Implications of co-infection of *Leptomonas* in visceral leishmaniasis in India

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SUMMARY

Protozoan parasites *Leishmania donovani* (family: Trypanosomatidae) cause fatal visceral leishmaniasis (VL) and the infection relapses in apparently cured population as post kala-azar dermal leishmaniasis (PKDL) in the Indian subcontinent. In recent years co-infection of another Trypanosomatid parasite *Leptomonas* with *L. donovani* during VL/PKDL in this region has become prominent. The observation of clinically lesser-known insect parasite, *Leptomonas* in leishmaniasis is intriguing to researchers. The presence of Leishmania look alike *Leptomonas* in the cultures of clinical isolates of *Leishmania* has been worrisome to those, who prefer to work with pure *Leishmania* cultures for drug and vaccine development or immune response studies. The exact implications of such a co-habitation, which might lead to a delay in the diagnostics of VL and elevate mortality, need a thorough investigation. Also whether *Leptomonas* is involved in leishmaniasis manifestation needs to be ascertained. Thus we are currently witnessing a new paradigm of a parasitic co-infection in VL/PKDL cases in India and this review outlines various opportunities for further research in understanding such emerging co-infection.

Key words: *Leishmania*, *Leptomonas*, co-infection, visceral leishmaniasis, post kala-azar leishmaniasis, sandfly.

INTRODUCTION

The causative organism of the fatal visceral leishmaniasis (VL; kala-azar) is *Leishmania donovani* and a sequel of VL after its treatment in certain cases in the Indian subcontinent is post kala-azar dermal leishmaniasis (PKDL) (Singh *et al*. 2006; Kumar *et al*. 2009; Ganguly *et al*. 2010). Infection of *L. donovani* simultaneously with other pathogens is not uncommon and in most cases leads to health deterioration (van den Bogaart *et al*. 2013; Patole *et al*. 2014; Singh, 2014). The secondary infection of VL mostly due to a compromised immunity of the host caused by different primary infections (Medrano *et al*. 1992; van Griensven *et al*. 2014). HIV, malaria and tuberculosis co-infections with *Leishmania* spp. has been reported. In recent years among the parasitic diseases, leishmaniasis has been increasingly displaying positivity to co-infection with both *Leishmania* and *Leptomonas* (Srivastava *et al*. 2010; Ghosh *et al*. 2012; Singh *et al*. 2013). Both are Trypanosomatid protozoan parasites. *Leptomonas* spp., until recently believed to be only an insect parasite, has been observed as a co-infectant with *L. donovani* in both VL and PKDL cases. To our knowledge there is no report of such co-infection in either cutaneous leishmaniasis (CL) or in mucocutaneous leishmaniasis (MCL). Since reports of such co-infection in VL in India are very recent, the relevance or clinical implications need to be investigated. The review here attempts to address/understand such issues by looking at the behavioural, epidemiological and genetic comparisons of *Leishmania* and *Leptomonas* parasites and the host immune status during co-infection.

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LEISHMANIA

The genus *Leishmania* is a protozoan parasite of the family Trypanosomatidae of the order Kinetoplastida. It is the causative agent of leishmaniasis in mammals in the tropical countries (Alvar *et al*. 2012). The three major forms of leishmaniases are visceral (VL), cutaneous (CS) and mucocutaneous (MCS) leishmaniases. Of these, VL is fatal if not treated. The typical symptoms of VL are chronic fever and enlargement of spleen and liver due to the parasite's survival and replication in these organs. Due to its endemicity more than 90% of deaths occur in the Indian sub-continent and in Sudan (Africa). About 10–15% and >50% of the treated populations in India and Sudan, respectively, develop PKDL. The migration of the residual parasites from infected visceral organs to the dermal site causes PKDL with macular and papular type of

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lesions (Kumar *et al*. 2009). PKDL patients are considered to be as human reservoirs to the continuing VL in these regions. It is transmitted by insect vector sandfly. The parasite is digenic with flagellated promastigote form that multiplies in the vector gut and the other non-motile amastigote form that replicates in the mammalian macrophages. Medications for leishmaniasis are available but expensive, take long time to cure and are threatened by development of drug resistance due to prolonged use (Sundar and Chatterjee, 2006). Although few licensed canine VL vaccines are in use, no vaccine is yet available for human leishmaniasis (Selvapandiyan *et al*. 2014; Jain and Jain, 2015).

LEPTOMONAS

Leptomonas is another Trypanosomatidae member known mostly as a monogenic form (promastigotes) living in the gut of certain insects (Yurchenko *et al*. 2006). Unlike *Leishmania, Leptomonas* was not a known habitant in vertebrates and hence not considered economically important. Its co-infection in the VL and PKDL cases in India through recent reports is a major concern. Presence of flagellated non-*Leishmania* Trypanosomatid parasites, most likely *Leptomonas*, has also been reported in the sandfly (vector in leishmaniasis) population in certain regions in Nepal (Bhattarai *et al*. 2009).

COMPARISON BETWEEN THE PARASITES

Except for minor differences, *Leishmania* and *Leptomonas* share many features like, most of the genome sequences, physiology and antigenicity (Tyzzer and Walker, 1919; Bacchi *et al*. 1975; Singh *et al*. 2013; Ferreira *et al*. 2014). In their promastigote stages, both the parasites have a single set of organelles, viz., flagellum, nucleus, kinetoplast (mitochondrium), Golgi apparatus and basal bodies etc. placed in the cells in identical polarity (McGhee and Cosgrove, 1980). *Leptomonas* promastigotes show optimal growth, gene transfection and clonal selection in *Leishmania* specific culture conditions *in vitro* (Bellofatto *et al*. 1991; Ahuja *et al*. 2015). Susceptibility of *Leptomonas* is known to many established anti-trypanosomatid agents. (Bacchi *et al*. 1974; Goldberg *et al*. 1974). The absence of drugs that can selectively eliminate *Leptomonas* renders it impossible to get rid of *Leptomonas* from the mixed cultures with *Leishmania*. This makes more challenging to develop drugs or vaccines against VL (Singh *et al*. 2013). Although *in vivo* in mammals the multiplication rate of *Leptomonas* has not been ascertained, in the *in vitro* cultures, this parasite's promastigote stage grows faster than that of *L. donovani* (Srivastava *et al*. 2010; Ahuja *et al*. 2015). Hence it became necessary for us to recognize the presence of *Leptomonas* in the *L. donovani* cultures originating from the clinical samples and eliminate it to have pure populations of *Leishmania* for downstream immunological and genetic studies. We and others after careful observation of the cultured *Leishmania* and *Leptomonas* promastigotes under bright field microscope reported the minor differences in their morphology (Tyzzer and Walker, 1919; Ahuja *et al*. 2015). In the *in vitro* cultures, *Leishmania* promastigotes were seen as heteromorphic cell population (rounded, ovoid or tadpole shaped), whereas *Leishmania seymouri* promastigotes were all uniform in shape (long ovoid). In the *in vitro* culture, *L. seymouri* promastigotes displayed faster (3·4-fold more) mobility than *L. donovani* promastigotes (Ahuja *et al*. 2015). We have also recently devised a simple procedure to selectively eliminate *Leptomonas* from the *in vitro* co-culture with *Leishmania* based on its differential growth at 37 ° C (Ahuja *et al*. 2015). At this temperature optimal growth of axenic amastigotes and reduced growth of promastigotes of *Leishmania* and a complete growth inhibition and death of *Leptomonas* were observed. This corroborates with the earlier observation by others where promastigotes of *Leptomonas costoris* infecting cultured macrophages were found to differentiate into amastigote like structures but failed to further replicate. *Leishmania donovani* on the other hand thrives in cultured macrophages and is even able to inhibit their capacity to digest this parasite (Kutish and Janovy, 1981). One report has also revealed the isolation of *Leptomonas* from the spleen of mice previously infected with *Leptomonas* spp. (Srivastava *et al*. 2010). These observations suggest the possible existence of *Leptomonas in vivo* in the macrophages even though it did not replicate in the vertebrate host free macrophages in culture. Both the parasites' promastigotes otherwise are grown optimally at 26 ° C in culture. The differences between these two parasites have been briefly summarized in Fig. 1.

There are several reports comparing the genomes of these two parasites. Complete genome sequence of several species of *Leishmania* (at: tritrypdb.org) including for *L. donovani* and *L. seymouri* (at: sanger.ac.uk) is available. Both *Leishmania* and *Leptomonas* reside in the common Trypanosomatid family cluster along with *Crithidia* and *Trypanosoma* in the phylogenetic analysis comparing several kinetoplast-bearing organisms based either on 18S rRNA or small subunit rRNA sequences (Moreira *et al*. 2004; Lukes *et al*. 2014). While comparing the DNA sequences of both ITS1 and GP63 of *Leishmania infantum, L. donovani* (both cause VL) and *Leptomonas*, the latter was found to be closer to *L. donovani* than *L. infantum* via unweighted pair group method with arithmetic mean (UPGMA) based phylogenetic analysis (Singh *et al*. 2013). However, close monitoring of DNA sequences of

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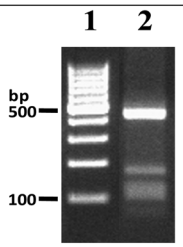
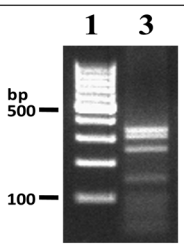
S. No.	Features	*Leptomonas seymouri*	*Leishmania donovani*	References
1	Type of hosts prior to knowledge of co-infection in leishmaniasis	Monoxenous in only invertebrates	Dixenous in invertebrates & vertebrates	Singh *et al.*, 2013, Yurchenko *et al.*, 2006
2	Promastigotes shape	monomorphic	heteromorphic	Ahuja *et al.*, 2015
3	Promastigotes cell doubling time *in vitro* culture	Less	High	Srivastava et al., 2010, Ahuja *et al.*, 2015
4	Promastigotes mobility *in vitro* culture	Fast	Slow	Ahuja *et al.*, 2015
5	Growth at 37°C *in vitro* culture	No	Yes	Ahuja *et al.*, 2015
6	PCR-RFLP of HSP70 gene Lane 1: Marker Lane 2: *L. seymouri* *Hae*III cut Lane 3: *L. donovani* *Hae*III cut			Srivastava et al., 2010, Singh *et al.*, 2013, Ahuja *et al.*, 2015
7	Infection in macrophages *in vitro*	No	Yes	Kutish *et al.*, 1981

Fig. 1. Differences between *Leishmania donovani* and *Leptomonas seymouri*.

heat shock protein-70 (HSP70) and internal transcribed spacer 1 (ITS1) genes of these parasites yielded genus specific polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) patterns, by which researchers now could differentiate between *Leishmania* and *Leptomonas* in their cultures (Fig. 1) (Srivastava *et al*. 2010; Ghosh *et al*. 2012; Singh *et al*. 2013). *Leptomonas* miniexon 1gene targeting Taqman real-time PCR was also suggested to specifically identify *Leptomonas* spp. in the clinical samples (Weirather *et al*. 2011). All such DNA based and microscopic methods are now useful tools to recognize *Leptomonas* when it appears as a contaminant with other intracellular pathogens in clinical samples.

The general perception among researchers is that the individuals once cured of leishmaniasis are protected thereafter. This is due to cellular (and humoral) immunological memory response generated against the primary infection of parasite (Selvapandiyan *et al*. 2012). Similarly immunity generated by either freeze-thaw-killed *Leptomonas collosoma* or live cultured *Leptomonas pessoai* cells was shown to protect the animals (including albino mice) from challenge against *Trypanosoma cruzi* (Johnson *et al*. 1963; Souza Mdo *et al*. 1974). These studies confirm that both the species of *Leptomonas,* which were not parasitic in vertebrates displayed stronger protective antigenicity against other trypanosomatids. In addition, comparison of cross-reactive epitopes of 6 different genera of Trypanosomatidae confirmed that the antigenicity of *Leptomonas seymouri* was similar to *Leishmania chagasi* (Ferreira *et al*. 2014). Otherwise detailed immune responses by either resistant or susceptible host during infection with different species of *Leishmania* in the animal models as well as in humans have been studied extensively (Selvapandiyan *et al*. 2009; Nylen and Gautam, 2010; Kedzierski and Evans, 2014). To our knowledge detailed immune responses due to *Leptomonas* infections have so far not been studied. With occurrence of *Leptomonas* co-infection in leishmaniasis, it becomes necessary now to know individually and in co-infection condition the responses generated by *Leptomonas* in the host during infection and how that will affect the disease outcome.

CO-INFECTION OF *LEPTOMONAS* WITH *LEISHMANIA* IN LEISHMANIASIS

Co-infection with other pathogens in leishmaniasis is on the rise in India and other countries. This poses an additional challenge to control VL. *Leishmania* (VL/PKDL)-HIV co-infection is known (Shah *et al*. 2010; Diro *et al*. 2014; Singh, 2014). Several cases of *Leishmania* co-infection with malaria, chagas, schistosomiasis and tuberculosis were also reported (Griemberg *et al*. 2006; Rathnayake *et al*. 2010; Gil *et al*. 2011; Cota *et al*. 2012; van den Bogaart *et al*. 2013; Vega Benedetti *et al*. 2013). Domestic cats and a dog showing co-infection with *Leishmania* spp. and *Toxoplasma gondii* were reported (Braga *et al*. 2014; da Silva *et al*. 2015). Although *Leptomonas* has been thought to be inhabitant in insects, recent reports revealed

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unusual 7–17% cases of VL and PKDL with clinical isolates of *L. donovani* showing co-infection with *Leptomonas* in India (Srivastava *et al*. 2010; Ghosh *et al*. 2012). The first such cases of VL were the splenic aspirates that yielded *Leptomonas* in addition to *Leishmania* in Bihar and Uttar Pradesh, India. The co-infection was confirmed by the sequencing of its HSP70 and 18S rRNA genes (Srivastava *et al*. 2010). Further confirmation of such co-infection in the Indian leishmaniasis was observed from a report revealing the presence of *L. seymouri* in 4 out of 29 VL and 2 out of 7 PKDL cases by aberrant internal transcribed spacer 1 (ITS1) RFLP (Ghosh *et al*. 2012). Further a next generation sequencing of oligonucleotide ligation and detection (SOLID)™ platform recognized the same non-*Leishmania* Trypanosomatid parasite during sequencing of clinical isolates of VL in India (Singh *et al*. 2013). *Leptomonas* contamination in the clinical isolates of *Leishmania* from Nepal and Sri Lanka has also been observed recently. Sandfly population from distinct regions in Nepal harbouring *Leptomonas* like non-*Leishmania* parasites in their body has also been confirmed using rRNA gene targeted PCR (Bhattarai *et al*. 2009). Such reports along with the clinical incidents of co-infection in VL in the Indian subcontinent indicate that the sandfly population itself might be harbouring *Leptomonas*. To its support a very recent report suggests that, *L. seymouri* stayed with *L. donovani* for several days in the *Leishmania* transmission vectors (sandflies), *Phlebotomus* spp. (*Phlebotomus orientalis* and *Phlebotomus argentipes*) under experimental condition (Kraeva *et al*. 2015).

CLINICAL IMPLICATIONS OF CO-INFECTION

The occurrence of co-infection of *Leptomonas* in the VL/PKDL cases has been inferred only after the observation of fast replicating *Leishmania* look alike *Leptomonas* in the culture of some of the clinical isolates. Such cultures were confirmed to be mixed cultures of *Leishmania* (slow moving) and *Leptomonas* [fast moving (Ahuja *et al*. 2015)] initially and after a few subcultures contained only *Leptomonas*. This was confirmed by RFLP analysis of either HSP70 or ITS1 genes with distinct patterns in *Leptomonas* and *Leishmania* (Srivastava *et al*. 2010; Ghosh *et al*. 2012).

Since such co-infection came into light only in recent years, its implications in the VL or PKDL disease severity, delay in the potential diagnostics and thereby increase in mortality in VL, when compared with patients infected only with *L. donovani* are yet to be determined. Since most of the leishmaniacides also kill *Leptomonas* (Singh *et al*. 2013) appropriate common drug treatment that could eliminate both the parasites together need to be established. Sandflies carrying either of these two parasites have been reported (Bhattarai *et al*. 2009) but there is no report as yet of a sandfly vector carrying both the parasites simultaneously, but such a scenario is possible. Hence at the moment it is not clear whether the entry and habitation of *Leptomonas* in humans is primary or secondary to *Leishmania* infection or it is a simultaneous infection due to bite by sandflies infected with both the parasites. An exclusive secondary infection due to immune suppressed state of VL/PKDL patients cannot be ruled out due to the fact that as an opportunistic infection *Leptomonas* amastigotes have also been noticed in the *in vitro* culture from bone marrow aspirate of a HIV patient (Pacheco *et al*. 1998). This patient had VL, but *Leishmania* promastigotes were not detected in the cultures. It is also possible that *Leishmania* parasites were also indeed present initially in the *in vitro* culture from bone marrow, not noticed and subsequently got eliminated due to rapid growth of *Leptomonas*. Upon VL cure, presumed residual *L. donovani* parasites in the body causing PKDL in certain cases are known. The observed co-infection also in the PKDL cases leads to the question whether *Leptomonas* too coexisted as residual parasites in the body before participating in the PKDL. In other occasions presumed monoxenous non-*Leishmania* trypanosomatid was noticed in HIV patients (Dedet *et al*. 1995; Jimenez *et al*. 1996; Morio *et al*. 2008). Especially Dedet *et al*. (1995) revealed that such an apparent lower trypanosomatid member (*Leptomonas*?) isoenzymatically different from *Leishmania* also developed a diffuse cutaneous nodular syndrome in those HIV patients. These cases lead to speculation that those parasites were indeed *Leptomonas*, which can infect immune compromised humans independently without association with *Leishmania*. The observation of *Leishmania-Leptomonas* co-infection occurring so far only in the Indian subcontinent raises the question, whether it is due to new adaptation of *Leptomonas* into the sandflies that so far harboured only *Leishmania* in this region. In addition we do not know if there is an increase of VL disease outcome in case of such co-infection.

In conclusion recently discovered incidents of *Leptomonas* co-infection in VL in the Indian subcontinent are a cause of concern and require further investigation. Both *Leishmania* and *Leptomonas* are mostly similar in their structures and antigenicity. These two have also been found to be genetically very close to each other based on several phylogenetic analyses. The reason for the monoxenous (in single host) parasite *Leptomonas* in the insect invertebrates, shifting to dixenous (in two hosts) parasite like *Leishmania* living in both invertebrates (insects) and vertebrates (mammals), is yet to be understood. Is it because of *Leptomonas*'s shifting its extracellular habitat into

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another insect, sandfly vector which also feeds on mammals is a moot point. In addition, it is not clear whether *Leptomonas* is seen as co-infectant only in recent years or it also existed in the past and was never explored. The reason for *Leptomonas*'*s* inability to grow in the *in vitro* media at 37 °C and in the cultured macrophages, although showing growth in the spleen of VL and skin of PKDL patients is not clear. Moreover, whether such co-infection is also observed in other parts of the world, where VL is endemic; whether *Leptomonas* infection is primary, secondary or simultaneous infection with *Leishmania*; whether there is any sandfly population that harbours both *Leishmania* and *Leptomonas* together; whether *Leptomonas* is involved in leishmaniasis outcome or exasperating the disease along with *Leishmania*; whether *Leptomonas* has its own host immune modulation or shares the same with *Leishmania* are worth exploring. Such knowledge will help researchers and clinicians to develop appropriate therapeutic strategies for combined elimination of the co-infecting parasites.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

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