Comparison of Some Molecular-genetic Techniques for Identification of *Leishmania* Circulating in Natural Foci of Zoonotic Cutaneous Leishmaniasis in the Central Asia Region

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Different molecular-genetic methods were used to identify a cohort of Leishmania *strains from natural foci of zoonotic cutaneous leishmaniasis located in Central Asia, on the former USSR territory. The results obtained using isoenzymes, PCR, restriction fragment length polymorphisms of kDNA and molecular hybridization techniques are discussed in terms of their applicability, discrimination power and feasibility for answering questions related to molecular epidemiological research and for detecting mixed* Leishmania *infections.*

Key words: Old World leishmaniasis - isoenzymes - kDNA - PCR - hybridization

The natural foci of *Leishmania major* infection comprise the vast territory of the Turan plain and coincide with the distribution of the reservoir host *Rhombomys opimus*. According to Dubrovsky (1978), very active leishmaniasis foci are found in irrigation oases in Turkmenistan, Uzbekistan and Kazakhstan (former USSR). It has, until recently, been assumed that leishmanial parasites circulating in the *R. opimus* population are considered to be *L. major*, displaying a great range of virulence and infectivity for humans. However, the introduction of new techniques for *Leishmania* characterization indicated that the parasites formerly identified as *L. major* in these areas belong to a polytype group comprising three independent species namely *L. major* sensu stricto, *L. turanica* (Strelkova et al. 1990b) and *L. gerbilli*. In fact, the three species are able to coexist in a single gerbil (*R. opimus*). *L. major* and *L. turanica* are generally found together even in the same skin lesion. Each species has its own relatively limited range of virulence; while *L. major* is found to be pathogenic for humans, *L. turanica* and *L. gerbilli* are not (Strelkova et al. 1990a, Strelkova 1990).

All the above mentioned stress the necessity to look for feasible methods for *Leishmania* identification both in a single or in mixed infections. The present paper attempts to evaluate some phenotypic and genotypic techniques for this purpose and discusses their relationship to epidemiological studies.

MATERIALS AND METHODS

Parasite strains - The strains studied are listed in Table. The isolates were obtained from patients with cutaneous leishmaniasis, from great gerbils (*R. opimus*) and from a sandfly (*Phlebotomus papatasi*) in Turkmenistan, Uzbekistan and Kazakhstan.

Isolation of the parasites - Parasite isolations were made by injecting as much sterile saline as possible containing penicillin (250 U/ml) and streptomycin (250 mg/ml) intradermally into gerbils' affected ears. The saline was aspirated and inoculated into tubes containing NNN medium. Fragments of human cutaneous lesions were obtained using a sterile scarifier or a scalpel and transferred into tubes of NNN medium containing antibiotics as above.

The tubes were incubated at 22-24°C and examined weekly for the presence of promastigotes for up to six weeks. The positive cultures were cryopreserved in liquid nitrogen.

Cloning - Clones were made using a Fonbrune micromanipulator (Alekseev & Safjanova 1977). With this micromanipulator a single promastigote

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TABLE

List of *Leishmania* strains analyzed in this study

Code	Geographical origin	Source	Virulence
MRHO/SU/88/KD704 *Leishmania major*	Uzbekistan	*Rhombomys opimus*	High
MHOM/SU/87/NARK *L. major*	Uzbekistan	Man	High
MRHO/SU/59/NEAL P *L. major*	Uzbekistan	*R. opimus*	Avirulent
IPAP/SU/91/M97 *L. major*	Turkmenistan	*Phlebotomus papatasi*	High
MRHO/SU/83/KD51 *L. turanica*	Uzbekistan	*R. opimus*	Low
MRHO/SU/80/CL3720 *L. turanica*	Uzbekistan	*R. opimus*	Low
MRHO/SU/87/DA11 *L. major/L. turanica*	Uzbekistan	*R. opimus*	Moderate
MRHO/SU/87/BK7 *L. turanica*	Kazakhstan	*R. opimus*	Moderate
MRHO/SU/88/E18 *L. gerbilli*	Kazakhstan	*R. opimus*	Low

can be aspirated and released into a separate drop of medium under microscopic control.

Development in experimental animals - The pathogenicity of the *Leishmania* isolates was assessed in hamsters (Kelina 1982). The inoculum consisted of 7.5 x 10^4 promastigotes from early stationary phase cultures, injected intradermally into the ears. The hamsters were examined weekly during the first month and every two weeks during the five subsequent months with registration of clinical signs of leishmaniasis. Parasite cultures which produced progressive ulcerative lesions in the hamsters within six weeks of inoculation were classified as highly virulent and those which produced only infiltrative lesions within six months were classified as of low virulence. Some parasite cultures produced abortive ulcerative lesions during five or six months of observation after inoculation and were considered as of moderate virulence. On the other hand, some cultures were found to be avirulent causing no infection in hamsters.

Isoenzyme electrophoresis - Promastigotes were harvested from seven-day cultures in MEM:EBLB:FCS medium (Evans 1987), washed three times in saline solution and centrifuged at 4ºC, 2.700 g for 10 min. Two volumes of 0.01 M aminocaproic acid were added to the sediment after the last washing and the cells were lysed by temperature shock. The lysates were centrifuged at 12,000 g for 20 min and the clear supernatants were analyzed by eletrophoresis. *Leishmania* isolates were identified by using the following marker strains: *L. major* (MRHO/SU/59/NEALP); *L. turanica* (MRHO/SU/83/KD51); *L. gerbilli* (MRHO/CN/60/GERBILLI). The electrophoresis analyses were carried out in polyacrylamide gels and a total of nine enzymes were used (PGI, PGM, 6PGD, MDH, ME, G6PD, ALAT, ASAT, Est.D). Conditions for electrophoresis and histochemical staining have been previously described (Harris & Hopkinson 1976, Altychov et al. 1981).

DNA isolation and PCR amplification - The DNA was obtained according to Sambrook et al. (1989). Promastigotes (10^4 - 10^5) were suspended in 200 µl of buffer containing 50 mM Tris-HCL pH 7.8, 50 mM EDTA, 100 mM NaCL, 1% Triton X-100, 1 µg/ml Proteinase K and incubated at 65ºC for 30 min. The cell residues were sedimented by centrifugation at 10,000 g for 2 min. PCR amplification was carried out according to Saiki et al. (1985) in 30 µl of the reaction buffer (20 mM Tris-HCL pH 8.8, 10 mM (NH4)2 SO4, 8 mM MgCL2, 100 µg/ml BSA) containing 0.25 mM of each dNTP, 2 units of Taq polymerase and 40-80 ng of primer. The PCR procedure was carried out in a thermocycler developed at St. Petersburg Institute of Nuclear Physics using 32 cycles regime: denaturation condition 92ºC for 50 sec, primer hybridization condition 55ºC for 4 sec and DNA extension 69ºC for 50 sec. PCR products were separated on 1.8% agarose gels. The gels were stained by ethidium bromide and photographed under an ultraviolet transiluminator. The oligonucleotide primer with the following sequence: 5'

TAAGGGCGGTGCCAGT was used (Bulat & Mironenko 1992).

kDNA extraction and digestion with restriction enzymes - The technique of extraction and analysis of kDNA restriction profiles (schizodeme analysis) has been previously described (Pacheco et al. 1986). Parasites were lysed using a strong detergent (sarkosyl) and digested by pronase at 60°C. The kDNA networks were collected by centrifugation at 33,000 rpm for 2 hr, extracted by phenol-chloroform followed by ethanol precipitation. Two micrograms of purified kDNA preparations were digested with the restriction enzymes Hae III and Alu I in the appropriate buffers according to the manufacturer's instructions. Maxi and minicircle kDNA fragments were separated in 5%-10% linear polyacrylamide gradient gels and photographed under ultraviolet light.

Dot blot hybridization - Purified kDNAs (20 ng/dot) were spotted onto nylon membranes (Zetaprobe, Biorad) after alkaline denaturation in 0.4 N NaOH using a microfiltration apparatus connected to a vacuum pump. After blotting the membranes were rinsed in 2X SSC (1X SSC is 150 mM sodium chloride/15 mM sodium citrate) and the DNA immobilized by ultraviolet light cross-link. Total kDNAs from *L. major* and *L. turanica* were radiolabelled with alfa 32P dATP by the technique of Random Primer (Feinberg & Volgelstein 1983) modified according to the protocol described by Pacheco et al. (1994) and used as probes. Hybridizations were carried out overnight at 65°C and the membranes were washed at 65°C in 0.1X SSC/ 0.5% SDS, 3 times 30 min each. Blots were exposed to Kodak X-OMAT X ray films with an intensifying screen overnight.

RESULTS

The isoenzyme analysis allowed us to separate the strains into four groups (Fig. 1). The first group was composed by the strains KD-704, NARK and M-97 of *L. major*, the second by the strains CL3720 and BK-7 of *L. turanica. L. gerbilli* (E-18) belonged to the third group and the last one was composed by a mixture of *L. major* and *L. turanica* (DA-11). After cloning procedures, 24 and 18 clones have been respectively obtained from strains BK-7 and DA-11. The isoenzyme analysis enabled us to identify 1 clone from the strain BK-7 as *L. major* and the remaining 23 clones as *L. turanica*, while among clones isolated from the strain DA-11, *L. turanica* (12 clones) and *L. major* (6 clones) have been identified (not shown).

Analysis of PCR patterns (Fig. 2) showed that the strains identified by isoenzymes as *L. major* and *L. turanica* displayed significant differences in their amplification products. Although, the sample composed by an artificial mixture of *L. major* and *L. turanica* promastigotes (1:1) was similar to *L. major*. The low molecular weight amplification product (320 bp) probably belong to *L. turanica*.

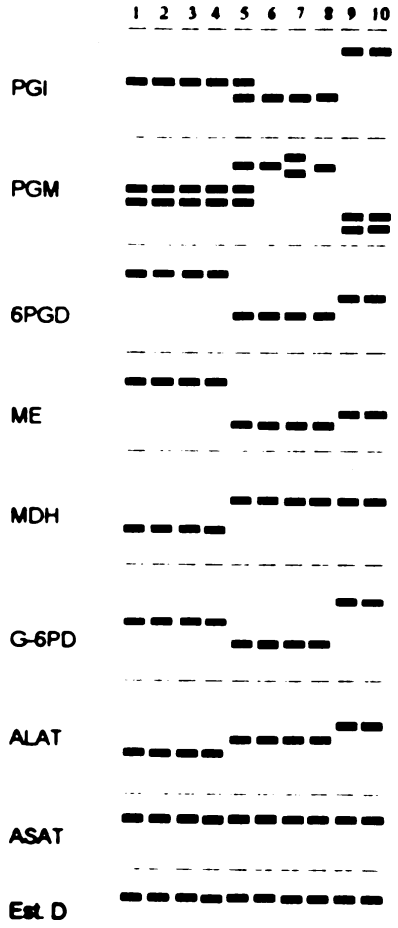

Fig.1: diagrammatic representation of the isoenzymatic patterns after electrophoresis in polyacrylamide gel. 1: *Leishmania major* (MRHO/SU/59/NEAL P)*, 2: *L. major* (MRHO/SU/87/KD-704), 3: *L. major* (MHOM/SU/87/NARK), 4: *L. major* (IPAP/SU/91/M-97), 5: mixed infection *L. major/L. turanica* (MRHO/SU/87/DA-11), 6: *L. turanica* (MRHO/SU/83/KD-51)*, 7: *L. turanica* (MRHO/SU/80/CL3720), 8: *L. turanica* (MRHO/SU/87/BK-7), 9: *L. gerbilli* (MRHO/CN/60/GERBILLI)*, 10: *L. gerbilli* (MRHO/SU/88/E-18)*, * marker strains.

Results of schizodeme analysis showed that NARK and KD-704 strains characterized as *L. major* displayed the same kDNA restriction profile, while the other strains analyzed each had a different genotypic profile. Strain DA-11 which is, according to the isoenzyme analysis, composed of a mixture of *L. turanica* and *L. major* showed a profile very closed to *L. major* (NARK and KD-704), although some slight microheterogeneities were observed in the kDNA minicircle regions. The isolate BK-7 displayed a more complex restriction profile; additional minicircle bands were observed

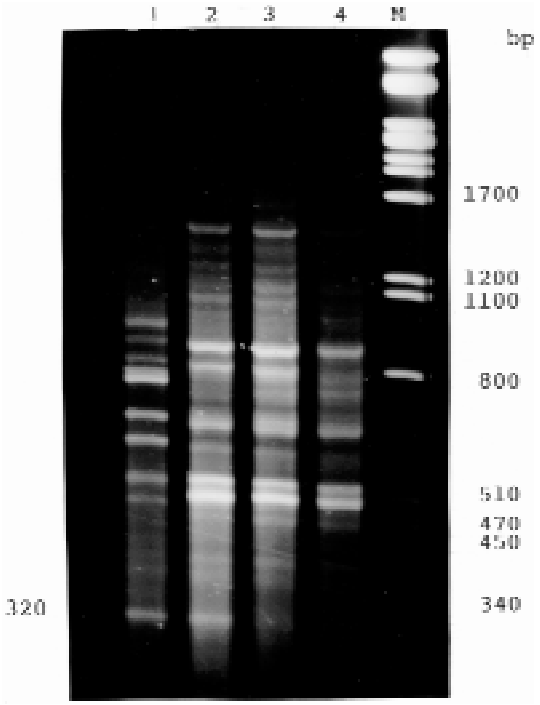

Fig. 2: agarose gel showing the PCR products after amplification reactions with the primer 5' TAAGGGCGGTGCCAGT. 1: *Leishmania turanica* (MRHO/SU/80/CL3720), 2: mixture (1:1) *L. major* (MHOM/SU/59/NEAL P) + *L. turanica* (MRHO/SU/80/CL3720), 3: *L. major* (IPAP/SU/91/M-97), 4: *L. major* (MHOM/SU/59/NEAL P), M: marker, lambda DNA digested with Pst I.

when compared with the other isolates. On the other hand, the isolate KD-51 was found to possess a very distinct profile. The results were confirmed by using a second enzyme, Alu I, which did not cut kDNA minicircles of this isolate suggesting that it is, in fact, a different parasite.

Molecular hybridization experiments have confirmed, in some aspects, the results of schizodeme analysis. By this approach, strains DA-11 and BK-7 are closely related to *L. major*. The radioactive labelled kDNA of *L. major* used as probe showed strong hybridization signals with the homologous kDNAs (NARK and KD-704) indicating a high degree of sequence homology and also with the isolates DA-11 and BK-7, although in different degrees. In addition, faint hybridization signals were detected with *L. turanica* (KD-51) and with *L. gerbilli* (E-18). Results of both methodologies are shown in Figs 3 and 4. By comparing the results of filter 1 with that of filter 3 one can observe that there is no strong cross-homology between *L. turanica* (KD-51) kDNA probe and kDNAs from *L. major* strains. These results indicate that strain KD-51 is indeed less closely related to *L. major*. No strong DNA homology was detected between the isolates KD-51 and DA-11 in cross-hybridization analysis. According to these results it is reasonable to establish the existence of a more closely genotypic relationship between *L. turanica* KD-51 and BK-7 than with the isolate DA-11 (Fig. 4).

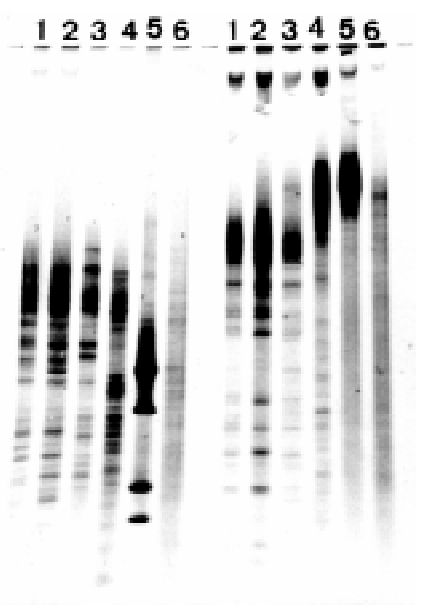

Fig. 3: polyacrylamide gradient gel (5%-10%) showing kDNA restriction fragments after digestion with the restriction endonucleases Hae III (left) and Alu I (right). 1: *Leishmania major* (MHOM/SU/87/NARK), 2: *L. major* (MRHO/SU/87/KD-704), 3: *L. major/L. turanica* (MRHO/SU/87/DA-11), 4: *L. turanica* (MRHO/SU/87/BK-7), 5: *L. turanica* (MRHO/SU/83/KD-51), 6: *L. gerbilli* (MRHO/SU/88/E-18).

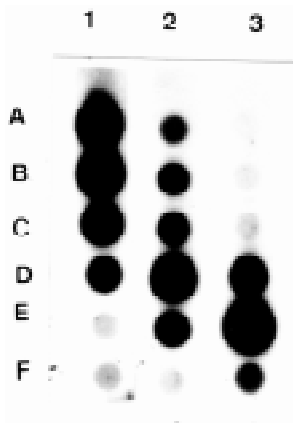

Fig. 4: autoradiograph showing dot-blot hybridization of purified kDNA from different isolates against radiolabelled total kDNAs of *Leishmania major* (MHOM/SU/87/NARK) (filter 1), *L. turanica* (MRHO/SU/87/BK-7) (filter 2) and *L. turanica* (MRHO/SU/83/KD-51) (filter 3) used as probes. A: *L. major* (MHOM/SU/87/NARK), B: *L. major* (MRHO/SU/87/KD-704), C: *L. major/L. turanica* (MRHO/SU/87/DA-11), D: *L. turanica* (MRHO/SU/87/BK-7), E: *L. turanica* (MRHO/SU/83/KD-51), F: *L. gerbilli* (MRHO/SU/88/E-18).

DISCUSSION

Until the 1970s, studies on natural leishmaniasis foci were based on methods used in clinical epidemiology and population ecology. Theses approaches allowed researchers to identify natural hosts and vectors of *Leishmania*, to analyse their biological and ecological features and to use this knowledge for initiating a series of programs for control of human leishmaniasis.

However, extensive data accumulated during the last two decades showed that these methods have incontestable limitations, particularly with respect to the identification of *Leishmania* species. At the same time, new molecular-genetic methods have been developed and subsequent progress in studies of *Leishmania* was associated with their introduction into laboratory routine. On the other hand, these methods proved to be not always adequated to the problems faced by epidemiologists and parasitologists.

The major goal of this report was to justify the systems that have been used for *Leishmania* identification by evaluating the potential of each technique as a discriminatory tool. Isoenzyme electrophoresis is considered a very discriminatory system for *Leishmania* identification (Le Blancq & Peters 1986, Le Blancq et al. 1986). Some authors use izoenzyme analysis to investigate variations at intraspecies level in *Leishmania* from different areas and hosts. By using nine enzymatic loci we were able to detect *L. major* and *L. turanica* as well as mixtures of both species in some isolates from endemic areas (for example DA-11). But, we were unable to distinguish in others as for example the isolate BK-7. Polymerase chain reaction, on the other hand, is a very promising technique made available for a rapid parasite identification. The results of PCR reaction in the present study suggest significant differentiation among *L. major, L. turanica* and *L. gerbilli* genomes, but mixture of *L. major/L. turanica* could hardly be identified. Such results encourage us to consider PCR amplification as a step for future development of specific primers for detecting sympatric disease agents which are relatively difficult to discriminate. Restriction fragment length polymorphisms of kDNA analyses also showed the feasibility of distinguishing among *L. major, L. turanica* and *L. gerbilli.* In addition, the technique revealed the presence of the same schizodeme of *L. major* circulating in two different hosts in the same geographic area, suggesting the wide spreading of this genotypic profile in humans and gerbils, as well as confirming the role of *R. opimus* as a reservoir host. Restriction endonuclease analyses showed genetic differences or similarities among the isolates emphasizing that is a valuable tool in epidemiological surveys. Mixed infections and/or clonal heterogeneity can be detected by schizodeme analysis (Pacheco et al. 1990, 1995) but the *L. major/L. turanica* infection in the present study was not recognized. Hybridization experiments gave an estimation of genetic proximity or distance of the strains.

In selecting suitable methods for a particular study, their properties and limitations need to be considered in relationship to the scope and purpose of the study. In endemic areas where distinct *Leishmania* species are concurrently transmitted, the possibility of mixed infections should not be discarded. In this work, we compared different methods of *Leishmania* identification and found that isoenzyme, PRC and restriction fragments of kDNA analyses are equally useful for discriminating single infections. However, in studies of mixed *Leishmania* infection or when difficulties in the taxonomic classification of an isolate occur these integrated methodologies in conjunction with biological data can produce reliable results. Nevertheless, in some particular cases, the analysis should be performed in several stages (identification - cloning - identification), which makes it much more labor-consuming.

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