Research Article

Application of kDNA Minicircle PCR-RFLP to Characterize Leishmania donovani Clinical Isolates Obtained from Post-Kala-Azar Dermal Leishmaniasis in Eastern Nepal

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1.Introduction

Post-kala-azar dermal leishmaniasis (PKDL) is a progression of visceral leishmaniasis (VL) that demonstrates lesions or hypopigmented skin rashes in patients even after successful treatment of VL [1]. This cryptic PKDL is characterised by papular, macular, and/or nodular lesions throughout the body, mainly demonstrated on the face, trunk, legs, arms, and genitals. About 5–10% of patients infected with VL develop PKDL in the Indian subcontinent [2]. In Nepal, PKDL occurs in 2.4% of previously treated cases of VL with 1.4% estimated to be at risk within 2 years of treatment [3] whereas in case of India, the interval ranges between 2 and 7 years [4].

The PKDL skin lesion has a tendency to become chronic and harbours the parasite, hence considered as a reservoir, especially challenging the elimination programme of VL from the Indian subcontinent [5, 6]. The clinical, epidemiological, parasitological, and immunological development of PKDL is not yet fully understood [7]. In fact, the eradication of PKDL could be an essential factor for the current VL elimination programme. Moreover, the asymptomatic VL infection is also considered as the reservoir for VL transmission that threatens for VL elimination in the Indian subcontinent [8]. The immunological responses are also found to be different in patients with VL and PKDL [2]. Hence, the molecular epidemiology of L. donovani causing different clinical manifestations is required to understand in order to minimize the risk of VL elimination in the Indian subcontinent. The genetic analysis has shown the significant heterogeneity among the Indian L. donovani isolates that cause different clinical presentations such as VL and PKDL [9, 10]. But no such data on genetic characterization of L.
donovani isolates from Nepal were available yet. Therefore, we focused on the study of the genetic characterization of VL and PKDL parasite isolates from Nepal using available highly efficient molecular assay kDNA minicircles PCR-RFLP together with haspB PCR assay.

2. Materials and Methods

2.1. Sample Collection and Parasite Isolation. The sample collection consisted of L. donovani clinical isolates from 14 VL and 4 PKDL patients who were presented between January 2013 and June 2014 at B. P. Koirala Institute of Health Sciences (BPKIHS), a tertiary care hospital in Dharan, Nepal. Ethical clearance was obtained from Institutional Ethical Review Board (IERB), BPKIHS, Nepal. Written consent was obtained from all patients or from parents or guardians in case of children. Promastigotes forms of L. donovani were isolated from bone marrow aspirates of VL patients and skin punch biopsy of PKDL patients. Parasite culture was performed by inoculating the clinical specimens in Tobie’s blood agar medium with Locke’s overlay, with 200 IU/ml penicillin and 200 μg/ml streptomycin [11]. Once the parasites were fully grown from the clinical material, they were transferred to M199 (Sigma-Aldrich, cat. no. 2520) with 20% fetal calf serum (Invitrogen, cat. no. 10270) + adenosine + haemin.

2.2. Parasite DNA Extraction and Species Identification. DNA was extracted from parasite cultures using the QIAamp DNA mini kit (Qiagen, http://www.qiagen.com). Parasites in late logarithmic growth phase were washed thrice with sterile PBS solution, and DNA was eluted in 200 μL AE buffer. DNA concentration and purity were verified by spectrophotometric measurement with the BioPhotometer plus (Eppendorf). The Leishmania donovani species was confirmed using PCR-RFLP analysis of the heat-shock protein 70 gene (hsp70). The fragments referred to as hsp70 were verified by spectrophotometric measurement with the BioPhotometer plus (Eppendorf). The Leishmania donovani species was confirmed using PCR-RFLP analysis of the heat-shock protein 70 gene (hsp70). The fragments referred to as hsp70 were confirmed using PCR-RFLP analysis of the heat-shock protein 70 gene (hsp70).

2.2.1. hspB PCR. The hspB PCR for K26 antigen detection was used as species screening for L. donovani by primers K26f (5′-ACGAAGGACTCCGCAAAAG-3′) and K26r (5′-TTCCCATCGTTTGTGCTG-3′) [18]. The PCR master mix was prepared in 50 μL containing 1X Qiangen PCR buffer, 1.5 mM MgCl2, 200 μM of each dNTP (Eurogentec), 0.5 μM of each primer (Sigma-Aldrich), and 1 U of HotStar Taq polymerase. Amplification was done with (i) initial denaturation step of 95°C for 5 minutes; (ii) 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds; and (iii) a final extension at 72°C for 10 minutes. Out of two haspB genotypes identified, genotype A has ampiclon size of 640 bp and genotype B has ampiclon size of 320 bp.

2.2.2. kDNA PCR-RFLP. Genotyping of L. donovani clinical isolates was also done by kDNA PCR-RFLP assay. First, the kDNA minicircle is amplified by PCR using the primer pairs BPKMINFOR (5′-CTGGGGTTTGGTGAATAATGGGC-3′) and BPKDNAMINREV (5′-CCCCATTGTTGGCATTTTTG-3′). The PCR master mix was prepared in 50 μL containing 1X PCR buffer, 2 mM MgCl2, 200 μM of each dNTP (Eurogentec), 0.5 μM of each primer (Sigma-Aldrich), 1 U of HotStar Taq polymerase, and about 1 ng of template DNA. Amplification was done with (i) initial denaturation step of 95°C for 5 minutes; (ii) 40 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute; and (iii) a final extension at 72°C for 10 minutes. The PCR amplicon verified at 800 bp was precipitated with 0.3 M sodium acetate and 100% ethanol at −20°C overnight. The pellets were washed with 70% ethanol, and restriction digestion was done on precipitated PCR amplicons. Restriction digestion was done in a total of 20 μL of the buffer with 10 units of HaeIII and incubated at 37°C overnight. The reaction was stopped by heating to 80°C for 20 minutes, and then the fragments were analysed by electrophoresis in 3.75 V/cm in a 3% metaphor agarose gel (Lonza, USA), after staining with ethidium bromide.

The patterns of fingerprint bands were analysed using Gel Compare II 6.6 (Applied Maths, Ghent, Belgium) software. The spectral analysis was used to remove the background noise using the gel picture without signal saturation. Other gel artefacts were filtered out using the DNA ladder at both sides and middle part of each gel. Only the region between 200 and 600 bp was considered for fingerprint analysis, following the digested fragments demonstrated by Bhattacharai et al. Similarity matrix was made and UPGMA dendrogram was built. The validity of each branch was evaluated using Pearson’s correlation coefficient between the dendrogram derived and the original curve similarities. To assure the reliability of the clusters, the
duplicate experiments were done. Furthermore, four consecutive subcultures of two parasites 63291-Fx and BPK-PKN468 were examined to minimize the experimental variations.

The Bayesian clustering approach was also employed to estimate the kDNA population structure using Structure v2.3.4 [19]. $L(K)$, $L(K)'$, and $\Delta K$ were computed from 50 runs for $1 \leq K \leq 10$ using a burn-in of $10^4$ and run length of $10^5$ iterations. For each RFLP band, allelic diversity for each population was calculated for the number of individuals.

### 3. Result

All parasite isolates were confirmed as *L. donovani* species by *hsp70* as shown in Figure 1 and rRNA assay. *haspB* assay was classified into group A ($n = 6$) and group B ($n = 12$) as shown in Figure 2. Most of the group A parasites were originated from hilly districts, namely, Bhojpur, Khotang, and Okhaldhunga, except the one parasite from Sunsari (Figure 3). In contrast, the group B parasites were isolated from low-land districts (Terai) such as Jhapa, Morang, Sunsari, Saptari, and Siraha. In case of clinical presentation in
patients, group A parasites were isolated only from VL patients whereas group B parasites were isolated from both VL and PKDL patients as shown in Table 1.

kDNA minicircle PCR-RFLP identified 18 different genotypes which were distributed as follows: six genotypes (K1, K2, K3, K4, K5, and K6) were isolated from hilly districts and remaining 12 genotypes (K7 to K18) were isolated from low-land districts as depicted in Figure 3. The interexperimental variability was controlled by testing the duplicate samples of parasite isolates in different gels and included in the dendrogram analysis as shown in Figure 4, and gel image of restriction fragments is shown in Figures 5 and 6. The distribution of kDNA genotypes is also depicted in Table 1. The output from the structure also showed the two distinct populations of kDNA (K = 2) as shown in Figure 7. Hence, two distinctly different L. donovani parasite populations were identified with kDNA genotyping.

Among two different approaches of genotyping, six kDNA genotypes (K1, K2, K3, K4, K5, and K6) were sharing same characteristics with group A haspB genotype. Other 12

| VL/PKDL | Isolates    | District | VDC   | haspB | Genotypes | kDNA RFLP |
|---------|-------------|----------|-------|-------|-----------|-----------|
| VL      | BPK806/0    | Bhojpur  | Pawala| A     | K4        |
| VL      | BPK811/0    | Saptari  | Rajbiraj| B    | K16       |
| VL      | BPK813/0    | Morang   | Biratnagar| B  | K10       |
| VL      | BPK814/0    | Jhapa    | Chandragadhi| B | K9        |
| VL      | 63232-Fx    | Bhojpur  | Mane Bhanjiyang| A | K1       |
| VL      | 63233-Fx    | Bhojpur  | Jarayotar| A   | K2        |
| VL      | 63238-Fx    | Jhapa    | Gauradaha| B   | K8        |
| VL      | 63241-Fx    | Jhapa    | Maheshpur| B  | K12       |
| VL      | 63245-Fx    | Morang   | Biratnagar| B  | K11       |
| VL      | 63256-Fx    | Morang   | Dewanganj| B  | K13       |
| VL      | 63269-Fx    | Khotang  | Wopung| A    | K6        |
| VL      | 63277-Fx    | Sunsari  | Dharan| A    | K3        |
| VL      | 63291-Fx    | Sunsari  | Chatara| B   | K14       |
| VL      | 63294-Fx    | Okhaldhunga| Obu | A    | K5        |
| PKDL    | BPK-PKN435  | Morang   | Jahada| B    | K18       |
| PKDL    | BPK-PKN466  | Morang   | Majhare| B  | K15       |
| PKDL    | BPK-PKN467  | Siraha   | Siraha| B   | K17       |
| PKDL    | BPK-PKN468  | Saptari  | Rupnagar| B | K7        |
kDNA genotypes (K7 to K18) had the same genetic characteristic with group B haspB genotype as shown in Figure 4.

4. Discussion

VL and PKDL are still considered as major public health problems in South Asian countries such as Nepal. PKDL cases are considered as reservoirs to maintain the endemicity during interepidemic period, but less attention has been given to explore the PKDL epidemiology. This may potentially jeopardize all the achievements of ongoing elimination programme. In fact, molecular epidemiology study has been limited to VL cases only [20, 21]. No such data are available in Nepalese PKDL cases. In this context, we aim to genetically characterize the L. donovani parasite causing VL and PKDL by the two assays (a) haspB PCR and (b) kDNA PCR-RFLP and explore the genetic similarity between these parasites with different clinical manifestations. This study differentiated the parasites into two different haspB genotypes, which is consistent with the finding of Bhattarai et al. We found that parasites isolated from hill districts had significantly different genotypes and PKDL were not identified in isolates from hill districts. The finding of this study indicates that the L. donovani genotypes have association...
with clinical features of leishmaniasis, and similar finding has also been demonstrated in Sri Lanka by Kariyaswami et al. [22]. This genotype diversity in these isolates needs further exploration to further confirm whether these clinical isolates are different, not only in their genetic makeup, but also in their clinical manifestation, epidemiology, antigenicity, and parasitic factor as well.

In addition, another molecular marker kDNA owing to its high amount of heterogeneity has been exploited by the molecular biologist for strain characterization in Leishmania to depict the microepidemiology of the parasite [20, 23, 24]. The kDNA RFLP is sensitive to interexperimental as well as interlaboratory variations, so robust protocol should be followed for reproducible result. To achieve reliable result, we used the standardized amount of DNA for PCR and RFLP assay, experimental control was included in each batch of RFLP, four consecutive subcultures of same isolates were studied, and for further robustness, all the samples were

**Figure 5:** Gel picture of kDNA PCR-RFLP using HaeIII restriction digested products. Note: M: DNA ladder, Lane 1: BPK806, Lane 2: BPK806 (duplicate sample), Lane 3: BPK813, Lane 4: BPK814, Lane 5: BPK815-Fx, Lane 6: 63241-Fx, Lane 7: 63245-Fx, Lane 8: 63256-Fx, Lane 9: 63269-Fx, Lane 10: 63277-Fx, Lane 11: 63291-Fx, Lane 12: 63294-Fx, Lane 13: BPK-PKN435, Lane 14: BPK-PKN467, Lane 15: BPK-PKN468, Lane 16: BPK811, and Lane 17: BPK-PKN466.

**Figure 6:** Gel picture of kDNA PCR-RFLP using HaeIII restriction digested products. Note: M: DNA ladder, Lane 1: BPK806, Lane 2: BPK813, Lane 3: BPK814, Lane 4: 63232-Fx, Lane 5: 63233-Fx, Lane 6: 63238-Fx, Lane 7: 63241-Fx, Lane 8: 63245-Fx, Lane 9: 63256-Fx, Lane 10: 63269-Fx, Lane 11: 63277-Fx, Lane 12: 63294-Fx, Lane 13: BPK-PKN435, Lane 14: BPK-PKN467, Lane 15: BPK-PKN468, Lane 16: BPK811, and Lane 17: BPK-PKN466.
studied in duplicates. In this study, kDNA fingerprint showed the distinct clusters of two separate genotypes which were consistent with group A and group B, as determined by haspB PCR. This indicates further proof of principle that these isolates had genetically separate entity that influences the different clinical manifestations such as visceral or dermal manifestation in patients. Similar findings have been reported from the Indian subcontinent using β-tubulin gene as a probe where parasites from VL and PKDL were randomly clustered in between different assigned genotypes [9]. Another study using probe Ldp13 also showed genetic heterogeneity among VL and PKDL isolates from the Indian subcontinent [10]. This is particularly interesting because the Sudanese isolates were found to have similar genetic makeup by various PCR fingerprinting methods [25]. The mechanism of parasite diversification between VL and PKDL is still a matter of research, but some evidence points towards the contributory role of immune response of patient [2].

Ostyn et al. demonstrated that the VL infections at hilly districts were locally transmitted, and in this study, we also found the different L. donovani genotypes circulating in hilly districts. Hence, Ostyn et al. documented the expansion of VL in new endemic hilly areas of Nepal which is verified by the evidence obtained from the study of epidemiology, microbiology, clinical, and entomology [16]. Moreover, Phlebotomus argentipes sandfly infected with L. donovani were identified, and hence, the asymptomatic infections were also reported in hilly districts by Ostyn et al. This might be the influence of recent increasing trends of temperature in hills due to the global warming that favours the breeding of sandfly vector populations. Therefore, it is an urgent need to explore the role of PKDL as a probable reservoir for VL in order to understand the transmission dynamic of VL together with global impact of climate change.

5. Conclusions

This study concludes that PKDL and VL clinical parasite isolates were genetically separated and most of them confined to particular geographic location of Nepal. However, different molecular tools are developed elsewhere [26]; the high discriminatory power of kDNA PCR-RFLP tool could be useful in the analysis of molecular epidemiology of VL and PKDL in future.

Data Availability

This is a hospital-based study. Samples were collected during the routine diagnostic and genotyping procedure. Therefore, the data of the analysis are available upon request from the corresponding author or Head, Department of Microbiology (hod.microbiology@bpkihs.edu), B. P. Koirala Institute of Health Sciences, Dharan, Nepal.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.
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