# Genetic fingerprinting and identification of differentially expressed genes in isolates of *Leishmania donovani* from Indian patients of post-kala-azar dermal leishmaniasis

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SUMMARY

Post-kala-azar dermal leishmaniasis (PKDL) is an unusual dermatosis that develops as a sequel in 5–15% of cured cases of kala-azar (KA) after months or years of treatment in India. Molecular differences are reported to exist between the KA and PKDL isolates which may underlie the diversity in clinical manifestations of the disease. Here, arbitrary primed-PCR (AP-PCR) has been used for genetic fingerprinting of parasite isolates from dermal lesions of PKDL patients ($n=14$) and compared with bone-marrow derived parasites from KA patients ($n=3$). All isolates showed an identical AP-PCR pattern with 4 arbitrary primers. Further, AP-PCR was exploited to identify the stage regulated genes of the parasite. Six polymorphic fragments were identified in PKDL in comparison with KA isolates, and were subjected to Northern blot analysis. Five polymorphic fragments represented transcribed sequences; 4 out of 5 drew differential expression in pro- and amastigote stages, although the expression was comparable between PKDL and KA isolates. The study led to the identification of genes, which exhibit stage-regulated expression in *Leishmania donovani* derived from PKDL or KA patients, including a putative phosphodiesterase, DEAD box RNA helicase, iron superoxide dismutase b (*fesodb*) and a hypothetical protein. Demonstration of transcripts of DEAD box RNA helicase in PKDL and KA diseased tissues implicates its role in disease pathogenesis.

Key words: *Leishmania donovani*, PKDL, visceral leishmaniasis, APPCR, differentially expressed genes, genomic fingerprinting.

## INTRODUCTION

Leishmaniasis is clinically and epidemiologically a diverse disease caused by obligatory, intracellular, haemoflagellate protozoan parasites of the genus *Leishmania* (family Trypanosomatidae). The disease, prevalent in 88 countries, manifests in a variety of clinical forms, ranging from the self-healing cutaneous disease to the more serious, potentially fatal visceralizing form. Visceral leishmaniasis (VL) or KA is of higher priority than cutaneous leishmaniasis (CL) as it is a fatal disease in the absence of treatment. There are 350 million people at risk with 500 000 new cases of VL and 1–1·5 million for CL per year and with DALYs: 2·4 millions (Desjeux, 2004). Anthroponotic foci of VL such as India and Sudan are of special concern as they are at the origin of frequent and deadly epidemics (Desjeux, 2004). Post-kala-azar dermal leishmaniasis (PKDL) is a dermatotropic form of leishmaniasis developed in cured KA patients (El Hassan *et al*. 1992; Ramesh and Mukherjee, 1995). The onset of PKDL is variable: PKDL may appear during or directly after treatment or up to 2 years post-treatment in Sudan, while in India the period between KA and PKDL is generally long ranging from 6 months to 20 years with an average of 3 years (Zijlstra *et al*. 2003). PKDL patients are considered to be an important source in VL transmission in the absence of an animal reservoir in India and are considered to contribute to the increasing antimony resistance in India (Addy and Nandy, 1992; Singh *et al*. 2006). The disease is characterized by the development of hypopigmented macules, papules and nodules, which first appear around the mouth; those which do not heal spontaneously become denser and spread over the entire body. A minimal mixed chronic inflammatory infiltrate consisting of plasma cells, lymphocytes and histiocytes in the perivascular areas of the upper dermis has been described in macules of PKDL (Ramesh, 1993; Ramesh and Mukherjee, 1995).

The causative organism isolated from PKDL patients has been shown to be *L. d. donovani*, though intrinsic differences in the PKDL and KA isolates have been demonstrated. Differences have been

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observed amongst PKDL and KA isolates at LdP13 and $\beta$-tubulin locus (Sreenivas *et al*. 2004*a*; Dey and Singh, 2006). Comparative gene expression profiling of PKDL and KA isolates revealed the upregulation of cell surface proteins in PKDL parasites (Salotra *et al*. 2006). For detection of genetic polymorphism among species and strains of *Leishmania* different methods have been used including PCR and PCR SSCP to scan single base differences (Noyes *et al*. 1996; Schonian *et al*. 1996). Genetic fingerprinting has been carried out for *Leishmania* parasites isolated from all forms of leishmaniasis reported from various geographical regions including India. Such analyses have been carried out on Indian KA isolates but as yet no studies are reported for the fingerprinting of *Leishmania* parasites isolated from PKDL patients.

Arbitrary primed PCR (AP-PCR) with random oligos is capable of producing unique genetic fingerprints from closely related organisms and this property has been utilized to differentiate several groups of parasites from trypanosomatids (Tibayrenc *et al*. 1993; Waitumbi and Murphy, 1993), coccidial (Procunier *et al*. 1993), to schistosome parasites (Neto *et al*. 1993). AP-PCR has been used to produce unique DNA fingerprints differentiating several *Leishmania* species or isolates (Pogue *et al*. 1995*a*). Due to the low complexity of the *Leishmania* genome the application of AP-PCR has been extended from DNA fingerprinting to surveying the genome for gene sequences of interest (Pogue *et al*. 1995*a*, *b*; Sreenivas *et al*. 2004*b*). The ability of AP-primers to amplify DNA from diverse portions of an organism's genome allows screening of genomic DNA from a wide range of gene sequences with little bias, and has led to identification of several differentially expressed RNAs (Pogue *et al*. 1995*b*).

*Leishmania* exists in 2 life-stages i.e. the promastigote present in the vector sandfly and the amastigote stage present in the vertebrate host. Identification of differentially expressed genes is important as those overexpressed in the amastigote stage are considered to be the virulence molecules which underlie the pathogenesis of the disease; thereby providing the necessary target for disruption of the disease cycle. A number of studies have been carried out to ascertain changes in gene expression amongst *Leishmania* stages, using methods such as differential or subtractive hybridization, differential display and cDNA microarrays derived from spliced leader libraries and genomic microarray (Heard *et al*. 1996; Liu *et al*. 2000; Wu *et al*. 2000; Almeida *et al*. 2002; Bellatin *et al*. 2002; McNicoll *et al*. 2006). AP-PCR has been used to identify polymorphic fragments which lead to identification of genes that are expressed differentially in the promastigote and amastigote stages of *Leishmania* (Pogue *et al*. 1995*a*, *b*; Sreenivas *et al*. 2004*b*). Here, we have exploited the AP-PCR technique to map genetic differences within the PKDL isolates of *L. donovani* and to identify DNA polymorphisms that distinguish geographical isolates. Further, we utilized the polymorphic DNA fragments to identify, isolate and characterize genes differentially expressed in promastigote and amastigote stages of the parasite.

## MATERIALS AND METHODS

### *Patients and parasite culture*

Parasite isolates were collected from dermal lesions of PKDL patients as described previously (Salotra *et al*. 2001; Singh *et al*. 2006). The patients presented with hypo-pigmented macules, papules and nodules. PKDL was confirmed by demonstration of amastigotes in skin biopsies. Informed consent was obtained from patients before collecting the biopsy material, according to the guidelines of the Ethical Committee, Safdarjung Hospital, New Delhi. Clinical isolates of *L. donovani* included in the study were prepared from 14 patients of PKDL (P1–P14) and 3 kala azar patients (*Ld*K1–*Ld*K3) that reported to Safdarjung Hospital between years 2001 and 2004. The reference stocks of *Leishmania* used in this study were *L. donovani* 1S from Sudan (WHO designation, MHOM/SD/00/1S-Cl2D), *L. donovani* WR 684 from Ethiopia (MHOM/ET/67/82), *L. donovani* AG83 from India (MHOM/IN/83/AG83) and *L. d. infantum* from Spain (MACAN/SP/100/XXX).

### *Cultures of promastigotes and axenic amastigotes*

Promastigotes were cultured at 24 °C in M199 medium with 25 mM HEPES (pH 7·4) supplemented with 10% heat-inactivated fetal calf serum, 100 IU and 100 $\mu$g/ml each of penicillin G and streptomycin, respectively. Promastigotes were propagated in the same medium at 24 °C for 4–5 passages, before harvesting for DNA extraction or transformation into amastigotes. Cytodifferentiation into axenic amastigotes was accomplished by inducing promastigotes to gradually adapt to altered growth conditions, as described earlier (Joshi *et al*. 1993; Sreenivas *et al*. 2004*b*).

### *Isolation of nucleic acids*

Genomic DNA and total RNA were prepared from minimally passaged parasites from kala-azar and PKDL isolates. Mid-log phase parasites ($1–2 \times 10^9$) were washed twice with cold PBS and used for DNA isolation by Wizard Genomic DNA kit (Promega). Total RNA from promastigotes and axenic amastigotes was prepared from parasites at identical time-points using Trizol reagent (Invitrogen). Bone marrow aspirates (BMA) and skin tissue samples from KA and PKDL patients were collected prior to treatment in Trizol, and stored in liquid nitrogen before isolation of total RNA.

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*AP-PCR reactions*

AP-PCR reactions were carried out under essentially similar conditions as described earlier (Pogue *et al.*1995*a*, *b*; Sreenivas *et al.* 2004*b*). The following AP primers were used: AP8-TGCCGAGCTG; AP9-GTTGCGATCC; AP-10-AGGTGACCGT; AP16-CAGCACCCAC. Briefly, the reaction was performed in a 50 $\mu$l reaction mixture containing 200 $\mu$M each of dNTP, 2 mM $MgCl_2$, 100 ng of oligonucleotide, 100 ng of template DNA (unless stated otherwise) and 2·5 units of *Taq* DNA polymerase overlaid with mineral oil. The reaction was amplified with the following cycling conditions: denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min for a total of 45 cycles followed by a final extension of 15 min at 72 °C. Amplification products from AP-PCR reactions were analysed by electrophoresis in 1% agarose gels and staining with ethidium bromide. The pattern of AP-PCR amplification fragments has been shown to be sensitive to the amount of genomic DNA template and the amount of AP oligo (Sreenivas *et al.* 2004*b*). The amounts of template and oligos were optimized for obtaining reproducible amplification from all *Leishmania* species tested. In the case of oligo AP-9, AP-10 amplification reactions were also carried out with 50 ng, 100 ng and 150 ng of template DNA to obtain additional unique DNA fragments.

*Cloning of polymorphic fragments*

Polymorphic DNA bands were sliced from the agarose gels and purified with QiaQuick gel extraction kit (Qiagen) following the manufacturer's protocol. The polymorphic bands were ligated in to pGEM-T Easy Vector-TA (Promega) according to the manufacturer's instructions.

*Northern hybridization*

To validate whether the polymorphic bands were inferring from the transcribed region, selected clones were subjected to Northern blot analysis. Total RNA (10 $\mu$g/lane) from PKDL and KA promastigotes/amastigotes was separated in formaldehyde gels, transferred to nylon membranes and hybridized with $^{32}$P labelled probe prepared from random labelling reaction using NEB blot kit (New England Biolabs). The stained rRNA signal intensities were used to normalize for RNA loading using AlphaEase Chemiimager V5.5. The quantitated hybridization intensity was used to calculate the relative expression of various genes in promastigotes/amastigotes of PKDL and KA.

*Nucleotide sequence analysis*

The cloned polymorphic fragments identified from AP-PCR were sequenced using automated sequencing machine single capillary node (ABI-310). Sequences were subjected to BLAST analysis to check for homology with known sequences in the database. The sequence generated was sufficient to find the homologous regions of the *L. major* and *L. infantum* genomes by BLAST searches of the databases (www.genedb.org). Sequences were submitted to GenBank and assigned Accession numbers.

*Expression analysis of FeSOD*

Total RNA (5 $\mu$g) from promastigote or axenic amastigotes was used to synthesize first strand cDNA in a reaction containing oligo dT20 primer, 2 $\mu$l of 0·1 M DTT, 1 $\mu$l of 10 mM dNTP mix, 10 U RNAsin and 200 units of superscript II reverse transcriptase following the manufacturer's instruction (Invitrogen). Amplification reactions were conducted with primers for the constitutively expressed housekeeping gene $\alpha$-tubulin for normalization of amounts of RNA. The amplification of FeSOD b1 and b2 UTRs was carried out under the following conditions: initial denaturation of 5 min at 94 °C followed by 94 °C for 45 sec, annealing 56 °C for 1 min and extension at 72 °C for 2 min for 36 cycles followed by final extension of 5 min. $\alpha$-Tubulin was amplified under the following conditions, initial denaturation for 2 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec 72 °C for 45 sec followed by final extension of 5 min at 72 °C. Primers were designed using Primer 3 software (http://wwwgenome.wi.mit.edu/genome_software/other/primer3.html). Primer sequences used in various experiments were as follows:

| | |
|---|---|
| *fesodb1* 3′ UTR | Forward-5′ GATGCGCAGTTTGGAGTTCAC 3′<br>Reverse-5′ GACAACGGTAGGGTCGCCAGAGAG 3′ |
| *fesodb2* 3′ UTR | Forward-5′ CTGGTGGCTCCCACTACGTCAAC 3′<br>Reverse-5′ CTCCTGCCTCTGCTTCCGTG 3′ |
| $\alpha$-tubulin | Forward-5′ TTTTTTTCGCCCTCGTCG 3′<br>Reverse-5′ GCCCCCCTCCACAGATACAC 3′ |

Control experiments were performed with amplification using total RNA in the absence of reverse transcriptase to check for DNA contamination. RT-PCR was carried out in RNAs from 3 different parasite isolates in promastigote and amastigote stages.

*Demonstration of transcripts of DEAD box RNA helicase in* Leishmania*-infected tissue lesion*

Total RNA (5 $\mu$g) from parasite infected tissue lesion of PKDL and KA patients was reverse transcribed

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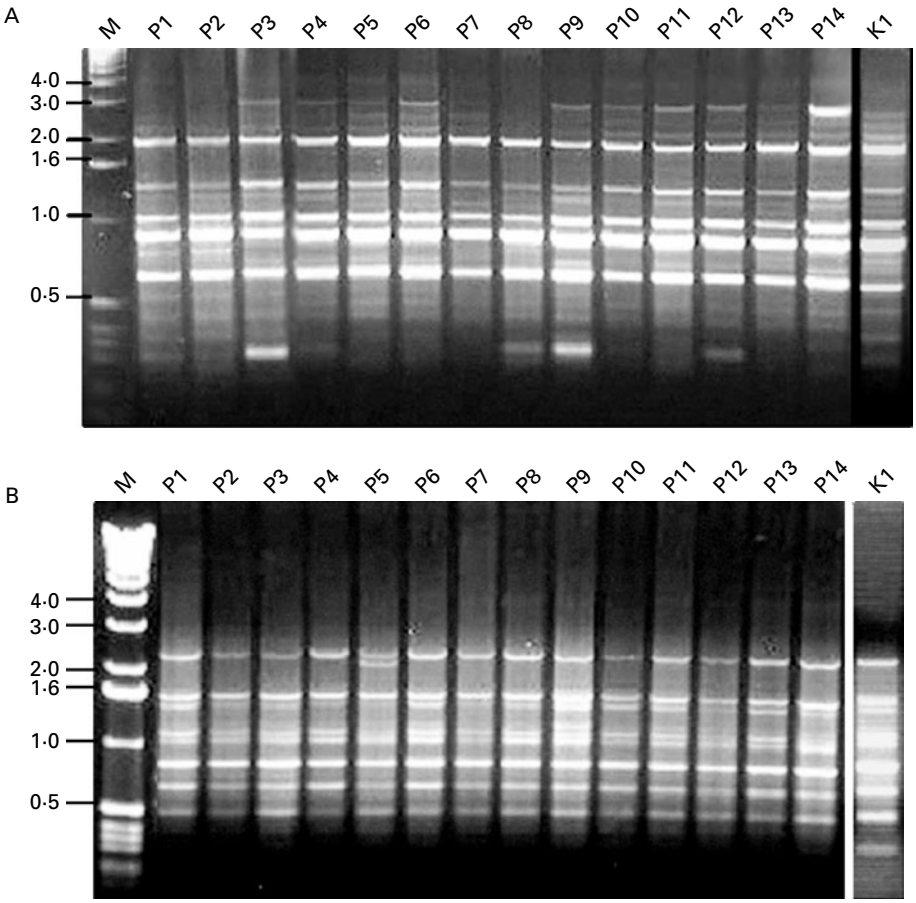


Fig. 1. Fingerprinting of Indian PKDL isolates of *Leishmania donovani* by AP-PCR using oligo AP-10 and AP-16. Genomic DNA (100 ng) from parasite cultures from PKDL lesions (P1–P14) and kala-azar isolate (*Ld*K1) was amplified and separated on agarose gel. M-DNA marker, 1 kb ladder. (A) Genomic fingerprint using AP-10; (B) fingerprinting pattern using AP-16 oligo.

into cDNA. The DEAD box RNA helicase ORF was amplified following the PCR conditions as described in the previous section except the annealing temperature was 63 °C for 1 min using the following primer sequence.

| | |
|---|---|
| DEAD box RNA helicase | Forward-5′ ATGGCAGAGCGTAACTACAGCCCCT 3′<br>Reverse-5′ CTATTGGCGCGCGCGCTTG 3′ |
| Hu HPRT | Forward-5′ CGAGATGTGATGAAGGAGATGGG 3′<br>Reverse-5′ CCTGACCAAGGAAAGCAAAGTCTG 3′ |

Human housekeeping gene HPRT was amplified for the normalization of lesion tissue RNA.

## RESULTS

### *Parasite isolation and characterization*

Initially, all *Leishmania* isolates derived from PKDL lesions were characterized as *Leishmania donovani* by immunofluorescence (IFA) using genus (G2D10) and species specific (D2, T2 and T10) monoclonal antibodies against *L. donovani*, *L. major* and *L. tropica*, obtained as a kind gift from TDR, WHO (Jaffe *et al*. 1984; Sreenivas *et al*. 2004*b*). In addition, all parasite isolates were characterized as *L. donovani* by isoenzyme analysis using a panel of enzymes (6PGD, G6PDH, NH, ME and MDH).

### *Fingerprinting of PKDL isolates by AP-PCR*

AP-PCR has been shown to be an attractive tool in assigning specific fingerprint to *L. donovani* geographical isolates that are morphologically and biochemically indistinguishable (Pogue *et al*. 1995*a*, *b*). In the current study the PKDL isolates ($n=14$) were subjected to genomic fingerprinting by AP-PCR analysis using primers AP-8, AP-9, AP-10 and AP-16. All PKDL isolates depicted almost identical fingerprinting patterns using primers AP-10 and AP-16 and the pattern was comparable with the predominant isotype of Indian KA isolate *Ld*K1 (Fig. 1A and B). The AP-PCR analysis with primer AP-8 and AP-9 also gave an identical fingerprinting pattern with all PKDL isolates (data not shown).

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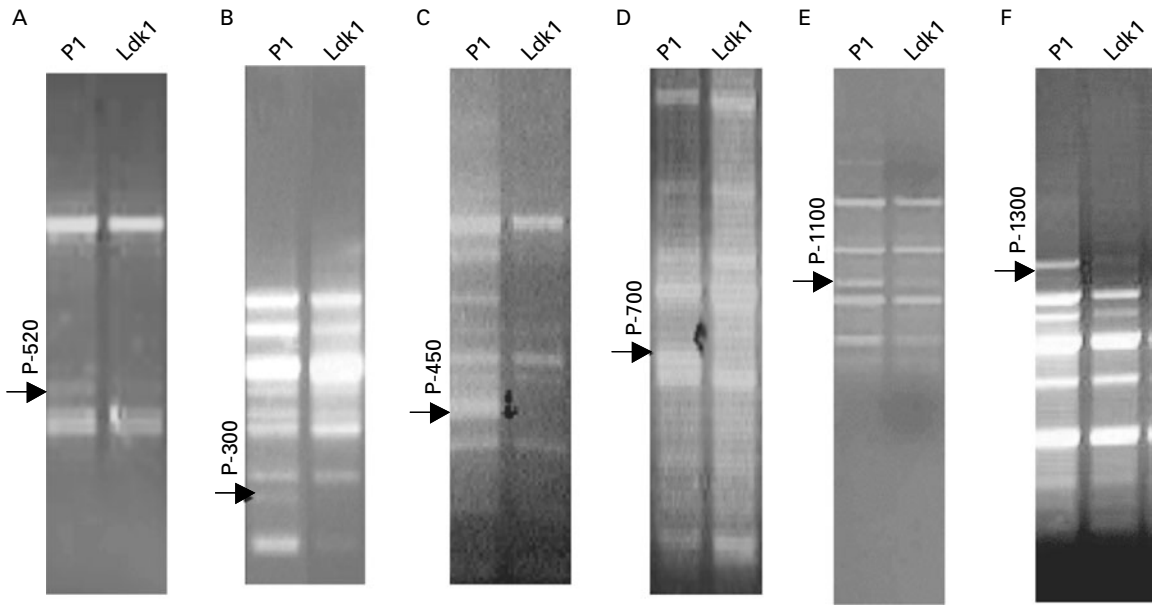


Fig. 2. AP-PCR analysis for identification of polymorphic fragment using oligo AP-9, AP-10 and AP-8. PCR was carried out using varying concentrations of DNA from parasites P1 and *Ld*K1. (A) 50 ng template DNA amplified with oligo AP-9; (B) 100 ng template DNA with AP-9; (C) 150 ng template DNA amplified with oligo AP-9; (D) 50 ng template DNA with oligo AP-10; (E) 150 ng template DNA with oligo AP-10; (F) 100 ng template DNA with AP-8 primer.

*L. donovani* isolates from different geographical regions i.e. *L. donovani* 1S from Sudan, *L. donovani* WR 684 from Ethiopia (MHOM/IN/83/AG83) and *L. d. infantum* from Spain (MACAN/SP/100/XXX) were also compared and revealed a distinct pattern in comparison with PKDL isolates as well as standard *L. donovani* AG83 from India (data not shown).

### *Identification of polymorphic fragments in PKDL* L. donovani

The pattern of AP-PCR is known to be highly dependent on the amount of genomic DNA template and primer (Pogue *et al.* 1995*a*, *b*; Sreenivas *et al.* 2004*b*). With altered template DNA concentration, different patterns were obtained with AP-9 and AP-10 with PKDL isolates in comparison with *L. donovani* isolates of KA. Attempts to identify fragments unique to PKDL isolates resulted in polymorphic bands in comparison with KA isolates with AP-9, AP-10 and AP-8 using different amounts of genomic DNA (50 ng, 100 ng, and 150 ng). The polymorphic bands were obtained reproducibly with all PKDL isolates and the representative data with P1 and *Ld*K1 are shown (Fig. 2).

The polymorphic bands of sizes approximately 520 bp, 300 bp and 450 bp obtained from AP PCR with AP-9 were designated as P-520, P-300 and P-450 based on the size of fragment (Fig. 2 A, B and C). AP-PCR analysis with oligo AP-10 using 100 ng genomic DNA did not reveal any polymorphic band in comparison to KA isolates (Fig. 1A); however, altered DNA concentration resulted in polymorphic bands. A distinct polymorphic band at 700 bp (designated P-700) was obtained with P1 when the DNA amount was 50 ng, while the corresponding band in KA isolates was very faint (Fig. 2D). Similarly, when tested with 150 ng DNA concentration with the same oligo AP-10, a distinct polymorphic band of 1·1 kb (designated P1100) was pictured with P1 isolate which was barely evident in KA isolate *Ld*K1 (Fig. 2E). AP-PCR analysis with AP-8 primer using 100 ng template illustrated a polymorphic fragment at about 1·3 kb in comparison to KA isolate (Fig. 2F).

### *Northern blot analysis using polymorphic fragments*

The polymorphic fragments were used in Northern analysis to determine if they represented transcribed sequences and if they corresponded to differentially expressed genes. P-520 hybridized to a 2·8 kb mRNA species that was found to be overexpressed ($1{\cdot}5 \pm 0{\cdot}2$-fold; mean ± S.D.) in promastigotes of both PKDL and KA isolates (Fig 3A). Another fragment P-300 that hybridized to 2·5 kb mRNA was also upregulated ($1{\cdot}7 \pm 0{\cdot}1$-fold) in the promastigote stage of the parasite (Fig. 3B). The third fragment P-450 hybridized to 2·3kb RNA that had $1{\cdot}8 \pm 0{\cdot}1$-fold higher expression in the amastigote stage compared to the promastigote stage (Fig. 3C).

Polymorphic fragments incurred from AP-10 were cloned and tested in Northern blot analysis. The probe P-700, when tested in Northern blot, hybridized to an ~2·9 kb size mRNA and revealed an

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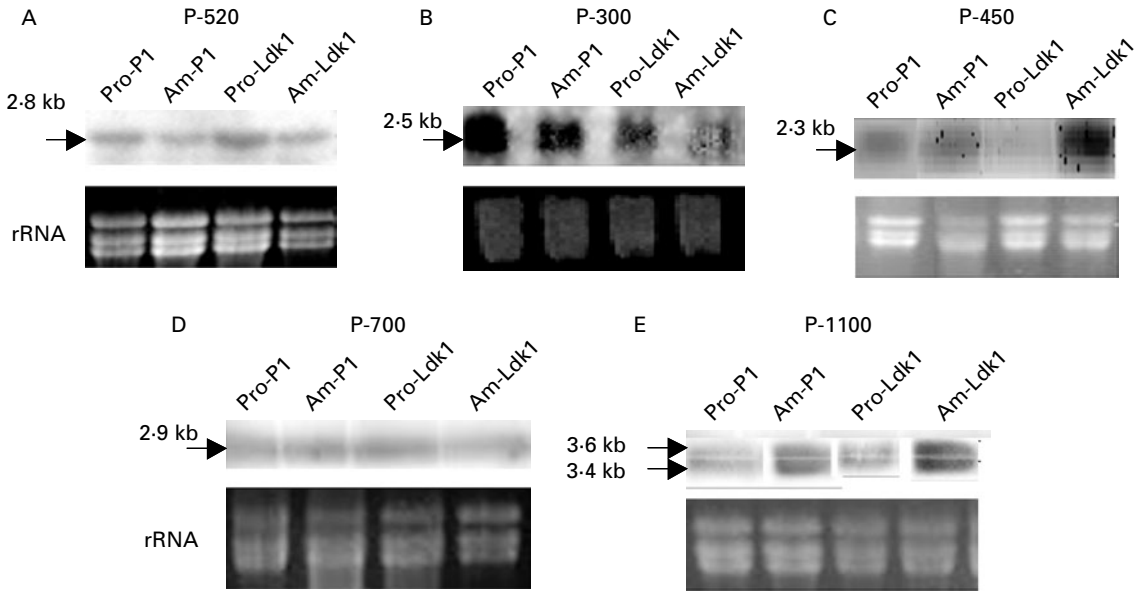


Fig. 3. Northern hybridization with total RNA isolated from promastigotes (Pro) or amastigotes (Am) of P1 and *Ld*K1, using as probes the polymorphic fragments identified in AP-PCR. (A) P-520; (B) P-300; (C) P-450; (D) P700; (E) P-1100. Ethidium bromide stained rRNA was used for normalization. Validation was carried out in RNAs from 3 different parasite isolates of each type and representative data are shown.

equal mRNA transcriptional level in both stages of parasites (Fig. 3D). Northern analysis showed that the P1.1 hybridized to 2 species of mRNA of size 3·6 and 3·4 kb. The expression was differential in 2 stages of both PKDL and KA isolates, being $2{\cdot}5 \pm 0{\cdot}35$-fold higher in the amastigote stage in comparison with promastigotes (Fig. 3E). Northern blot analysis with the polymorphic fragment 1·3 kb obtained from AP-8 did not yield any signal when blotted, indicating that the fragment obtained from our analysis probably belonged to a non-transcriptional region (data not shown). The current study demonstrated that a majority of AP-PCR fragments incurred from PKDL isolates belonged to transcribed regions that may code for proteins.

### *Nucleotide sequence analysis*

The cloned fragments which were found to contain transcribed sequences were subjected to sequence analysis and the BLAST analysis revealed that transcripts were homologous with known or predicted genes in the *L. infantum* annotated genome database.

Sequences of P-520, P-300 and P-450 drew homology with iron superoxide dismutase *fesod* (LinJ32.2230), 3′ UTR of a putative gene phosphodiesterase (LinJ18·1100) and 5′ UTR of DEAD box RNA helicase (LinJ36.4680), respectively. These sequences were submitted to GenBank and assigned Accession numbers EF032817, EF032816, EF032815 respectively. Sequences of P-1.1 and P-0.7 in BLAST analysis depicted homology to 3′ UTR of LinJ33.2760 coding for a hypothetical protein of unknown function and to 5′ UTR and partial ORF of LinJ18.1040 another conserved hypothetical protein, respectively (GenBank Accession nos. EF032814; EF032818) (Table 1).

### *Expression analysis of FeSOD*

The *Fesodb* gene is classified into 2 types, *fesodb1* and *fesodb2*, both having homologous sequences and are distinguishable by their UTRs (Plewes *et al*. 2003). The expression of both was found to be stage specific; *fesodb1* expression was upregulated in the amastigote stage by $1{\cdot}5 \pm 0{\cdot}1$-fold while *fesodb2* was overexpressed in the promastigote stage by $3{\cdot}0 \pm 0{\cdot}2$-fold (Fig. 4A). Investigation of *fesodb1* and *fesodb2* expression at mid-log, late-log and stationary phase of the PKDL promastigotes, using UTR specific primers revealed that *fesodb1* expression increased in intensity from the mid-log promastigotes to the stationary stage as shown by the 1·8 kb amplification product; in contrast, RT-PCR analysis using the specific 3′ UTR of fesodb2 revealed a single band of 1·6 kb that increased in intensity from mid-log to late-log phase and then decreased in the stationary stage. cDNA was normalized using the constitutively expressed $\alpha$-tubulin gene of *Leishmania* (Fig. 4B).

### *Detection of DEAD box RNA helicase in lesion tissue*

Presence of gene transcripts coding for DEAD box RNA helicase in parasite-infected lesion tissues was investigated since the gene had shown upregulation in the amastigote stage. The transcripts corresponding to ORF of *Leishmania* DEAD box RNA helicase

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Table 1. Identity and expression pattern of genes corresponding to polymorphic fragments generated by AP-PCR analysis

| Fragment ID | NCBI Accession numbers | Leish Gene DB *L. infantum* | Gene Identity | Region of Homology of the fragment | Expression pattern |
|---|---|---|---|---|---|
| P-520 (AP-9) | EF032817 | LinJ32.2230 | Iron superoxide dismustase Putative (FeSODB) | 3′ UTR, ORF | Pro Up regulated |
| P-300 (AP-9) | EF032816 | LinJ18.1100 | Phosphodiesterase putative gene | 3′ UTR | Pro Up regulated |
| P-450 (AP-9) | EF032815 | LinJ36.4680 | DEAD box RNA helicase, putative | 5′ UTR | Amastigotes up regulated |
| P-700 (AP-10) | EF032818 | LinJ18.1040 | Hypothetical protein, conserved | 5′ UTR and ORF | Equal Linear expression |
| P-1100 (AP-10) | EF032814 | LinJ33.2760 | Hypothetical protein, conserved (Unknown function) | 3′ UTR | Amastigotes Up regulated |

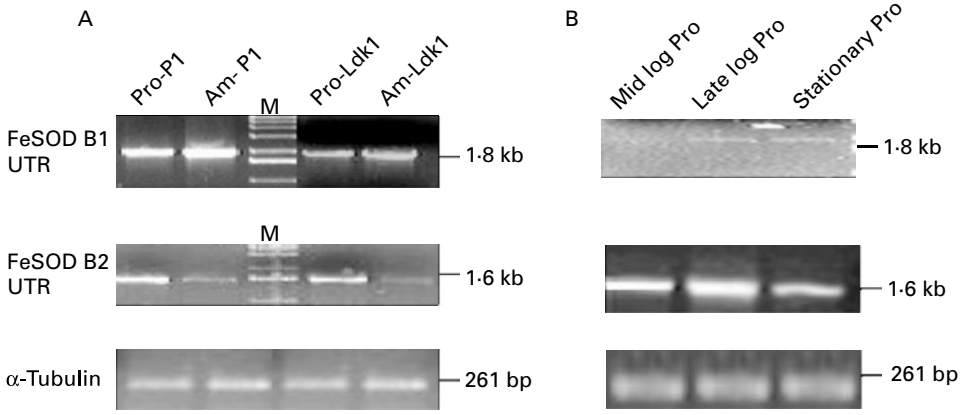


Fig. 4. Differential expression analysis of *fesodb1* and *b2* by semi-quantitative RT-PCR. (A) RTPCR analysis using cDNA from promastigotes and amastigotes of P1 and *Ld*K1. (B) Analysis at mid-log, late-log and stationary phase of the PKDL promastigotes. cDNA was PCR amplified with α-tubulin for normalization of RNA levels.

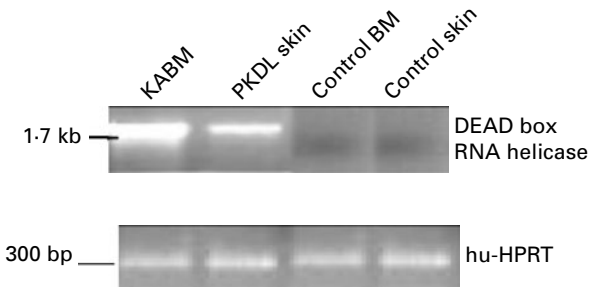


Fig. 5. Expression analysis of DEAD box RNA helicase by semi-quantitative RT-PCR in lesion tissue. *Leishmania* specific DEAD box RNA helicase was amplified from cDNA synthesized using total RNA isolated from bone marrow of KA (KABM) and skin lesions of PKDL (PKDL skin). Control BM and skin samples were taken from uninfected individual's bone marrow and skin biopsy, respectively. HPRT human house keeping gene (huHPRT) was used for normalization.

(1·7 kb) were demonstrated by RT-PCR in RNA extracted from human bone marrow of KA patients and in skin lesions of PKDL patients, while the uninfected control samples did not show the amplification of parasite-specific DEAD box RNA helicase (Fig. 5).

## DISCUSSION

Leishmaniasis has forced itself upon medical attention as an increasingly significant problem over the last decade and is considered by WHO as one of the 6 tropical diseases in Category 1 for which the most important research needs are new basic knowledge and better intervention tools (Remme *et al*. 2002). Parasites belonging to the genus *Leishmania* have a wide geographical distribution and cause different clinical manifestations including cutaneous, mucocutaneous and visceral pathology. Considerable research has focused mainly on pathogenesis and management of PKDL, leaving unexplored the important issues such as the parasite genetic factors responsible for the pathology. The gross differences in the spectrum of clinical manifestations (Ramesh and Mukherjee, 1995; Zijlstra *et al*. 2003), humoral immune responses apparent in the KA and PKDL phenotypes (Salotra *et al*. 1999; Saha *et al*. 2005) are, at least in part, a consequence of the alterations in the

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expression pattern of a multitude of parasite genes. In the present report we have employed AP-PCR for the genomic fingerprinting of Indian isolates of *L. donovani* obtained from PKDL patients originating from the endemic region and to identify differentially expressed genes in promastigote and amastigote stages of the parasite. Our earlier study using arbitrary primers for fingerprinting of Indian KA isolates revealed 2 divergent classes with the majority of KA isolates giving the same pattern as standard isolates *Ld*AG83 and *Ld*DD8 (Sreenivas *et al.* 2004*b*). The present study revealed that AP-PCR profiles with 4 oligos displayed a high degree of similarity within the Indian isolates of PKDL, which were quite similar to the predominant KA isolates.

An earlier AP-PCR approach has been exploited in surveying the *Leishmania* genome for gene sequences of interest and several classes of differentially expressed genes in *L. donovani* strains have been identified (Pogue 1995*a*, *b*; Selvapandiyan *et al.* 2001; Sreenivas *et al.* 2004*b*). The amplification patterns in AP-PCR are sensitive to the primer and template concentration (Pogue *et al.* 1995*a*). We identified polymorphic fragments in PKDL isolates in comparison with KA isolates upon changing the template concentration. The bands identified by AP-PCR were considered polymorphic if they were unique or quantitatively different in PKDL isolates in comparison with KA isolates. This approach led to identification of 6 polymorphic fragments, of which 5 were found to be representing transcribed sequences upon Northern analysis. Comparative expression analysis of all the fragments by Northern blot revealed that the majority (4/5) of them were differentially expressed in pro- and amastigote stages of the parasite. The fold differences observed for various genes studied here were found to be between 1·5- and 2·5-fold, which are similar to those reported earlier using genomic microarray studies for transcriptome analysis at promastigotes and amastigote stages in *Leishmania* (Akopyants *et al.* 2004; Srividya *et al.* 2007).

One such gene that showed significantly higher expression in the promastigote stage of the parasite corresponded to iron superoxide dismutase (*fesod*). *Leishmania* species possess 2 types of *fesod*, *fesoda* and *fesodb* (Paramchuk, 1997). *fesoda*, is a single copy gene, whereas the other gene, *fesodb*, belongs to a multi-gene family. Transcript levels and enzyme activities of *fesoda* and *fesodb* show differential stage expression, with higher levels present in the amastigote compared to the promastigote stage (Ghosh *et al.* 2003). Further, *fesoda* deficiency has been shown to markedly reduce the survival of *Leishmania* amastigotes in mouse macrophages (Ghosh *et al.* 2003). The role of superoxide dismutases in the intra-macrophage survival of *Leishmania* spp. remains to be established. Our observation of the high level of expression of *Ldfesodb2* in the early logarithmic promastigote stage in Indian isolates of *L. donovani* suggests that the gene may be important for survival within the mid-gut of the sandfly, and the high level of expression of *Ldfesodb1* in the stationary promastigote and amastigote stages suggests that it may be important for survival within macrophages by controlling endogenous superoxide levels.

Another stage-regulated gene identified was homologous to DEAD box RNA helicase. RNA helicases play a central role in the control of ribonucleic acid metabolism, regulate RNA unwinding and secondary structure in an ATP-dependent manner *in vitro* and control mRNA stability and protein translation. A recent study at the proteomic level has demonstrated a 2-fold upregulation of RNA helicase at protein level in the amastigote stage of *L. infantum*, similar to our observation at the transcriptomic level (McNicoll *et al.* 2006). The presence of its transcripts in the clinical samples derived from bone marrow of KA and dermal lesions of PKDL patients demonstrated in the present study indicate its role in the pathogenesis of the disease and its potential as a diagnostic tool. The lower expression of this gene in PKDL tissue lesion in comparison to KA bone marrow may be attributed to the scanty parasite load in the skin.

The gene corresponding to putative 3′-5′ cyclic nucleotide phosphodiesterase, was found to be upregulated in the promastigote stage in *L. donovani*. TbPDE1, a non-essential enzyme in *Trypanosoma brucei* (Gong *et al.* 2001), may represent a modulatory element of the cAMP signalling pathways of *T. brucei*, and its physiological roles remain to be explored both in *Trypanosoma* and *Leishmania*. Confocal laser scanning of *T. cruzi* epimastigotes showed its association with the plasma membrane and concentrated in the flagellum of the parasite. The localization of this enzyme is a unique feature that distinguishes it from all the trypanosomatid phosphodiesterases described so far and indicates that compartmentalization of cAMP phosphodiesterases could be important in these parasites (D'Angelo *et al.* 2004). The polymorphic fragment P-1100 corresponded to a hypothetical protein of 82·2 kDa with a transmembrane domain that showed significantly higher expression in the amastigote stage in comparison to the promastigote stage.

The stage-regulated expression of genes reported here in both PKDL and KA isolates, suggests that their in-depth study would provide important insights into the differences in the parasite factors responsible for manifestation of disease by *Leishmania donovani*. Further investigation on their function and contribution in the parasite life-cycle and infection process are warranted.

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