Asymptomatic *Leishmania* Infection: A New Challenge for *Leishmania* Control

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Visceral leishmaniasis (VL) is a serious parasitic disease, causing high morbidity and mortality in the developing world. The pathogenesis of VL is complex, and the clinical presentation ranges from asymptomatic infection to severe and fatal disease. Despite a wealth of research on the full-blown "clinical VL" syndrome, asymptomatic leishmania infections remain poorly understood. Asymptomatic infection could present a major challenge for control programs if its infectiousness is confirmed. In this viewpoint, we highlight the crucial knowledge gaps as well as the obstacles in research on asymptomatic leishmanial infection. Research in this area is essential for the development of more-effective VL control strategies.

Keywords. visceral leishmaniasis; asymptomatic infection; immunity.

Visceral leishmaniasis (VL), also known as kala-azar, is a life-threatening vector-borne disease caused by the *Leishmania donovani* species complex and resulting in an estimated 200 000–400 000 new cases per year worldwide [1]. India, Nepal, and Bangladesh harbor an estimated 67% of the global VL disease burden, and the governments of these countries have committed to eliminate VL by 2015 and aim to reduce the incidence of VL to <1 per 10 000 population in endemic districts [2]. One of the challenges faced by this elimination initiative is that only a small proportion of all *L. donovani* infection manifests as clinical disease. The outcome of *L. donovani* infection ranges from asymptomatic carriership to full-blown symptomatic disease characterized by prolonged fever, splenohepatomegaly, pancytopenia, and hypergammaglobulinemia. In Brazil, where *Leishmania infantum* is the causative species, subclinical forms were described in serologically positive individuals with at least 1 clinical manifestation, such as lymphadenopathy or mild symptoms that were often self-resolving [3]. Asymptomatic leishmanial infection is not well defined, but is usually ascertained by a positive serological test, polymerase chain reaction (PCR), or leishmanin skin test (LST) in individuals who are otherwise in a healthy condition [4, 5]. Mathematical modeling suggests that these asymptomatic carriers constitute a reservoir of parasites driving the epidemic [6], although their infectiousness to sand flies is not yet formally established. Several prospective studies have documented the ratio of incident asymptomatic infections with *L. donovani* or *L. infantum* (known as *L. chagasi*) infection to incident clinical cases as 1:2.4 in Sudan [7], 4:1 in Kenya [8], 5.6:1 in Ethiopia [9], 18:1 in Brazil [10], 50:1 in Spain [11], 4:1 in Bangladesh [12], and 8.9:1 in India and Nepal [13, 14], demonstrating that many people infected with *Leishmania* species develop an effective immune response and do not manifest clinical disease. Neither the role of these asymptomatic carriers in transmission nor the prognosis of asymptomatic infection at the individual level is fully elucidated. Research in this area is challenged by the fact that the precise immune mechanisms underlying human VL are still not fully understood, and that the responses necessary for protection by vaccination are not as clear as in the mouse model

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[15]. Another problem is the absence of validated markers for asymptomatic *L. donovani* infection, as so far diagnostic assays for VL have been evaluated primarily on their capacity to detect clinical disease. In the present viewpoint article, we discuss obstacles to research in the domain of asymptomatic leishmanial infection and identify the knowledge gaps hampering more effective control of transmission.

IMMUNOBIOLOGY OF ASYMPTOMATIC INFECTION

The factors determining whether or not an infected individual progresses to clinical VL have not been fully identified, but a *Leishmania*-specific cellular immune response determined by both parasite and host factors seems to play a pivotal role. The macrophage is the cellular target of *Leishmania* parasites in the mammalian host. Studies from Colombia showed that responses of monocyte-derived macrophages from naturally infected humans to infection with *Leishmania (Viannia) panamensis* were closely associated to clinical outcome. Macrophages from LST-positive asymptomatic/subclinical individuals were less permissive to in vitro infection than cells from individuals presenting recurrent or chronic disease (clinical susceptibility) [16–18]. Whether such intrinsic differences in macrophages might control the clinical outcome of infection with *L. donovani* has so far not been reported. Much attention is now focused on the role of cytokines that modify the immune response by their actions on the macrophages. Few studies are available in the literature regarding the role of cytokines in asymptomatic infections and/or subclinical cases of VL, and these investigations typically determined cytokine levels only once, before the occurrence of any clinical manifestations. Prospective studies elucidating the role of cytokines in pathogenesis of the disease and their relation with the different clinical forms are lacking. Carvalho et al showed that peripheral blood mononuclear cells (PBMCs) from some (but not all) individuals with subclinical or asymptomatic infection (serology and skin test positive with *Leishmania* antigen) respond typically to stimulation with *Leishmania* antigen with the production of interleukin (IL) 2, interferon (IFN)–γ, and IL-12 [19]. We reported that active disease produced a mixed IFN-γ/IL-10 response, but asymptomatic infections (IFN-γ release assay [IGRA]–positive endemic healthy controls) did not lead to an antigen-induced whole-blood IL-10 response [20]. Interestingly, numbers of $CD4^+$ T cells are increased in person with asymptomatic infection having positive LST [21], and $CD8^+$ T cells isolated from asymptomatic subjects produce high amounts of IFN-γ, which strongly suggests a role of $CD8^+$ cells in human resistance to *Leishmania* infection. Moreover, a unique population of $CD4^+$ cells producing both IFN-γ and IL-5 was found to play a role in the control of infection in asymptomatic subjects [22]. More recently, a protective role for Th17 cells in human VL was suggested by a longitudinal study carried out in Sudan; it was reported that *L. donovani* induces production of IL-17 and IL-22 by PBMCs from exposed healthy and resistant subjects who did not develop VL either before or after evaluation of their cytokine response [23].

Further in-depth studies focused on the immune modulation in both subclinical and asymptomatic individuals are required to understand the nature of infection with *Leishmania* parasite and to develop better therapeutic control strategies.

CLINICAL AND EPIDEMIOLOGICAL ASPECTS

It is at present not possible to predict exactly who among the asymptomatically infected people will develop VL disease and when [24]. Whether an infection remains asymptomatic or progresses toward VL probably results from a complex interaction between environmental, parasite, and host-related factors (Figure 1). Understanding how the environmental and genetic risk factors associated with exposure to infection differ in susceptibility could provide important leads for improved therapies. Candidate gene and genome-wide linkage studies have highlighted a number of genes or gene regions contributing to disease susceptibility (reviewed in [25]). More recently, we have reported that common variants in the HLA-DRB1-HLA-QA1 HLA class II region contribute to susceptibility to *L. donovani* and *L. infantum/chagasi* [26], suggesting shared genetic risk factors for VL that cross the epidemiological divides of geography and parasite species. Polymorphism at *SLC11A1* has been shown to be linked [27, 28] and associated [28] in regulating susceptibility with human VL in Sudan. However, no evidence of such an association was found in an Indian population [29]. In addition to genetic factors, poor nutritional status has been shown to increase the risk of progression from infection to clinical VL [30–33]; children with moderate to severe protein energy malnutrition were found to have about a 9-fold increased risk of developing VL [30]. Malnutrition is considered to associated with impaired immune responses against the parasite and can weaken both innate as well as T-cell immunity [31, 34]. The relationship between malnutrition and the course of VL at the cellular level is poorly understood. A better understanding of these mechanisms might open new opportunities for prevention or therapeutic dietary intervention.

Several epidemiological studies have been conducted on the Indian subcontinent (ie, Bangladesh, India, Nepal) trying to identify epidemiological risk factors associated with VL, some using *L. donovani* infection as an endpoint, and others looking at VL disease. Asymptomatic infection is frequently associated in family members or the vicinity of clinical VL cases, suggesting that family members are exposed to the same risk of infection [35–38]. Ranjan et al showed a similar finding in a study

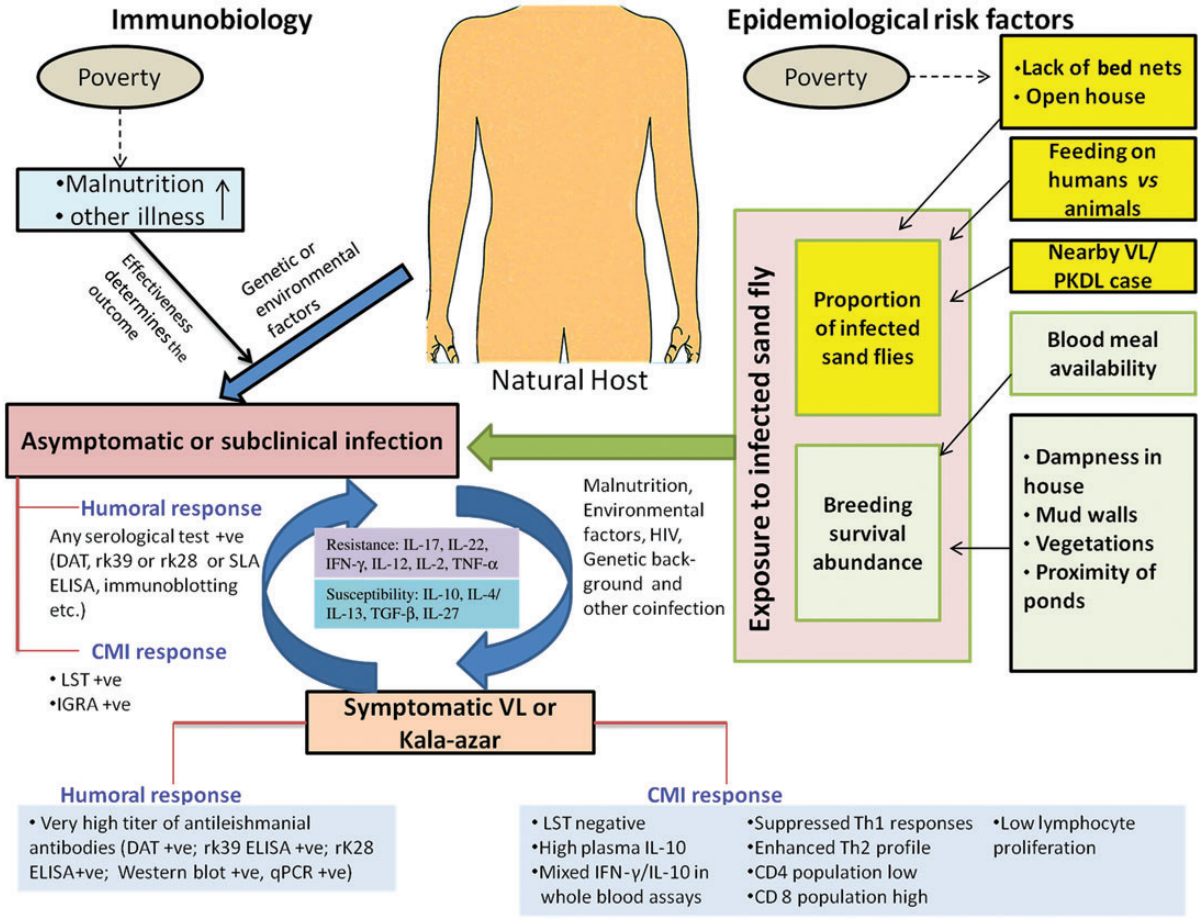

Figure 1. Generalized scheme of immune status and interplay of factors that affect risk of visceral leishmaniasis (VL) and asymptomatic *Leishmania donovani* infection. Infection with the *Leishmania* parasite causing visceral disease leads to different pathological conditions. A considerable percentage of the infected population undergoes asymptomatic or subclinical infection and remains immune to symptomatic disease, whereas some infected individuals develop symptomatic VL. There is a balance between effector responses, which control the parasites, and regulatory cytokines, which limit collateral tissue damage. As a result of combinations of genetic and environmental factors, *L. donovani* infection ensues when innate or acquired immune responses are inadequate to clear or control the infection. Infection risk is increased by exposure to infected sand flies, by proximity to the infectious reservoir host (eg, untreated post–kala-azar dermal leishmaniasis and kala-azar patients) and host factors such as nutrition that affect the immune response, but may be decreased by behaviors such as bed-net use that interrupt human–sand fly contact. Cattle may affect risk in complex ways, through their effect on sand fly abundance, breeding, infection rates, and feeding frequency on humans. Abbreviations: +ve, positive; CMI, cell-mediated immunity; DAT, direct agglutination test; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IFN-γ, interferon gamma; IGRA, interferon-γ release assay; IL, interleukin; LST, leishmanin skin test; PKDL, post–kala-azar dermal leishmaniasis; qPCR, quantitative polymerase chain reaction; SLA, soluble leishmania antigen; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VL, visceral leishmaniasis.

from India, where family members of VL patients showed 1.8 times the odds of being infected, compared with those without VL in the household [39]. Poor housing conditions (that are conducive to sand fly breeding and resting) were found to be associated with increased risk in several studies [40–45]. A strong association between VL disease and poverty was documented in a study by Boelaert et al [46]. For ownership of animals, conflicting results have been reported. Bern et al found a protective effect of ownership of bovines in Nepal [42], whereas Barnett et al in India identified ownership of cows as a risk factor [47]. In a later study in Bangladesh, Bern found a protective effect of increasing numbers of bovines in close proximity to a household [12]. Singh et al conducted a case-control study specifically looking into the association between VL and domestic animals but found no evidence for animals being either a risk factor or a protective factor [45]. In a large cohort study in the same area, Hasker et al found only a weak effect of ownership of goats (odds ratio, 1.4) but no associations with other animals [44]. Conflicting results were also obtained about the protective effect of bed-net use on VL. Whereas several observational studies reported a protective effect, no effect was found in a large randomized controlled trial conducted in India and

Table 1. Research Agenda for Future Work on Asymptomatic Infection

Research Questions
1. Can people without clinical symptoms infect sand flies? If, yes, then how many people fit into that category (infectious but asymptomatic), and what are their characteristics?
2. Can we identify the infectious subset of persons in a population affected by *Leishmania donovani*? Which assay markers for this infectious subset of persons can be used?
3. How does the number of infectious people change over time and over the course of their infections?
4. Can the identification and validation of novel *Leishmania*-specific biomarkers help to distinguish between latent *Leishmania* infection and active disease?
5. How does test assay performance vary between high- and low-VL-incidence settings? In addition to geographical variability, are there racial/ethnic differences in tests performance and accuracy?
6. Will treating or vaccinating these asymptomatically infected individuals affect the intensity of transmission, and will it have a significant impact on the VL epidemic?

Abbreviation: VL, visceral leishmaniasis.

Nepal [48]. More recently, Mondal et al reported a 66.5% reduction of VL in a nonrandomized intervention study that compared one intervention area with a single control area in Bangladesh [49]. Some of the conflicting results could be related to the importance of controlling for socioeconomic status when studying risk factors in observational studies as well as to the specific epidemiology of VL that needs to be considered in intervention studies. VL incidence is highly clustered and tends to cause epidemics in those small clusters. The epidemic cycle in that small area dampens after a period of 4–5 years, and the disease will then be observed in a neighboring cluster. This phenomenon of "microepidemics" requires a sufficient number of clusters to be observed in an intervention study and may lead to differences in associations over time in observational studies, as was also observed by Bucheton et al in Sudan [50]. Better understanding of the risk factors/determinants of VL transmission at the village, household, and individual levels is important as it may allow for better targeting of control measures. This, however, requires a longitudinal prospective follow-up design that allows for the exploration of the combination of epidemiological, parasitological, immunological, and genetic risk factors in the same population. There are a number of specific questions to be answered to clarify the role of asymptomatic infections, and these are outlined in Table 1.

An additional challenge is the complexity of measuring leishmanial infection. Serological tests detecting *L. donovani* antibodies were so far mainly evaluated on their capacity to diagnose clinical VL, but they have also been used in many studies to identify asymptomatically infected individuals [51, 52]. Recently, we have observed a statistically significant increase of seroprevalence with age, although average antibody titers among asymptomatic seropositive individuals were much lower than those among recent clinical VL cases [4]. Moreover, strong associations were found between seropositivity or seroconversion and progression to clinical VL, particularly for those with high DAT or rK39 titers [53]. However, this strong association does not preclude that the vast majority of healthy people with a positive VL serology will never progress to disease, as the base rate of developing VL is low. In the absence of a gold standard to measure infection, it is hard to know whether those seropositive individuals who remained healthy were truly infected with *L. donovani* or whether the serology results were simply false-positive results or prior infection that cleared. Moreno et al [54] suggest that serological methods are inaccurate in identifying asymptomatic infection compared with molecular techniques and could underestimate the infection rates in population studies. On the other hand, the fluctuating nature of PCR positivity has also been observed, depending on the choice of target sequences [55] and due to the short half-life of DNA in the body (24 hours) [56]. Despite the wealth of existing PCR assays, very few have been validated on larger numbers of clinical samples, and none of them has become a reference tool in *Leishmania* diagnosis [5, 57]. Furthermore, feasibility of documenting cellular immune status through the LST is low [58]. A prototype of whole-blood IGRAs has recently been developed as an alternative to the LST to screen naturally exposed immune individuals [20]. The current evidence suggest that the IGRA has higher sensitivity and specificity than LST and could become in the future a suitable marker for intervention studies employing vaccine or vector control [59] (reviewed in [60]); but one of the greatest advantages of the LST is that assessment of T-cell–mediated immunity has been established in many cohort studies for various populations and associated clinical conditions. Currently, there are no equivalent data for IGRAs. Therefore, scaling up efforts to improve the characterization of asymptomatic infection in endemic regions and establishing a standard case definition for leishmanial infection should be a priority. The determinants of a self-clearing infection vs progression to full-blown disease must be fully investigated. It is important to expand beyond the traditional association and correlation analyses to include more comprehensive whole-blood transcriptional profiling methods. Concerted efforts should also be directed toward the development of highly sensitive, cheap, and easily obtained rapid diagnostic detection kits, which can be used to detect parasitemia at submicroscopic densities.

For VL control, the single most important question remains whether asymptomatic infected persons are infectious to the sand fly vector. Xenodiagnosis is a proof of principle which confirms whether asymptomatic infected persons can be infectious to sand flies, and could be an important step in deciding whether or not adjustments should be made to the current VL control

strategy. Molina et al [61] used xenodiagnosis as a method to diagnose VL infection in human immunodeficiency virus (HIV)–infected patients; even asymptomatic patients in early stages of HIV infection were able to infect. More entomological studies are needed in order to clarify the vector's behavior in the endemic area. Developing a method that is easy to apply and directly measures exposure to sand flies at the level of the individual has great potential benefits. The sand fly saliva antibody test developed by Clements et al [62] may provide a promising tool for this purpose. Better knowledge in this field will also be of crucial importance to monitor and improve the currently inadequate vector control efforts.

One of the most important features of asymptomatic infection is that people do not seek for treatment, and current drugs are too toxic to justify their use in these otherwise healthy individuals. Drug development efforts currently do not target the asymptomatically infected people. This is partly because the asymptomatic status is not well defined, but mainly because an intervention is less of a priority as long as asymptomatically infected persons' role in transmission is not elucidated. By generating this evidence, we will be able to inform the disease control programs whether or not they need to address the issue of asymptomatic infection.

CONCLUSIONS

Leishmaniasis is a global health problem and a major killer in endemic countries. The morbidity and mortality burden of VL could be reduced by strengthening prevention, improving VL diagnosis, and adopting strategies aimed at preventing asymptomatic/subclinical infection. The identification and management of asymptomatic carriers has become a new and increasingly important challenge for VL control programs, as infected hosts might serve as silent reservoirs, and as such jeopardize the sustainability of elimination if their role in transmission is confirmed. Moreover, a better knowledge of the factors that mediate visceral disease might offer the identification of better epidemiologic markers for potential vaccines and immunomodulators, which will support disease elimination efforts that would have a significant impact in the poorest regions of endemic countries.

Notes

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