu Raunk
KMS 14	*L. (L.) mj*	MHOM/PK/03/SHD2	Multiple (3), dry and wet ulcerative	Ankle	60	M	30	Larkana, Shadadkot
KMS 15	*L. (L.) mj*	MHOM/PK/03/GD2	Multiple (3), dry ulcerative	Ankle, foot	180	M	50	Larkana, Gaibi Dero
KMS 16	*L. (L.) mj*	MHOM/PK/03/SHD7	Single, dry ulcerative	Hand	30	F	3	Larkana, Shahdadkot
KMS 17	*L. (L.) mj*	MHOM/PK/03/SB1	Single, wet ulcerative	Leg	60	F	20	Sibi city
KMS 18	*L. (L.) mj*	MHOM/PK/03/WR1	Multiple (2), wet ulcerative	Ankle, foot	90	M	24	Larkana, Warah
KMS 25	*L. (L.) tr*	MHOM/PK/03/MA14	Single, dry active	Face	30	F	9	Quetta, Mari Abad
KMS 26	*L. (L.) tr*	MHOM/PK/03/Q5	Single, wet ulcerative	Face	360	F	7	Quetta city
KMS 26	*L. (L.) tr*	MHOM/PK/03/MA2	Multiple (2), wet ulcerative	Face, leg	60	F	16	Quetta, Mari Abad
KMS 26	*L. (L.) tr*	MHOM/PK/03/MA4	Multiple (2) wet ulcerative	Face, wrist	360	M	50	Quetta, Mari Abad
KMS 26	*L. (L.) tr*	MHOM/PK/03/MA20	Multiple (2), dry active	Face, forehead	360	F	3	Quetta, Mari Abad
KMS 26	*L. (L.) tr*	MHOM/PK/03/QH4	Multiple (2), dry active	Face	60	F	7	Quetta city

* Done separately by multilocus enzyme electrophoresis and cytochrome *b* gene sequencing.
KMS, Kochi Medical School; *L. (L.) mj, Leishmania (Leishmania) major; L. (L.) tr, L. (L.) tropica;* F, female; M, male.

have been reported.[19] Only *L. (L.) majo*r, a causal agent of ZCL, was reported from Quetta, Balochistan, by using monoclonal antibodies, without indicating the detailed data of isolates tested, such as origins, altitudes of localities, lesion types, etc.[19]

This paper reports for the first time on the application of numerical zymotaxonomy and the *Cyt* b gene sequencing, in a double blind assay, in the characterization and identification of *Leishmania* isolated from CL patients who were actively recruited in endemic areas of Balochistan and Sindh provinces, Pakistan, and gives a brief discussion on the implications of the findings on the clinical and epidemiologic aspects of this disease.

MATERIALS AND METHODS

Parasites and study area. Seventeen stocks of *Leishmania* were isolated from lesions of patients with CL and analyzed enzymatically in this study. They were collected in two campaigns during January and December of 2003 in several villages in highlands (1,600–1,800 m, above sea level [a.s.l.]) and lowlands (~100 m, a.s.l.) of Balochistan and Sindh provinces of Pakistan (Table 1; Figure 1).

All subjects who were recruited in an active search using house-to-house visits agreed to participate voluntarily in this survey. Those who were diagnosed to have a *Leishmania* infection were treated with meglumine antimonate by local physicians, as was previously reported.[12]

Six World Health Organization (WHO) reference strains of *Leishmania* were included in the analysis: *L. (L.) major*, MHOM/SU/73/5ASKH and MHOM/IL/80/Friedlin; *L (L.) aethiopica,* MHOM/ET/72/L100; *L (L.) infantum*, MHOM/TN/80/IPT1; *L. (L.) donovani*, MHOM/IN/80/DD8; and *L. (L.) tropica*, MHOM/SU/74/K27.

Isolation, mass cultivation, soluble enzyme extract, and sample for DNA analysis preparation. Seventy-three aspirated materials were taken from lesion edges of suspected CL patients using a syringe with 0.5 mL of sterile proline balanced salts solution (PBSS) containing 100 U/mL penicillin and 50 μg/mL streptomycin (PS). The materials obtained were inoculated into "Difco" Blood agar (USMARU) medium containing 20% defibrinated rabbit blood.[20,21] Approximately 1 mL of PBSS was added after 4 days of cultivation. The cultures were maintained at ~25°C during field transportation. In the laboratory, the liquid phases of cultures were

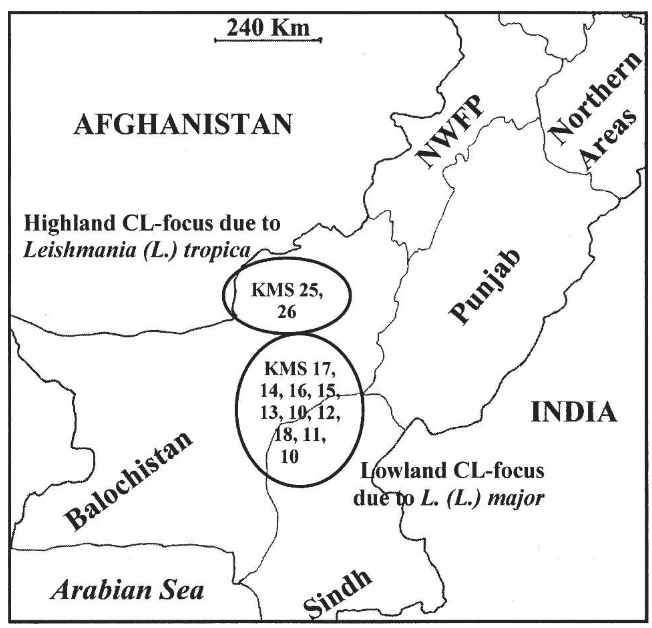

FIGURE 1. Map of Pakistan showing the geographical origin of the *Leishmania* zymodemes in Balochistan (highland CL focus, 1,600–1,800 m, a.s.l.: Quetta city and Mari Abad) and Sindh provinces (lowland CL focus, ~100 m, a.s.l.: Sono Khan, Tharri Hajra, Warah, Lalu Raunk, Shadadkot, and Gabi Dero; Sibi city, Balochistan, also belongs to this lowland focus). NWFP, Northwest Frontier Province; CL, Old World cutaneous leishmaniasis.

centrifuged at 2,500 rpm for 10 minutes, and the used USMARU mediums were replaced with fresh ones after washing the pellets twice with 5 mL of PBSS. The cultures were maintained at 23°C and examined regularly for 1 month.

All *Leishmania* stocks isolated were mass cultured using Schneider Drosophila medium supplemented with PS and 20% heat inactivated fetal bovine serum, after a maximum of two subcultures in USMARU. The soluble enzyme extracts were prepared from the promastigotes pellets obtained following the protocols previously described.[21] For the DNA analysis, a small amount of the promastigotes pellets was resuspended in 2 mL of TE buffer (10 mmol/L Tris-HCl, pH = 8, 1 mmol/L EDTA). These samples were aliquoted and stored at –20°C until use.

Enzyme electrophoresis. The electrophoresis, on cellulose acetate support (Sebiagel; Moulineux, France), staining, and photographic procedures were performed following the methods previously described.[21] Each enzyme extract was analyzed by 11 enzyme systems for 12 putative enzymatic loci: alanine aminotransferase (E.C.2.6.1.2; ALAT), aspartate aminotransferase (E.C.2.6.1.1; ASAT), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49; G6PDH), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44; 6GPDH), glucose-phosphate isomerase (E.C.5.3.1.9; GPI), malate dehydrogenase (E.C.1.1.1.37; MDH), malic enzyme (E.C.1.1.1.40; ME), mannose-phosphate isomerase (E.C.5.3.1.8; MPI), nucleoside hydrolase (inosine) (E.C.2.4.2; NHi1 and NHi2), phosphoglucomutase (E.C.2.7.5.1; PGM), and pyruvate kinase (E.C.2.7.1.40; PK).

Electrophoretic data analysis. The distance that each reproducible enzyme band migrated from the origin (anode) was measured. The bands were numerated from the fastest (the one nearest to the cathode) to the slowest for each enzyme gel or zymogram. The obtained set of bands defined an electrophoretic profile for each extract. Stocks with the same profile were grouped in the same zymodeme; the species attribution was made comparing the profiles with the WHO reference *Leishmania* strains. To examine the relationship between the zymodemes, Jaccard distances were obtained for all pairwise comparisons, and a distance matrix was built using software designed on practical extraction and report language (Perl) for this work and transformed into a phenogram by the UPGMA method using the MEGA version 2.1 software.[21,22]

Detection of cytochrome *b* gene using PCR. To extract the genomic DNA from each of the samples prepared, a genomic extraction kit (i.e., Genomic Prep™ Cell and Tissue DNA Isolation Kit; Amersham Biosciences, Piscataway, NJ) were used, following the protocol and methods described by the company. PCR was performed with Ex-Taq polymerase (Takara Bio Inc., Takara, Japan) under the following conditions: initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for one minute, 50°C for one minute, and 72°C for one minute. Two hundred nanograms of parasite DNA as a template, fragments of LCBF1 forward primer (5′-GGTGTAGGTTTTAGTYTAGG-3′), and LCBR2 reverse primer (5′-CTACAATAAACAAATCATAATATRCAATT-3′) were used for *Cyt* b gene amplification. The PCR products were visualized on a 1% agarose gel.

Cytochrome *b* gene direct sequencing analysis. The *Cyt* b gene sequences for each stock were determined following the procedure described previously.[4,5] Briefly, the amplified fragments were electrophoresed on 1% Seakam GTG agarose gel (FMC, Germany), the band was excised, and the DNA was purified by using the QIA quick Gel Extraction kit (Qiagen, Valencia, CA). The PCR products were examined by direct sequencing with the BigDye Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA) and ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing primers used were LCBF1, LCBR2, LCYT B F4L (5′-TGTTATTGAATATGAGGTAGTG-3′), and LCYT B R4 (5′-GAACTCATAAAATAATGTAAACAAAA-3′). The obtained sequences, assembled and edited by Genetyx Mac 11.0.0 (Software Development Co. Ltd., Japan) were compared with the reference strains, available from DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers from AB095957 to AB095970.

Statistical analysis. The Fisher exact test was used for analyzing the relation between clinical features (wet or dry type lesions) and *Leishmania* species involved.

RESULTS

Multi-Locus enzyme electrophoresis analysis. Seventeen *Leishmania* stocks isolated from patients infected in Pakistani CL-endemic areas were analyzed studying 12 putative enzymatic loci. The denomination, species assignation of the isolates, the clinical data of the patients and their geographic origin are shown in Table 1 and Figure 1. Based on phenotypic comparison, 11 stocks that expressed nine zymodemes were assigned to *L. (L.) major*. All were isolated from the lowlands (~100 m, a.s.l.) of the Larkana district (Sono Khan, Tharri Hajra, Warah, Lalu Raunk, Shadadkot, and Gaibi Dero), Sindh, and Sibi city, Balochistan.

The remaining six, distributed in two zymodemes (five and one for each) isolated from the highland (1,600–1,800 m, a.s.l.) of Quetta (Quetta city and Mari Abad), Balochistan, were identified as *L. (L.) tropica*. None of the Pakistani isolates showed the same enzymatic profile as the WHO reference strains used (Table 2; Figure 2). The ALAT, PK, and MPI were the loci that had clearly differentiated between *L. (L.) major* and *L. (L.) tropica* (Figure 3A). The GPI, PGM, and G6PDH loci showed the phenotypic diversity among the *L. (L.) major* stocks (Figure 3B), whereas GPI and MDH loci were polymorphous for *L. (L.) tropica* (Figure 3C). The enzymatic profiles are shown in Table 2.

Species identification based on cytochrome *b* gene sequencing. The species assignation based on this recently established technique was made comparing each one of the *Cyt* b sequences of the 17 Pakistani *Leishmania* stocks with the reference strains previously published with accession numbers from AB095957 to AB095970.[4] Eleven of these stocks were identified as *L. (L.) major*, because their sequences showed the highest similarity with the reference strain MHOM/SU/73/5ASKH (AB095961), 99.70% for 10 of them, and 99.60% for MHOM/PK/03/SK2. The remaining six stocks were assigned to *L. (L.) tropica*, because their sequences were indistinguishable (100% of similarity) with the reference strain MHOM/SU/58/strain OD (AB095960).

There was complete agreement on the *Leishmania* species identification between MLEE and *Cyt* b gene sequencing (Table 1).

No association was found between *Leishmania* species and the induction of clinical features (wet or dry type lesions; $P > 0.05$).

TABLE 2
Enzymatic profiles for Pakistani *Leishmania* stocks and zymodemes of WHO reference strain

	Enzymatic loci											
Zymodeme	ALAT	ASAT	G6PDH	6GPDH	GPI	MDH	ME	NH_i1	NH_i2	PGM	PK	MPI
KMS 10	1	3	2,6	2	2,4,6	2,5	3	2	2	5,8	3	1
KMS 11	1	3	2,6	3	2,6	2,5	3	2	2	3,8	3	1
KMS 12	1	3	2,5	2	2,4,6	2,5	3	2	2	3,7	3	1
KMS 13	1	3	5	2	3,6	2,5	3	2	2	7	3	1
KMS 14	1	3	2	2	3,6	2,5	3	2	2	3	3	1
KMS 15	1	3	2	2	1,6	2,5	3	2	2	3	3	1
KMS 16	1	3	2	2	2	2,5	3	2	2	2,7	3	1
KMS 17	1	3	5	2	2,5	2,5	3	2	2	6	3	1
KMS 18	1	3	2,6	2	2,5	2,5	3	2	2	2,6	3	1
KMS 19	1	3	2,5	2	2,5	1,5	3	2	2	2,6	3	1
KMS 20	1	3	2,5	2	2	1,5	3	2	2	6	3	1
KMS 21	3	2	4	3	1,6	4,5,8	1	1	–	4	2	2
KMS 22	3	1,3	3,6	1	3,6	2,6,8	2	2	3	2,7	1	2
KMS 23	3	3	1,4	3	3,6	2,4,6,8	2	2	4	1,4		2
KMS 24	2	1,3	6	3	3,6	3,8	2	2	1	7	4	3
KMS 25	2	1	6	3	2,6	3,7	2	2	1	7	4	3
KMS 26	2	1	6	3	3,6	3,7	2	2	1	7	4	3

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; G6PDH, glucose-6-phosphate dehydrogenase; 6GPDH, 6-phosphogluconate dehydrogenase; GPI, glucose-phosphate isomerase; MDH, malate dehydrogenase; ME, malic enzyme; NH_i1 and NH_i2, nucleoside hydrolase (inosine); PGM, phosphoglucomutase; PK, pyruvate kinase; MPI, mannose-phosphate isomerase; KMS, Kochi Medical School; KMS 10–18, *Leishmania (Leishmania) major*; KMS 25 and 26, *L. (L.) tropica* from Pakistan (Table 1); KMS 19, MHOM/IL/80/Friedlin, *L. (L.) major* reference strain; KMS 20, MHOM/SU/73/5ASKH, *L. (L.) major* reference strain; KMS 21, *L. (L.) aethiopica* reference strain; KMS 22, *L. (L.) infantum* reference strain; KMS 23, *L. (L.) donovani* reference strain; KMS 24, *L. (L.) tropica*.

DISCUSSION

Accurate identification of etiologic agents for *Leishmania* spp. by molecular approaches in the transmission foci of the disease is highly relevant not only for clinical aspects but also for eco-epidemiologic features, because of the diversity of putative vectors and reservoirs associated with *Leishmania* transmission.[3]

In this study, two *Leishmania* spp., *L. (L.) tropica* and *L. (L.) major,* were found at different altitudes in Pakistan; the former from highlands and the latter from lowlands. This is the first report of the incrimination by molecular techniques of *L. (L.) major* as a causal agent of CL in Sindh province, Pakistan. The species was also found in Sibi, a lowland city of Balochistan province. *L. (L.) major* has been reported from the highlands (Quetta city) of Balochistan,[19] without indicating, however, the clinico-epidemiologic data of the parasite sources including locality origins of the patients and the basic features of the technique used. In contrast, we only found a single species, *L. (L.) tropica,* in the highland areas (1,600–1,800 m, a.s.l.) of Quetta city, Balochistan.

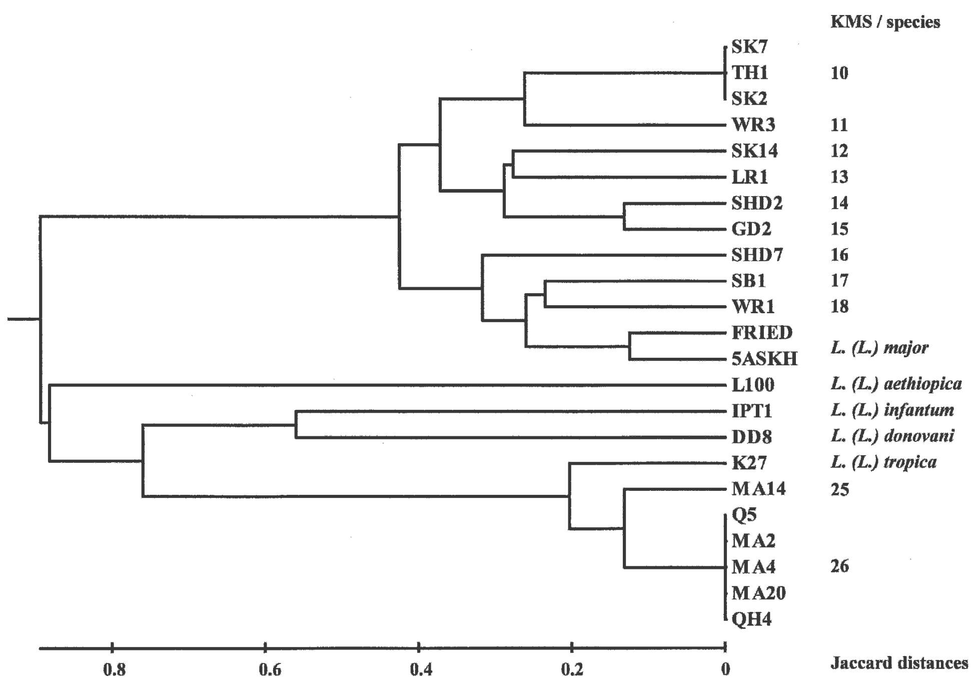

FIGURE 2. Phenogram built by the UPGMA method showing the species assignation based on the phylogenetic relationship among the zymodemes expressed by Pakistani and WHO reference strains of *Leishmania*. The denomination and characteristics of the stocks and zymodemes are shown in Tables 1 and 2.

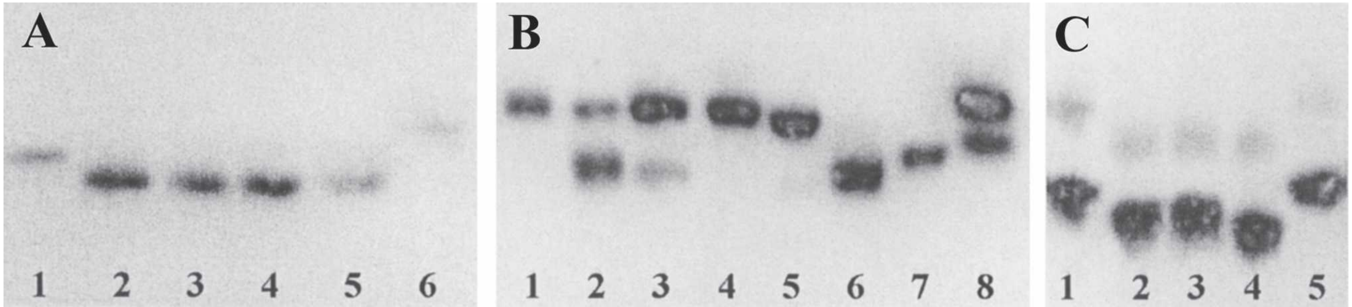

FIGURE 3. (**A**) Mannose-phosphate isomerase (MPI) zymogram showing the *Leishmania* species assignation of Pakistani stocks. Line 1, band expressed indifferently by *L. (L.) aethiopica*, *L. (L.) infantum,* and *L. (L.) donovani* reference strains; lines 2–5, Pakistani and reference strains of *L. (L.) tropica*; line 6, Pakistani and reference strains of *L. (L.) major*. (**B**) Glucose-6-phosphate dehydrogenase (G6PDH) zymogram. The intraspecific polymorphism of *L. (L.) major* is shown. Lines 1–5 and 7, zymodemes KMS 16, 18, 11, 15, 10 (local), and reference strain MHOM/SU/73/5ASKH of *L. (L.) major,* respectively; line 6, *L. (L.) tropica* reference strain; line 8, *L. (L.) donovani* reference strain. (**C**) Malate dehydrogenase (MDH) zymogram. Intraspecific polymorphism of *L. (L.) tropica*. Lines 1 and 5, zymodemes KMS 13 and reference strain of *L. (L.) major*; lines 2 and 3, local zymodeme KMS 26 of *L. (L.) tropica*; line 4, reference strain of *L. (L.) tropica*.

Although *L. (L.) major* was the only species found in the lowland CL-endemic foci, and *L. (L.) tropica* the only one in the highland foci of these study sites, the clinical features of CL described as wet and dry type lesions were detected in both foci. Because no statistical association between these clinical presentations and the *Leishmania* species involved were found, the lesion types such as dry and wet clinical forms might be affected by the secondary infections, environmental factors, and/or host-related factors. Therefore, this lack of association suggests that the identification or estimation of the etiological *Leishmania* species from the clinical features may not be reliable.[11,12,18,23] This criterion might be supported by the work of Aljeboori and Evans[24] in which *L. (L.) major* was isolated from dry type lesions (usually associated with *L. (L.) tropica*) in Iraq.

A great intraspecific polymorphism was found by MLEE among the Pakistani *L. (L.) major* isolates, whereas the *L. (L.) tropica* stocks have been shown to be a homogeneous group. Although genetic diversity can be an intrinsic characteristic for each species, it may be correlated with the number of vectors and/or animal reservoirs involved in the transmission cycles.[3] Thus, although many wild mammals can be the reservoirs of *L. (L.) major* in a zoonotic cycle, humans may act as a sole reservoir of *L. (L.) tropica* in a restricted anthroponotic cycle.[13,14] This also has implications for the occurrence of CL in Balochistan and Sindh provinces. Whereas the dispersion for *L. (L.) tropica* is connected with humans and their activities, for *L. (L.) major,* it may be more related with the movement or ecological aspects of the animal primary reservoirs than the human activities, because this species conforms to zoonotic cycles.[25]

The little intra-specific variations found on the *Cyt* b gene among Pakistani zymodemes for both of the *Leishmania* species analyzed have indicated that the former is highly conserved sequences inside these species. On the other hand, there are clear inter-specific differences in the *Cyt* b gene sequences.[4] Thus, these data validate and highlight the usefulness of *Cyt* b gene sequencing on *Leishmania* species identification among wild Pakistani stocks.

In conclusion, the accurate identification by the gold standard and a novel molecular technique of *L. (L.) tropica* and *L. (L.) major* in two different foci of Balochistan and Sindh provinces, CL-endemic areas in Pakistan, reported here contributes not only to the clinical aspect but also to the eco-epidemiology of the diseases. Such data represent a first step for future incrimination of vectors and/or reservoirs, disclosing the eco-epidemiologic picture of these diseases.

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