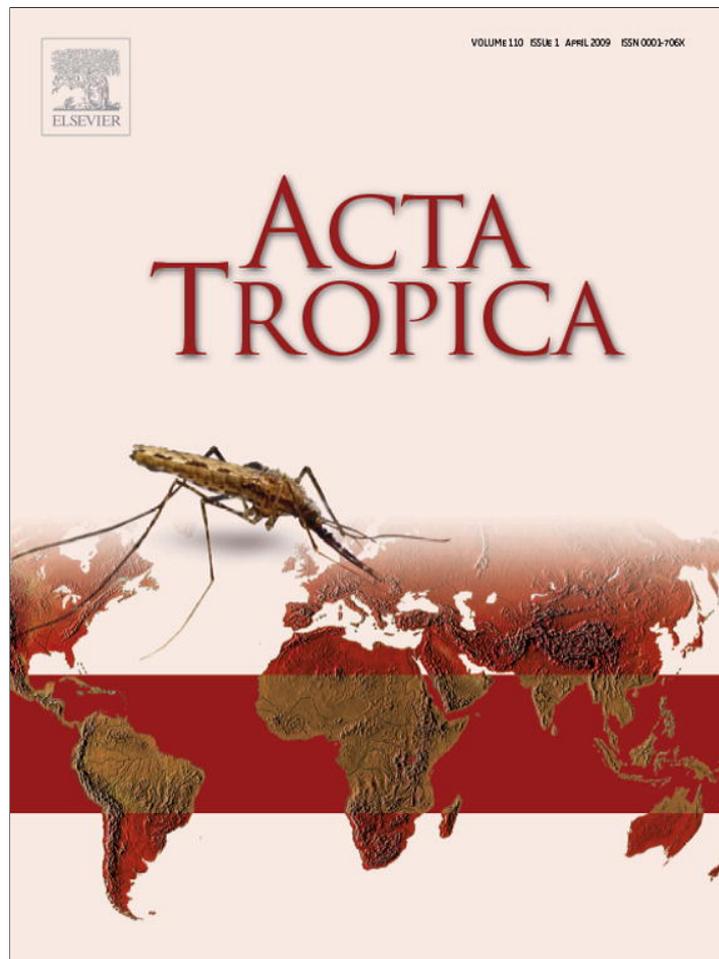


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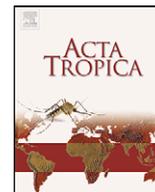
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Short communication

Identification of genetic markers in Sodium Antimony Gluconate (SAG) sensitive and resistant Indian clinical isolates of *Leishmania donovani* through amplified fragment length polymorphism (AFLP)

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ABSTRACT

Sodium Antimony Gluconate (SAG) is currently used worldwide as the first-line drugs for the treatment of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) since 1940s. Unfortunately, the resistance of *Leishmania* parasite to this drug is increasing in several parts of the world. The mechanism of drug resistance in clinical isolates is still not very clear. Earlier, we have established a differentiation between six clinical isolates as sensitive and resistant on the basis of their sensitivity to SAG *in vitro* and *in vivo* as well as expression of proteophosphoglycan contents. In this preliminary study, we have further analyzed these isolates on the basis of their genetic diversity, molecular variance and phylogenetic structure using for the first time, a fingerprinting approach – amplified fragment length polymorphism (AFLP). Altogether 2338 informative AFLP bands were generated using 10 selective primer combinations. Percentage of polymorphism was 55.35%. A number of unique AFLP markers (217) were also identified in these strains. It was deduced that a higher rate of variations occurred among *Leishmania* clinical isolates which indicate the shifting of drug sensitive nature of parasite towards resistant condition.

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1. Introduction

Leishmania donovani, a causative agent of visceral leishmaniasis (VL) or kala-azar poses a serious health threat on human populations and endemic areas in large parts of the world (Murray et al., 2005). The disease is usually fatal if not treated properly. The life cycle of *Leishmania* is digenetic with vertebrates as definitive host and *Phlebotomine* sand fly as intermediate one. Due to the lack of an effective vaccine, the control of VL relies mostly on chemotherapy. The pentavalent antimonials such as Sodium Antimony Gluconate (SAG) are the main drugs used for the treatment of the infection of *L. donovani* but unfortunately, the incidence of parasite becoming resistant to these drugs is increasing in several parts of the world including South America (Rojas et al., 2006), Europe (Carrio et al., 2001), Middle East (Hadighi et al., 2006) and most notably in India (Sundar et al., 2000). In India it is most prevalent in Bihar, West Bengal, Orissa, Assam, and Eastern Uttar Pradesh. The mechanism of resistance is now partly understood in laboratory bred isolates

but it is still lagging behind in respect of field or clinical isolates. Recent studies with field isolates (Decuypere et al., 2005; Singh et al., 2003) suggest similarities and differences with laboratory resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates.

The previous studies in our laboratory have established the sensitivity of recently isolated Indian clinical isolates of *L. donovani* to SAG the first-line antileishmanial drug. On the basis of their responses to SAG both *in vitro* and *in vivo* three resistant and three sensitive isolates were identified (Dube et al., 2005). Further characterization also revealed that a mucin like glycoconjugate proteophosphoglycan (PPG) is differentially expressed in these isolates (Samant et al., 2007) suggesting the possibilities of involvement of PPG in drug-resistant mechanisms and of using PPG abundance as a marker for identifying drug-resistant clinical isolates in Indian kala-azar. In this study we have further tried to explore these SAG sensitive and resistant clinical isolates at genomic level. A comparative analysis of genomic study of SAG sensitive and resistant *L. donovani* strains isolated from kala-azar patients was done using amplified fragment length polymorphism (AFLP) which is a DNA fingerprinting technique, for the study of genetic polymorphism across the whole genome. Since, prior sequence knowledge is not necessary to conduct AFLP experiments; hence, this method is particularly applicable to organisms for which no substantia-

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Table 1
Showing polymorphism on the basis of 10 selective primer combinations.

Selective primer combinations	Number of all bands	Number of monomorphic bands	Number of polymorphic bands	Number of unique bands	Percentage of polymorphism
(1) MseI-CAA/EcoRI-ACA	253	174	79	7	31.22
MseI-CAA/EcoRI-ACG	49	30	19	1	38.77
MseI-CAA/EcoRI-AGC	119	42	77	19	64.70
(2) MseI-CAC/EcoRI-ACA	92	54	38	2	41.30
MseI-CAC/EcoRI-ACG	45	24	21	2	46.66
MseI-CAC/EcoRI-AGC	62	42	20	5	32.25
(3) MseI-CAA/EcoRI-ACT	86	48	38	13	44.18
MseI-CAA/EcoRI-AGG	87	6	81	5	93.10
MseI-CAA/EcoRI-AAC	25	18	7	2	28.00
(4) MseI-CAC/EcoRI-ACT	67	30	37	7	55.22
MseI-CAC/EcoRI-AAG	86	54	32	6	37.20
MseI-CAC/EcoRI-AAC	20	12	8	0	40.00
(5) MseI-CAT/EcoRI-ACA	92	12	80	11	86.95
MseI-CAT/EcoRI-ACG	32	6	26	12	81.25
MseI-CAT/EcoRI-AGC	35	0	35	10	100
(6) MseI-CTC/EcoRI-ACA	46	6	40	6	86.95
MseI-CTC/EcoRI-ACG	47	12	35	5	74.46
MseI-CTC/EcoRI-AGC	54	12	42	12	77.77
(7) MseI-CAG/EcoRI-ACA	80	30	50	7	62.50
MseI-CAG/EcoRI-ACG	43	12	31	2	72.09
MseI-CAG/EcoRI-AGC	89	30	59	8	66.29
(8) MseI-CTA/EcoRI-ACA	174	42	132	11	75.86
MseI-CTA/EcoRI-ACG	135	66	69	9	51.11
MseI-CTA/EcoRI-AGC	118	42	76	11	64.40
(9) MseI-CTG/EcoRI-ACA	82	42	40	9	48.78
MseI-CTG/EcoRI-ACG	95	60	35	9	36.84
MseI-CTG/EcoRI-AGC	59	36	23	9	38.98
(10) MseI-CTT/EcoRI-ACT	63	42	21	5	33.33
MseI-CTT/EcoRI-AAG	43	18	25	7	58.13
MseI-CTT/EcoRI-AAC	60	42	18	5	30.00
Total number of bands	2338	1044	1294	217	

tive DNA sequence data are available (Vos et al., 1995). AFLP has been used widely to investigate organisms ranging from very simple unicellular bacteria to complex multicellular organisms (Hill et al., 2004; Ravel et al., 2001). Recently, AFLP has been used in genetic studies of several protozoan parasites (Agbo et al., 2002; Martinelli et al., 2005) but no such data are available for *Leishmania*. Since, AFLP techniques are able to extract considerable information about genome-wide nucleotide variability observations of this study would provide an evidence of genomic variations responsible for drug resistance. Documentation of genetic variations of SAG sensitive and resistant strains of *L. donovani* would be a significant impediment to advances in studying these pathogens. To our knowledge, this is the first time that AFLP has been used to characterize genomic variability of clinical isolates of VL.

2. Materials and methods

2.1. *Leishmania* parasites culture and maintenance

L. donovani strain Dd8 (MHOM/IN/80/Dd8) and the new clinical isolates of *L. donovani* of which two as SAG sensitive like Dd8 (2001, 2087) and the three strains identified as SAG resistant (2039, 2041, and 2093) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Sigma, USA) at 25 °C as described previously (Dube et al., 2005).

2.2. Genomic DNA extraction from *Leishmania* parasite

Genomic DNA of all *L. donovani* strains was isolated as per protocol described previously (Kelly, 1993). Briefly, logarithmic phase

promastigotes were disrupted in NET lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0), and then incubated overnight with proteinase K (100 mg/ml, Sigma-Aldrich) at 37 °C. DNA was purified further by phenol-chloroform extraction and ethanol precipitation.

2.3. AFLP reactions

AFLP was performed as described by Vos et al. (1995). Briefly, 500 ng genomic DNA was digested using two restriction endonucleases, EcoRI and Tru9I (an isoschizomer of MseI) for 4 h at 37 °C. Adapter ligation was performed in a 50 µl reaction mixtures containing EcoRI and MseI adapter primers, T4 DNA ligase and digested template DNA at 16 °C over night. After ligation, the pre-selective PCR amplification reaction was performed using template DNA, a pair of primers based on the sequences of the EcoRI and MseI adapters, including one additional selective nucleotide at the 3' end of the MseI primer (MseI + C) and the EcoRI primer (EcoRI + A). The preamplified PCR products was diluted 20-fold with T10E0.1 buffer and selective PCR amplifications were carried out using 10 different MseI and EcoRI primer combinations (Applied Biosystems, USA). The selective PCR products were loaded on a 5% denaturing polyacrylamide gel using an ABI PRISM® 377 DNA sequencer. For AFLP reactions, the AFLP amplification modules and the guidelines supplied by Applied Biosystems, USA were used.

2.4. Data analysis of AFLP

Analysis of AFLP output was performed using the ABI GENESCAN Analysis Software (Applied Biosystems, USA) which automatically

detects the DNA fragments. Different DNA fragments amplified with each primer were treated as discrete characters and numbered sequentially. For diversity, analysis bands were scored as present (1) or absent (0) to form a raw data matrix. Matrix of similarity was then obtained using Jaccard similarity coefficient (Jaccard, 1908) by SPSS 10.0.1 software. The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (Unweighted Pair Group Method with Arithmetic average) method using NTSys v2.1. Principal component analysis was carried out with help of SPSS software. The support values for the degree of confidence at the nodes of the dendrogram were analyzed by BOOTSTRAP analysis (Felsenstein, 1985) using two softwares WINBOOT (Yap and Nelson, 1996) and FREETREE (Pavlicek et al., 1999).

3. Results

3.1. AFLP analysis

A total of 2338 informative AFLP markers were generated using 10 selective primer combinations (Table 1). Out of 2338 AFLP bands, number of polymorphic ones identified were 1294 and that of monomorphic 1044. On the basis of all the 10 primer combinations used the percentage of polymorphism was calculated to be 55.35.

3.2. Dendrogram and phylogeny

The phylogenetic tree was obtained by using two softwares WINBOOT (Fig. 1A) and FREETREE (Fig. 1B). Bootstrap value and dendrogram pattern of both the softwares were found to be almost similar. The dendrogram clearly separated clinical isolates of *L. donovani* into three main clusters. The first cluster from the top constitutes of Dd8 and 2001 sharing 50.8% similarity. The second cluster was divided into two sub-clusters, the first one comprising of isolate 2041 sharing 83.2% similarity with the second one having isolates 2087 and 2093. Interestingly, the later isolates too shared 56.5% similarity among themselves. The first and second main clusters have shown 55.7% similarity. The third cluster 2039 isolate was observed to be completely out-grouped from others, showing high rate of polymorphism.

3.3. Principal component plot

The principal component plots generated from AFLP data using SPSS software have been shown in Fig. 2. On the basis of principal component analysis, the six strains of *L. donovani* were placed in three quadrants. In the first quadrant, there was occurrence of three species – 2041, 2087, and 2093. In the second one, both drug sensitive isolates – Dd8 and 2001 were placed but interestingly again, SAG resistant strain 2039 was placed as totally distinct from all other strains in the third quadrant.

3.4. Polymorphism analysis

Fingerprinting of *Leishmania* using 10 combinations of EcoRI/MseI primers revealed a total number of 1294 (55.35%) unambiguous polymorphic AFLP fragments (Table 1) with an average of 129.4 polymorphic loci per primer combination. Results confirmed that AFLP was capable of detecting substantial numbers of polymorphic loci. The primer combination MseI-CAT/EcoRI-AGC revealed the highest percentage of polymorphic fragments (100%), while the lowest percentage (30%) was generated by the primer combination of MseI-CTT/EcoRI-AAC (Table 1).

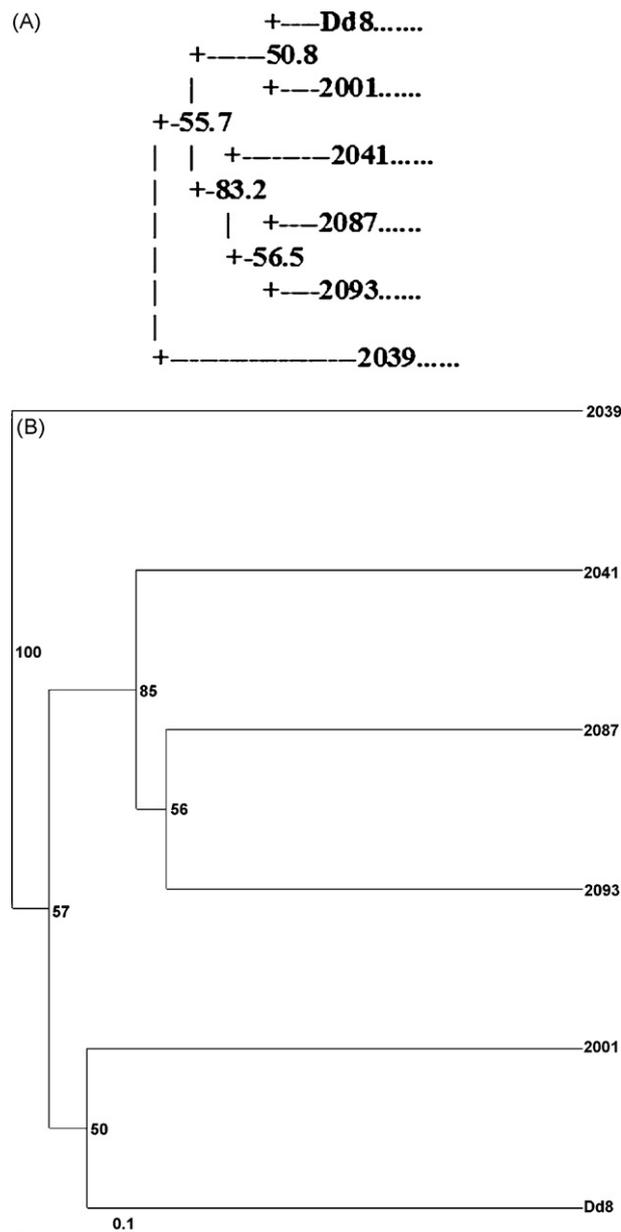


Fig. 1. (A) UPGMA cluster analysis of AFLP data generated by 10 selective primer combinations for 6 clinical isolates of *L. donovani* depicting patterns of genetic diversity through WINBOOT. (B) Dendrogram of *L. donovani* strains showing phylogenetic relationship among them using FREETREE.

3.5. SAG sensitive and resistant strain specific AFLP markers

A number of unique molecular marker specific for each strain of *Leishmania* were detected through AFLP analysis which has been listed in Table 2. Maximum number of unique bands (12) was observed in drug sensitive isolate – 2087 using primer combination of MseI-CAA/EcoRI-ACG. However, among the drug-resistant isolates, using 3 different primer combinations MseI-CTA/EcoRI-ACA, MseI-CTA/EcoRI-ACG and MseI-CTT/EcoRI-AAC maximum (5) unique bands were noticed with each primer combination in 2039 strain. The primer combinations, as evident in Table 3, showed a number of group specific (SAG sensitive/resistant) AFLP markers which could be particularly helpful in detection of SAG resistant cases. The following selective primer combinations i.e. MseI-CAA/EcoRI-ACA, MseI-CAC/EcoRI-ACG, MseI-CAA/EcoRI-ACT, MseI-CAC/EcoRI-ACT, MseI-CAC/EcoRI-AAG, MseI-CAT/EcoRI-AGC,

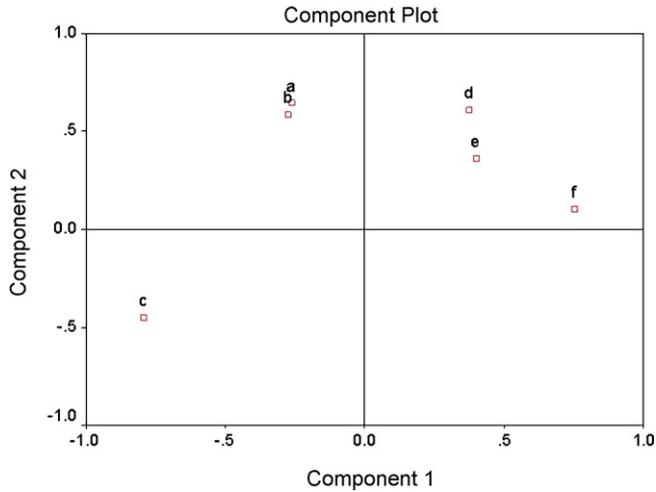


Fig. 2. Principal component plot based on the AFLP data of six clinical isolates of *L. donovani* (a – Dd8, b – 2001, c – 2039, d – 2041, e – 2087, f – 2093).

and MseI-CAG/EcoRI-AGC were found to be important for detection in respect of SAG resistant isolates, and MseI-CAA/EcoRI-AGG, MseI-CTC/EcoRI-ACA, MseI-CTA/EcoRI-AGC, and MseI-CTG/EcoRI-AGC for SAG sensitive ones.

4. Discussion

In our earlier study, using six clinical isolates of *L. donovani*, we have demonstrated the differential expression and intensity of PPG

Table 3

Group specific AFLP markers (size in bp) of SAG sensitive and resistance strain of *L. donovani*.

Primer combinations	SAG sensitive strains specific bands (bp)	SAG resistant strains specific bands (bp)
MseI-CAA/EcoRI-ACA		110, 240, 262, 390
MseI-CAA/EcoRI-AGC	143, 215	273, 346, 352
MseI-CAC/EcoRI-ACG		320
MseI-CAA/EcoRI-ACT		269, 276, 346
MseI-CAA/EcoRI-AGG	160, 240	
MseI-CAC/EcoRI-ACT		277
MseI-CAC/EcoRI-AAG		174, 264, 299
MseI-CAT/EcoRI-ACA	202	332, 337, 340
MseI-CAT/EcoRI-AGC		179, 218
MseI-CTC/EcoRI-ACA	117	
MseI-CAG/EcoRI-AGC		274
MseI-CTA/EcoRI-ACA	250	281, 314, 366
MseI-CTA/EcoRI-ACG	135, 316	198
MseI-CTA/EcoRI-AGC	113	
MseI-CTG/EcoRI-ACA	161	202
MseI-CTG/EcoRI-ACG	165	212
MseI-CTG/EcoRI-AGC	378	
MseI-CTT/EcoRI-ACT	174	206

by FACS and Western blotting which was higher in SSG resistant isolates (Samant et al., 2007). The observation of differential expression of PPG in drug-resistant and sensitive strains gave an indication of genomic variations that are responsible for drug resistance. The difficulty involved in the understanding related to SAG resistance of *Leishmania*, highlighted the need for a DNA marker system for easy detection and diagnosis against leishmaniasis. Exploration of these genetic variations could provide a way for marker identi-

Table 2

Unique molecular marker bands (size in bp) specific for each SAG sensitive and resistance strains of *L. donovani* detected through AFLP.

Primer combinations	Drug sensitive isolates			Drug-resistant isolates		
	Dd8	2001	2087	2041	2039	2093
(1) MseI-CAA/EcoRI-ACA MseI-CAA/EcoRI-ACG MseI-CAA/EcoRI-AGC	124, 153, 184, 284		101, 247, 370 261 105,109,110, 112,113,119, 128,129,130,131,183,223	345	142, 392	382
(2) MseI-CAC/EcoRI-ACA MseI-CAC/EcoRI-ACG MseI-CAC/EcoRI-AGC	379 347	102	114	276, 277	212	321 245
(3) MseI-CAA/EcoRI-ACT MseI-CAA/EcoRI-AGG MseI-CAA/EcoRI-AAC	134, 248, 274, 319 319	222, 280 227, 228, 259	119 203		121, 218, 275, 317	135,242, 250 223
(4) MseI-CAC/EcoRI-ACT MseI-CAC/EcoRI-AAG MseI-CAC/EcoRI-AAC	183, 251, 347 260	172, 284	221 288		267 127,141	157, 215
(5) MseI-CAT/EcoRI-ACA MseI-CAT/EcoRI-ACG MseI-CAT/EcoRI-AGC	115, 251 253, 260, 318, 319, 321 321, 326, 334	333 112	101, 262, 304, 348 235, 263 215, 219, 307	256 259	200 202, 213 203, 247, 336	334, 339 233 344
(6) MseI-CTC/EcoRI-ACA MseI-CTC/EcoRI-ACG MseI-CTC/EcoRI-AGC	165, 337 185 190	123 187, 311, 322 311, 313, 321, 317, 318,321	173, 216	179, 274	213	211 232
(7) MseI-CAG/EcoRI-ACA MseI-CAG/EcoRI-ACG MseI-CAG/EcoRI-AGC	171, 223, 242 172	107,164, 267	137, 343	107, 161		243
(8) MseI-CTA/EcoRI-ACA MseI-CTA/EcoRI-ACG MseI-CTA/EcoRI-AGC	175, 277 112, 162 378	217, 344, 367	379 210 102	123	116,310, 312 363, 364 113,124,307, 310, 314 262, 274, 315, 326	238, 297 119
(9) MseI-CTG/EcoRI-ACA MseI-CTG/EcoRI-ACG MseI-CTG/EcoRI-AGC	119, 179, 216, 217 166, 180, 197, 379 112, 199, 308, 369	210 217	101	162 179	205 136, 208, 228, 374 163, 277, 283	207 195
(10) MseI-CTT/EcoRI-ACT MseI-CTT/EcoRI-AAG MseI-CTT/EcoRI-AAC	208 189, 205, 209, 375				348, 349, 350, 351 140, 348, 350 202,348,349, 350,353	

fication responsible for drug resistance which could be used for detection and diagnosis against VL. Therefore, this technique was utilized to evaluate the polymorphism and phylogenetic relationships in SAG sensitive and resistant strains of *L. donovani* isolated from VL patients that are being maintained in our laboratory (Dube et al., 2005; Samant et al., 2007). Since we have observed a differential expression of PPG in SAG resistant and sensitive isolates (Samant et al., 2007) it was concluded that this differential expression may be due to genetic variation in between these isolates. In this study we carried out AFLP analysis on similar six isolates to study the genetic variation responsible for drug resistance. Number of primer combinations taken for this study is also an important issue to produce an accurate estimate of genetic relatedness (Ellis et al., 1997). It is believed that by choosing at least six combinations of primers, it is possible to explain the expected relatedness by more than 80% (Chen et al., 2004). We have taken here more than 6 i.e. 10 combinations of EcoRI/MseI primers in our study for more accuracy, reliability and distinguishing genetic relations in our result (Vos et al., 1995). These combinations yielded 20–40 DNA fragments after the PCR, within the size range of 100–400 bp that were used to screen all the clinical isolates. The results thus obtained from a large number of primer combinations were utilized to check the robustness of the dendrogram and estimates of phylogeny that clearly establish the polymorphism revealed by AFLP which is not only abundant but also statistically reliable (Chen et al., 2004).

This study supports the hypothesis that SAG resistance strains evolve from the sensitive ones. From dendrogram it is clear that 2041 and 2093 strain (resistant) share homology with 2087 strain (sensitive) but their response against SAG is different. It indicates that due to point mutations, insertions, deletions, and/or other genetic rearrangements (Vos et al., 1995) there is change in the genetic make-up of *Leishmania* strain which facilitates the conversion of parasite from sensitive to resistance condition. Indeed, this study too provides evidence suggesting that the *Leishmania* populations originated from a common ancestral species. This phylogenetic relationship among *Leishmania* species shows that all the lineages in this species converge to a single point before separating from other related species included as out-grouped. These results further ascertain that 2039 strain of *Leishmania* is totally out-group indicating that this strain is highly polymorphic and develop resistance against SAG. The variability observed among SAG sensitive and resistant populations based on AFLP analysis suggests that the SAG resistant strain may be a composite group. We may say that due to this genomic differentiation they acquire drug resistance. Further, it was evident from the component plots that the SAG resistant accessions 2039 are remarkably distinct from other SAG resistant accessions as it falls at the extremes of the plot. This further proved the highly polymorphic nature of 2039 and correlates well to our previous findings in which, while using the same clinical isolates, we have shown that expression of proteophosphoglycan is highly upregulated in 2039 strain (Samant et al., 2007).

Moreover, these results demonstrate that genetic resolution provided by AFLP is amenable to phylogenetic analysis of closely related *Leishmania* species. The high sensitivity of the AFLP technique, which provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity (Vos et al., 1995), along with its rich banding pattern (20–40 bands for each pair of selective primers) provided more information about polymorphisms among *Leishmania* clinical isolates. A noteworthy observation, as evident from dendrogram, was that AFLP analysis, while displaying a higher rate of polymorphisms among *Leishmania* clinical isolates, further indicates towards the shifting of drug sensitive parasite to resistant condition.

In conclusion, our approach using AFLP revealed genetic polymorphism among the *L. donovani* clinical isolates obtained from the

patients of endemic area and maintained in our laboratory. This preliminary exercise of AFLP fingerprinting could be further extended to a broad survey of populations in order to evaluate the impact of co-evolution among various strain of parasite. Future studies may also include molecular cloning and recovery of AFLP marker bands for the identification of disease specific markers of VL. The genomic fingerprinting data of *Leishmania* obtained from AFLP study serves as a basic framework in order to obtain more detailed information on the genome and markers linked to genes responsible for resistance and disease.

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