

Characteristics of *Leishmania* spp. isolated from a mixed focus of cutaneous and visceral Leishmaniasis in Himachal Pradesh (India)

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Abstract

Background: A mixed endemic focus of cutaneous and visceral leishmaniasis has emerged along the Sutluj river valley of Himachal Pradesh (India).

Methods: The laboratory work up of 218 new consecutive cases of localized cutaneous leishmaniasis (LCL) and 14 patients of visceral leishmaniasis (VL) from this focus is analyzed.

Results: Tissue smears were positive in 44.42% of LCL and 78.57% of VL patients. Positivity was higher (73.97%) in LCL lesions of <6 months duration as compared to (36.53%) those of >6 months duration. Only modified NNN medium supplemented with RPMI 1640 and 10% heat inactivated fetal bovine serum was found to be the most suitable for primary isolation of the parasite while other seven media used did not yield significant growth. Culture for the organism was positive in 38 of 112 (33.92%) patients of LCL and 9 of 14 (64.28%) patients of VL. The cultured organisms from 4 each of LCL and VL samples were identified by PCR as *Leishmania donovani*. Although subculture/bulk cultivation of this *Leishmania* spp. was unsuccessful in the various media tried, the two of the primary cultures could be maintained for 50 days by just replenishing the liquid overlay. **Conclusion:** This new focus of leishmaniasis appears peculiar where cutaneous and visceral forms co-exist, and both *Leishmania donovani* and *Leishmania tropica* are producing LCL all the while *L. donovani* being the predominant pathogen. The difficulty in culturing the isolates is also reminiscent of LCL caused by *Leishmania infantum* suggesting that these isolates, both from LCL and VL patients, perhaps belong to *Leishmania donovani*-*infantum* complex.

INTRODUCTION

Leishmaniasis is a protozoal disease capable of producing a spectrum of clinical syndromes ranging from cutaneous lesions to systemic infections. With the exception of Australia and Antarctica, the parasites have been identified throughout the world with an estimated worldwide annual incidence of about 2 million cases primarily of cutaneous leishmaniasis [1,2]. In the Old World, localized cutaneous leishmaniasis (LCL) has been reported to result from the infection by *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica* and *Leishmania infantum*. Recently, isolated reports have appeared implicating *Leishmania donovani* as the causative agent for LCL [3,4,5,6].

The diagnosis of leishmaniasis is complicated by the fact that different strains and species are associated with various forms of the disease, and their geographical variations in clinical presentation. The cultural characteristics of different strains may also vary in certain foci and some difficult to

culture strains have been observed. Characterization of the isolated parasites is necessary for epidemiological purposes as well as for tracing their phylogenetic origins while species identification is important in view of variable therapeutic response observed in different species [7]. Thus parasitologic confirmation of the diagnosis becomes imperative for correct and early treatment.

A new focus of LCL has recently emerged in Himachal Pradesh, a small hill state in North-West India [8]. Sporadic cases of visceral leishmaniasis (VL) have been reported from here in the past [9,10] and are still being observed as well. Peculiarly both *L. donovani* and *L. tropica* have been assigned as the species to the isolates from the LCL patients of this new focus, preliminary studies showed that this being predominantly *L. donovani* [6]. The characterization of the cultured parasite from any new endemic focus by genotyping e.g. by zymodeme analysis or PCR studies is an essential prerequisite. However, earlier we had mainly studied the

clinico-epidemiologic profile and strain characteristics of the *Leishmania* spp. responsible for the disease in this area [6,8]. In this communication, we report cultural characteristics of the parasites isolated from this mixed focus of LCL and VL highlighting some cultural variability of the parasites. The results of PCR done on strains cultured from 9 LCL and 4 VL patients are also presented.

MATERIAL AND METHODS

The LCL and VL patients reporting from April 2003 to December 2005 were selected for the culture studies. Specimens of VL were provided either by Medicine or Pediatrics departments in the form of smears and bone marrow or splenic aspirates. Some of the patients were examined in survey-cum- treatment field camps, where smear/ culture was not carried out for all the patients. After informed consent punch biopsies (4 mm) were obtained from the indurated margin of the lesions under local anesthesia while observing all aseptic measures. In a sterile petri dish the biopsy tissue was cleared of blood with sterile gauze and cut into two parts. Touch smears were prepared from the smaller part. The smears were stained with Giemsa stain and examined under oil immersion for amastigotes. The other larger part of the biopsy was teased thoroughly with a sterile needle and inoculated into the liquid phase of the different media. As the nutritional requirements of the *Leishmania* spp. from this new focus were not known, the culture was attempted on different media as shown in Table-1. All these culture media were supplied by Hi-Media Laboratories, Mumbai, India.

Penicillin (100units/ml) and Gentamycin (50mg/100ml) were added to all these media to check bacterial contamination. They were used within 28 days of preparation. Primary isolation was mainly attempted on media I-IV, while subculture/maintenance was attempted on media V-VIII. When the subculture failed in media V-VIII, it was attempted in media I-IV. The inoculated media were incubated at $25 \pm 1^\circ \text{C}$. Wet preparations were prepared from liquid overlay of these culture media every other day from 3rd day onwards and screened thoroughly for promastigotes under phase contrast microscope. All cultures were held for 21 days before labeling them as negative. In case of a positive culture the subculture was attempted on every 7th or 8th day. A rough estimate of positivity was made by counting the number of promastigotes in 20 fields and then calculating the average number/field. Smears for promastigotes were made from highly positive cultures and

stained with Giemsa for permanent record.

PREPARATION OF DNA SAMPLES FOR PCR

Thirteen samples of cultured organisms from 9 LCL and 4 VL patients were processed at Molecular Immunology Department, Postgraduate Institute of Medical Education & Research, Chandigarh (India), for *L. donovani* specific PCR studies. The total genomic DNA from the promastigotes of *Leishmania* was isolated by the method of Palmiter et al [11]. Briefly the pelleted parasites were suspended in 500 μl of lysis buffer containing 1% SDS and 0.01% proteinase K in Tris-EDTA (TE) buffer (pH 8.0) and incubated at 55°C overnight. After phenol-chloroform extraction, the DNA was precipitated with chilled isopropanol, resuspended in TE buffer and quantitated spectrophotometrically. The control genomic DNA from promastigotes of a known strain of *Leishmania donovani* (Dd8) was also isolated by the same method.

PCR REACTION

For PCR, a 20 μl reaction mixture comprised of 2 μl of 10x PCR buffer (0.1M Tris-HCl, pH8.8, 15mM MgCl_2 , 0.5M KCl and 1% Triton-X 100), 250 μM dNTPs (Roche, Germany), 10 pmol of each primer, and 1.0 U of Taq DNA polymerase (Roche, Germany). The LDS and LDK are specifically designed primers which are specific for only *Leishmania donovani* the sequence of primers has been determined on the basis of B-cell epitope mapping [12] of *Leishmania* heat shock protein gene cloned in this laboratory [13]. The sequence of primers is withheld as the patent application filed is under consideration. The primers were custom-synthesized from Sigma-Genosys, USA. The genomic DNA from promastigotes cultured from dermal lesions or visceral organs was used as template DNA. The genomic DNA from promastigotes of Dd8 *L. donovani* was used as the positive control. These primers would amplify an *L. donovani* specific gene fragment of 243bp. The thermo cycling was carried out for 30 cycles with denaturation at 95°C for 1 min., annealing at 60°C for 1 min. and extension at 72°C for 1 min. in a temperature gradient thermo cycler (Eppendorf AG, Germany) with initial denaturation for 5 min and extension in the last cycle for 10 min.

POST-PCR DETECTION

After amplification, 8 μl samples of the products were analyzed by electrophoresis through 1.5% agarose gel containing 0.5 $\mu\text{g/ml}$ ethidium bromide and visualized in a gel-doc system (Image Master, Pharmacia Protech, Sweden).

RESULTS

CLINICAL PICTURE

A total of 218 patients of LCL comprised 126 males and 92 females aged between 11 months and 70 (mean 26.02) years. The majority 139 (63.76%) of patients were in the age group of 10-30 years. There were 62 (29.44%) children ≤ 14 years of age. The total numbers of lesions in these patients were 338 (range 1-8 lesions) with 150 (68.80%) patients having single lesion. Most of the lesions (98%) were nodulo-ulcerative plaques over exposed body parts especially face.

Fourteen (M: F 10: 4) patients of VL were referred to us between April-Nov 2005 by physicians and pediatricians. They were between 6-65 years of age and 4 were children ≤ 14 years. Ten of them belonged to Himachal Pradesh and had acquired the infection indigenously.

TOUCH SMEARS

In Giemsa stained smears from 165 lesions only 73 (44.42%) showed amastigotes (Leishman- Donovan bodies). The positivity was higher (73.97%) from 54 lesions of ≤ 6 months' duration while out of 52 lesions of ≥ 6 months' duration only 19 (36.53%) were positive for amastigotes. A distinct morphological variation was seen in two smears where the amastigotes were relatively larger (4-5 μ m), had a very clearly defined cell membrane and a vacuolated cytoplasm pushing the nucleus and kinetoplast to one side producing signet ring appearance. Eleven of the VL patients showed amastigotes in smears from bone marrow and / or splenic aspirates.

CULTURE CHARACTERISTICS

Out of the 112 attempted cultures from LCL cases 38 (33.92%) were positive for promastigotes and 9 of 14 (64.28%) positive cultures were from bone marrow/splenic aspirate samples from VL patients. The maximum positivity was attained with culture medium II i.e. Modified NNN medium with RPMI 1640 and 10% heat inactivated fetal bovine serum (HIFBS) Table-1. Positive cultures obtained from LCL lesions of 1-8 (mean 4.23) months duration. Out of the 28 lesions of ≥ 6 months duration only 6 (21.4%) were culture positive as compared to 32 (38%) from 84 lesions of ≤ 6 months duration. Thirty eight (33.92%) cultures developed bacterial or fungal contamination within 7 days of inoculation. Positivity of cultures was obtained as early as 3rd day and latest by 7th day. The number of promastigotes was highest between 11-13 days of inoculation.

Figure 1

Table 1: Culture media used and Culture positivity results

| Culture Media | | Cultures attempted | Positive Cultures (%) |
|---------------|---|--------------------|-----------------------|
| I | Modified NNN Medium (Part A, Part B). | 17 | 2 (11.76) |
| II | Modified NNN medium with RPMI 1640 and 10% (vol/vol) heat inactivated (at 56° C for 30 minutes) fetal bovine serum | 112 | 38 (33.92) |
| III | Modified NNN medium with medium 199 (with Hepes buffer) with 20 % heat inactivated fetal bovine serum ²⁵ . | 11 | 1 (9.09) |
| IV | RPMI 1640 with 30% heat inactivated fetal bovine serum. | 16 | 2 (12.5) |
| V | Schneider's Insect Medium with 20% heat inactivated fetal bovine serum [14]. | 10 | 0 |
| VI | P-Y medium [15]. | 7 | 0 |
| VII | Beef extract, peptone, sodium chloride, bactoagar and fetal bovine serum [16]. | 5 | 0 |
| VIII | Nutrient Blood broth with 20% heat inactivated fetal bovine serum [17]. | 4 | 0 |

A luxuriant growth was obtained in medium II in most of the positive cultures. Medium I did not give a very good yield. One of the cultures was highly positive in medium III. An interesting phenomenon was observed in two culture specimens. When we took out 1-2 ml of liquid phase from the primary culture from medium II for subculturing and added an equal amount of fresh liquid phase to it, the promastigotes in the primary medium could be maintained for about 50 days.

Subculturing was mostly unsuccessful with these particular strains of *Leishmania*. On subculture in Medium II promastigotes could be observed only for 2-3 days and that too in decreasing number. It could not be ascertained whether promastigotes were from the original inoculums or had actually multiplied in the subculture. All subcultures attempted in media V, VI, VII, and VIII were unsuccessful.

PROMASTIGOTE MORPHOLOGY

Under phase contrast the promastigotes were seen either singly or in groups comprising about 50 promastigotes. The body size ranged from 15-20 μ m x 2-4 μ m with broader end bearing a thin flagellum of about the same length as that of the body or even longer (Fig.1). The free flowing promastigotes showed characteristic whiplash movements resulting in movement of the body. The groups often produced rosette-like arrangement with their broader flagellated ends pointing inwards (Fig. 2).

Figure 2

Figure 1: Typical promastigotes (Geimsa x100)



Figure 3

Figure 2: Promastigotes forming a rosette (Geimsa x100)



The bodies of aberrant forms were small or very small and had either round, oval or elliptical shape reminiscent of metacyclic stage. Some of the promastigotes were having abnormally long or multiple flagella indicative of failed mitosis or cell division. By 17-18th day, the growth started declining evidenced by the presence of some non-motile or sluggish promastigotes in the wet preparation. By 21st day, it was observed that almost all promastigotes became non-motile.

CHARACTERISATION OF STRAINS BY PCR AMPLIFICATION WITH LDS AND LDK PRIMERS

A unique 243 bp band was amplified from the promastigote DNA of only *L. donovani* strains with LDS and LDK primers (Fig. 3). The PCR was positive in all the

promastigote isolates from VL patients showing the presence of *L. donovani* parasite, while only 4 out of 9 isolates from the LCL lesions showed a positive amplification for *L. donovani*.

Figure 3: PCR amplification with LDS and LDK primers.

2.0% agarose gel stained with ethidium bromide showing amplification of hsp70 gene fragment (243 bp) using the primers LDS and LDK from promastigotes DNA. Lane 2, 3, 4, 5: Promastigote DNA from dermal lesions; Lane 6 & 7: Promastigote DNA from visceral leishmaniasis. Lane 8: Promastigote DNA from cultured *L. donovani* (Positive control); Lane 1: Negative control; Lane 9: DNA marker (100 bp ladder)

DISCUSSION

In the present study the demographic and clinical features of the disease were similar to its known patterns. The course of LCL is long, often unpredictable and the lesions often heal with ugly scars on the face. Thus an early and definite diagnosis and specific treatment is imperative. Furthermore, VL may cause serious morbidity or mortality. Although confirmation of diagnosis by tissue smears is often sufficient, culture of organism and its characterization is important to study epidemiological aspects of any new endemic focus. The positivity of Giemsa stained smears varies between 50-80% depending upon the age of LCL lesion and the technique used. It was 44.42% in the present series; the positivity was higher (73.97%) in the lesions of ≤ 6 months' duration. These observations are similar to those made by other workers [18].

Initial isolation of a strain is apt to be difficult for any new focus [19]. Various studies have reported culture positivity varying from 42-55.3% in LCL [20,21]. Purohit et al [22] were able to culture *L. tropica* from skin biopsies only in 20% patients from Bikaner in Rajasthan, a known endemic focus for LCL in India. In the present study the low culture positivity rate of 33.92% could be either due to high rate of contamination or variable nutritional requirements of the strains. Contamination of the cultures by bacteria or yeast from skin flora is a major limiting factor [22] and was also observed by us especially for samples collected under field conditions which perhaps further increased the chances of contamination. It is also well known that some members of the genus may be difficult to culture [23,24] and inoculation in hamsters may be required to maintain strains in the laboratory for zymodeme or genetic studies in such

situations. Variation is also found within species as to the ability to grow in particular media and some strains being more readily amenable to in vitro cultivation [19].

Furthermore, lesions with low smear positivity in this study gave negative cultures more often indicating that the size of inoculums is an important determinant for better yields in cultures.

Although culture medium II was most successful for primary isolation, it was not found ideal for subculture. However, other standard cultivation media such as P-Y medium, Schneider's *Drosophila* medium or other cell culture media too did not support the growth of this particular strain. Despite all efforts we had limited success to subculture and maintain *Leishmania* promastigotes even after their successful isolation initially perhaps due to change in their nutritional requirement. The characteristic peak growth of promastigotes observed at 11-13 days of inoculation followed by a decline is a well known phenomenon [25]. It was a useful and interesting observation in maintenance of primary culture for 50 days with replenished RPMI 1640+HIFBS. However, it remains speculative as how the promastigotes could multiply in this and not in a similar fresh culture medium or why subsequent attempt to subculture in RPMI 1640 +HIFBS also failed. Normally as the growth of organisms in cultures progresses, the pH and the accumulated toxic products in the media may be deleterious for them. Similarly, the nutritionally rich media too do not appear suitable for maintenance of their growth.

Many molecular techniques like PCR, specific DNA probes and nucleic acid amplification have been developed in the last decade. These techniques can be carried out not only on cultured organisms but also on direct biopsy specimens, archived smears or even sandflies [26,27,28]. The isoenzyme analysis is an accepted technique for *Leishmania* (sub) species identification at present [2]. Direct sequencing of the genes encoding the isoenzymes are however less informative than expected. Although *L. donovani* specific PCR-negative samples could not be processed for other *Leishmania* species, the PCR studies on cultured organisms from biopsy samples of 4 LCL patients identified a predominant presence of *L. donovani* on account of the specificity of the primers used.

In India, the LCL due to *L. tropica* and *L. major* had been confined mainly to the northwest state of Rajasthan in Thar Desert [29]. These species have also been reported as the cause of LCL from the neighboring country Pakistan [30].

There are also reports from other countries of *L. donovani* causing LCL [3,4,5]. The *Leishmania* strains isolated previously from 4 LCL patients in this focus, 3 were similar to *L. donovani* and one was *L. tropica* [6]. However, these *L. donovani* strains did not resemble the known zymodeme MON-2 strains which have been implicated for visceral leishmaniasis and post kala azar dermal leishmaniasis in eastern India.

The positive *L. donovani* specific PCR (which picks up only *L. donovani* and not *L. infantum*) on our 4 VL samples is suggestive of *L. donovani* VL in this region. Though it could not be ascertained as yet whether *L. donovani* strains isolated from these VL patients are similar to or different from other Indian strains causing VL, but our recent studies are indicative of presence of other species as well in this mixed VL/ LCL focus.

Although some features are very similar to those of the endemic focus in Turkey [31], this new focus of leishmaniasis appears peculiar where endemic cutaneous and sporadic visceral forms co-exist, and both *L. donovani* and *L. tropica* are producing LCL all the while *L. donovani* being the predominant pathogen. The difficulty in culturing the isolates is also reminiscent of LCL caused by *Leishmania infantum* [32] suggesting that these isolates, both from LCL and VL patients, perhaps belong to *Leishmania donovani*-*infantum* complex.

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