Conclusions
Visceral Leishmaniasis (VL), or kala-azar, is a vector-borne disease that is caused by the protozoan parasites *Leishmania donovani* and *L. infantum*. The disease is transmitted to humans by the bite of infected phlebotomine sandflies. Patients present with fever, weight loss, fatigue, and general weakness as also hepatosplenomegaly. As VL is potentially fatal if left untreated and treatment is associated with high toxicity, a diagnostic test that is sensitive and specific is the need of the hour. Moreover, eradication of VL is presently a national priority, the WHO target of eliminating VL (annual incidence of less than one per 10,000 population) being 2017 in India, Bangladesh, Bhutan and Nepal (http://apps.who.int/iris/handle/10665/148778, p128), it is very important to be able to examine critically each and every suspected case. Therefore, very sensitive and specific diagnostic methods are urgently needed which will also be less invasive than spleen/bone marrow aspiration procedure.

PCR is a very powerful tool for sensitive and specific diagnosis as it detects nucleic acid of the parasite itself. The benefits of the PCR-methodology are undoubtedly enormous. One of the focuses of this work was to use the ITS1-PCR for diagnosis of Leishmaniasis in India, where the methods have been established and are ready now for routine use. The ITS-1 amplification in combination with RFLP analysis is clearly superior to other PCR based methods known as no study has been found in the literature so far which is simultaneously sensitive and species specific for the whole spectrum of *Leishmania* species. With this ITS1 PCR-RFLP method, nearly every *Leishmania* species can be identified with high sensitivity. Additionally, patients with relapse of VL or PKDL can also be diagnosed by this PCR which could not be achievable by serological tests as antibodies generated during the disease, remained in the circulation for a long time period (Ghosh et al., 2015).

We reported 4/60 patients (VL and PKDL) were concomitantly infected with *Leishmania donovani* and *Leptomonas seymouri* as also 2/10 isolates. It may be envisaged that as VL induces a strong immunosuppression, it possibly allows non-human trypanosomatids to be installed in mammalian hosts. The occurrence of insect trypanosomatids in humans is exceptional, but reports are available that HIV-positive patients are additionally infected with nonpathogenic insect trypanosomatids. As the *in vitro* susceptibility toward antimony of both the *Leptomonas* isolates was lower than others, it raised the possibility of the potential contribution of *Leptomonas* to the growing incidence of unresponsiveness to antimonials reported from the Indian subcontinent. However, more studies are needed to understand the functional implication of these opportunistic *Leptomonas* infections which may influence the epidemiology and pathology of VL.

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Along with diagnosis, parasite load was also measured in patients with VL and PKDL by amplifying a small region of kDNA by qPCR. Since, VL is a systemic disease and PKDL is a dermal disease, parasite load was quantified in blood and dermal skin biopsies of patients with VL and PKDL respectively. The parasite load was correlated with clinical and immunological markers to check whether this parasite load is a parameter for disease severity. Since no disease severity marker is available for VL, it was felt worthwhile to monitor parasite load with clinical parameters. Among the clinical parameters studied, only disease duration was associated with parasite load in both diseases. To find out an appropriate disease severity marker for VL as well as PKDL, a well designed study must be carried out in future including more clinical parameters like splenomegaly, biochemical markers for VL and lesional score for PKDL.

Among immunological markers, no correlation was found between humoral markers and parasite load in both diseases. According to the cell mediated immune response in terms of cytokines and chemokines, varied responses were noticed between VL and PKDL. Pro-inflammatory cytokines TNFα and IFNγ along with anti-inflammatory cytokines IL-4 positively correlated with parasite load in patients with VL.

Regarding the chemokine responses, levels of neutrophil chemoattractants were significantly higher in patients with VL as compared to healthy individuals which positively correlated with parasite load. This strong positive correlation indicated that neutrophils are playing an important role in replication of parasites to sustain the disease suggesting that neutrophils are involved in active disease of VL and perhaps are acting as a ‘Trojan horse’ to sustain infection and facilitates parasite entry into macrophages.

Additionally, there were raised levels of circulatory chemokines responsible for monocyte/macrophage migration in patients with VL as compared to healthy individuals, among which levels of CCL7 and MIF positively correlated with parasite load. In patients with PKDL, levels of CCL2, CCL7 and CCL8 were significantly higher as compared to healthy individuals and only levels of CCL8 negatively correlated with parasite load. Taken together, although macrophages are the main host cells for amastigote development, no remarkable correlation was evident between parasite load and level of chemoattractants in both diseases.

Chemoattractants for B cells and T cells were significantly higher in patients with VL as compared to healthy individuals but there were minimal alterations in patients with PKDL.
Conclusions

However, only skin tropic T lymphocyte chemoattractant CCL17 positively correlated with parasite load in patients with PKDL which accounted for the huge infiltration of immune cells at the lesional site (Mukherjee et al., 2015). Taken together our data suggests that immune response due to *L. donovani* infection in humans do not mirror the number of parasites infected. Moreover, as parasites are only found in the dermal lesions during PKDL, more studies are needed to correlate the mRNA expression of immune regulatory genes at lesional site with parasite load.

Since, immune response and disease severity varied among individuals and did not proportionally change with parasite load, some host specific genetic factors might play a role in disease pathogenesis. TLR4 being the important receptor molecule in innate immunity, two non-synonymous *TLR4* polymorphisms, an A/G transition at SNP rs4986790 that causes an Asp/Gly polymorphism at amino acid 299 and a C/T transition at SNP rs4986791 that causes a Thr/Ile polymorphism at amino acid 399 were studied in Indian VL and PKDL patients. The derived states (G and T, or Gly and Ile, respectively) have been shown to change the ligand-binding site of the receptor (Rallabhandi et al., 2006). We found a high proportion of Gly-Ile haplotype in Indian VL and PKDL patients and total absence of a Gly-Thr haplotype. According to the HapMap data, African population have a higher proportion of the Gly-Thr haplotype, but not the Gly-Ile haplotype. This radical difference in TLR4 gene in two populations might be a clue towards the difference in disease progression of VL to PKDL in Indian and Sudanese (African) population. More studies are needed in high number of samples to draw a firm conclusion and also in-vitro studies are required to understand functional implication due to the change in gene sequence.

In summary, this study gave an overview on occurrence and progression of Indian Leishmaniasis ([Figure 1](#)). In endemic areas, although many people get a sand fly bite, not all present with VL and remain asymptomatic or healthy. There are very few studies regarding polymorphisms in *Leishmania* strains which may be a factor for differential disease incidence (Subba Raju et al., 2008). Simultaneously, another hypothesis is host genetic factors which may also play a role in this differential disease incidence. Another step in the disease progression in India ([Figure 1](#)) is the incidence of PKDL wherein very few people (5-10% of cured VL) present with PKDL after curing from VL. Here too, parasite genetic factors and host genetic factors may come into play.
Collectively, the key findings of this study are:

1. There were no difference in frequency of polymorphic variant (in selective SNPs) of TLR2 and TLR4 between healthy and patients with VL or PKDL. Therefore, no TLR2 polymorphisms or TLR4 polymorphisms studied here were associated with VL or PKDL occurrence.

2. After having VL, 6.7% of the patients were found to be co-infected with *Leptomonas seymouri* which is an insect parasite. Due to immunosuppression, some patients got infected with opportunistic pathogens though consequences due to this infection were not clearly understood yet.

3. After treatment with anti-leishmanials, most patients were cured; this was confirmed by PCR and also by clinical features. In this study, 14.89% patients (7 out of 47) presented at the OPD with relapse.

4. The interval between cure of VL and occurrence of PKDL ranged from 3 months to 42 years. In PKDL, (3 out of 25, 12%) presented with recurrence.
5. There was a huge variation in parasite load between patients with VL and PKDL which correlated with disease duration among patients with a lower parasite load being inversely proportional in VL and directly proportional in PKDL).

6. Parasite load was not representative of the humoral immune response in VL and PKDL. However, during VL, to a large extent, it represented the cell mediated immune response in terms of chemokines and cytokines.