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Virulence Diversity of *Leishmania Tropica* Strains Isolated from Human Lesions in Syria

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Abstract: Virulence variability was investigated by analyzing the experimental pathogenicity of seven *Leishmania tropica* strains isolated from human lesions in Syria, through the evaluation of the infective ability, growth pattern and pathogenicity. Two of the strains presented higher levels of macrophage infection, increased promastigote replication in culture medium and as well as amastigote multiplication within macrophages were classified as the most virulent. The other strains exhibited either low virulence, with reduced infective capability and low replication levels, or an intermediate virulent phenotype showing mixed features similar to low and high virulent phenotypes. A correlation between the infectivity and growth dynamics of each strain and NO production was demonstrated. Strains with accentuated virulent phenotype induced lower levels of NO production and parasite killing. These results suggest that *L. tropica* isolates from human lesions may differ in Virulence phenotype which seems to be a characteristic of each strain.
Keywords : *L. tropica*; virulence; macrophages; NO production.

Introduction:

Parasites from the Leishmania genus are trypanosomatid protozoans responsible for a group of diseases with a broad range of clinical manifestations collectively known as leishmaniasis. *Leishmania tropica* parasites are one of the causative agents of the Old World zoonotic cutaneous leishmaniasis (ZCL)[1-3]. ZCL is endemic in North Africa, Central Asia and Middle East, including Syria and is a major health problem in different parts of the country. Evidence shows that polymorphism is present in human ZCL which may express various clinical patterns, ranging from asymptomatic infection featuring a conversion of the leishmanin skin test without apparent lesion to benign self-limited cutaneous sore(s) or to more protracted and extensive lesion(s), which may cause severe disfiguring. This clinical polymorphism, which may be observed even within small endemic foci, may reflect either variability in the host immune response and/or variability in the parasite virulence[4].

In experimental models, the outcome of the disease correlates with genetic background of the host and their immunological condition. Most inbred mice (i.e., C57BL/6 mice) show ability to control the disease. In contrast, BALB/c mice are susceptible to the parasites and sub-cutaneous inoculation of these mice with metacyclic promastigote results in uncontrolled infection[5,6]. Several studies have addressed the important role of $CD4^+$ T-cell subsets in immunity against Leishmania. The resistance is developed by T-helper type-1 (Th1) cells producing IFN-γ which is induced via secretion of IL-12 by dendritic cells, while the susceptibility is conferred by Th2 cells producing IL-4, IL-5 and IL-10[7,8]. It has been shown that the production of IFN-γ activates macrophages to kill the intracellular amastigotes via the production of nitric oxide (NO) and other reactive nitrogen intermediates. In contrast, Th2 immune response limits the action of Th1 functions via induction of IL-4 and IL-10 which results in deactivation of macrophages and growth of intracellular parasites, exacerbating the disease progression[8-12].

Evidence shows that different strains of Leishmania species elicit distinct levels of pathogenicity and various patterns of the immune responses. Data obtained from different studies using genotypically distinct strains of *L. major*[13], *L. infantum*[14], *L. braziliensis*[15] and *L. amazonensis*[16], have shown different levels of susceptibility to infection along with distinct patterns in immune responses in inoculated BALB/c mice.

In this study, virulence of seven *L. tropica* isolates was evaluated in *in vitro* experiment using human monocytes-derived macrophages. We demonstrate strain differences in virulence correlating with differences in *in vitro* parasite growth and in parasite burden as well as in induction of NO the principal effector in killing intracellular Amastigotes which may reflect virulence variations between these wild strains.

Materials and Methods:

Parasites Culture

Seven strains of *L. tropica* isolated from skin lesions of seven ZCL patients were used for this study. Parasites were cultured in semisolid culture medium (Agar, NaCl) containing penicillin/streptomycin 100 U/ml (Cytogen, Germany), And then were grown in RPMI-1640 medium (Sigma, USA) supplemented with L-glutamine (Sigma, Germany), penicillin/streptomycin 100 U/ml, and 10 % heat-inactivated fetal bovine serum (FBS, Cytogen, Germany) with less than five passages. All these isolates were previously identified as *L. tropica* by Nested-PCR. Infective metacyclic forms were selected from stationary phase (5th day) and used for macrophage infection.

Mononuclear Cells Isolation and Macrophage Differentiation

Buffy coat were obtained at the Blood Center in Damascus from healthy volunteer donors without previous leishmaniasis using the classical method with some adaptations. Briefly, Buffy coat was diluted 1:4 with 0.15 M NaCl, and separated on a Ficoll Hypaque gradient (1.077 g/ml; GE Healthcare). Mononuclear cells were collected from the interface between the plasma and the Ficoll, washed once with Phosphate buffered saline (PBS) without Ca^{+2} and Mg^{+2} (Sigma, Germany). The red blood cells were treated with lysis buffer (0.15 M NH_4Cl, 0.1 M $KHCO_3$, pH=7.2-7.4) and then washed thrice with PBS. The monocytes were collected by centrifugation and were cultured in a 12-well plate (Cellstar, Greiner, Germany) at a concentration of 3×10^6 cell/well in 2 ml IMDM culture medium (Sigma, Germany) containing 2 % heat-inactivated human AB serum (Sigma, Germany), penicillin/streptomycin 10 U/ml (Cytogen, Germany) and maintained at 37 °C in 5% CO2–95% air. After 2 hours of incubation, the non-adherent lymphocytes were removed by rinsing with warm culture medium and the resultant adherent monocytes were allowed to differentiate to monocyte-derived macrophages over six days in IMDM culture medium containing 10% heat-inactivated human AB serum at 37 °C in 5 % CO_2.

Infection of Macrophages

Macrophages were stimulated with 5 ng/mL recombinant human interferon gamma (rHu-IFN-γ) (Sigma). After two days, macrophages were infected with metacyclic promastigotes from each strain by removing the medium and replenishing with 0.5 ml of complete IMDM medium containing stationary phase (5th day) promastigotes at a final ratio of five parasites per macrophage. The cultures were incubated at 37°C in 5 % CO_2–95% air for 2 h. After this time, cells were extensively washed with PBS twice to eliminate non-phagocytosed parasites and IMDM culture medium containing 10 % heat-inactivated human AB serum and antibiotics was added. The percentage of infected cells (i.e. number of infected cells per 100 macrophages) and the intensity of infection (i.e. number of parasites per macrophages) were monitored by light microscopy under oil (×1000) by counting 300 cells per well, after fixing and staining with Giemsa at different time points: 2, 24 and 48 h post-infection. Production of NO was also quantified from supernatants at 48 h by Griess reagent kit (Oxford Biomedical, USA) according to the protocols supplied by the manufacturer. Every experiment was repeated three times.

Statistical Analysis

All the experiments were repeated at least three times on different occasions. The results are presented as means ± standard deviations (SD). To compare statistical differences between means two-sided t test or one-way ANOVA followed by Dunnett's multiple comparison test were run when comparing 2 or more groups, respectively, unless otherwise stated. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results:

In Vitro Human Monocytes-Derived Macrophages Infection with *L. Tropica* Promastigotes

Infection of macrophages derived from human monocytes was used to test for differences in the rate of virulence of the seven *L. tropica* isolates selected. Two hours after addition of the parasites, cells were washed to remove unbound promastigotes, and the rate of macrophage infection was measured at 2, 24 and 48 h of culture. Results are expressed as the percentage of infected macrophages (Fig. 1 A) and the mean number of amastigotes detectable within one infected macrophage (Fig. 1 B). Generally, the levels of macrophage infection reached a peak at 48 h post infection. Syr-9 and Syr-14 strains presented higher infectivity (about 95%). At 48 h Syr-8, Syr-10 and Syr-11 strains showed lower percentages of infected macrophages (70%, 72% and 75% respectively), and Syr-12 and Syr-13 presented the lowest levels of infection (about 40 %) (Fig. 1 A). Higher levels of parasitized macrophages were obtained at 48 h post infection by all strains, being Syr-9 and Syr-14, the most aggressive strain with an average of 23 parasite/macrophage, followed by Syr-8, Syr-10 and Syr-11 with 16, 14.5 and 15.8 parasite/macrophage respectively. On the contrary, strains Syr-12 (8.5 parasite/macrophage) and Syr-13 (7.7 parasite/macrophage) presented reduced number of intracellular amastigotes (Fig. 1 B). Thus, a large heterogeneity in the percentages of macrophages infected with different strains as well as in the parasite burden was observed. Some strains (Syr-12 and Syr-13) infected a small percentage of macrophages with a low number of intracellular amastigotes. Other strains showed great potential to infect higher percentage of macrophages with a high parasite burden levels.

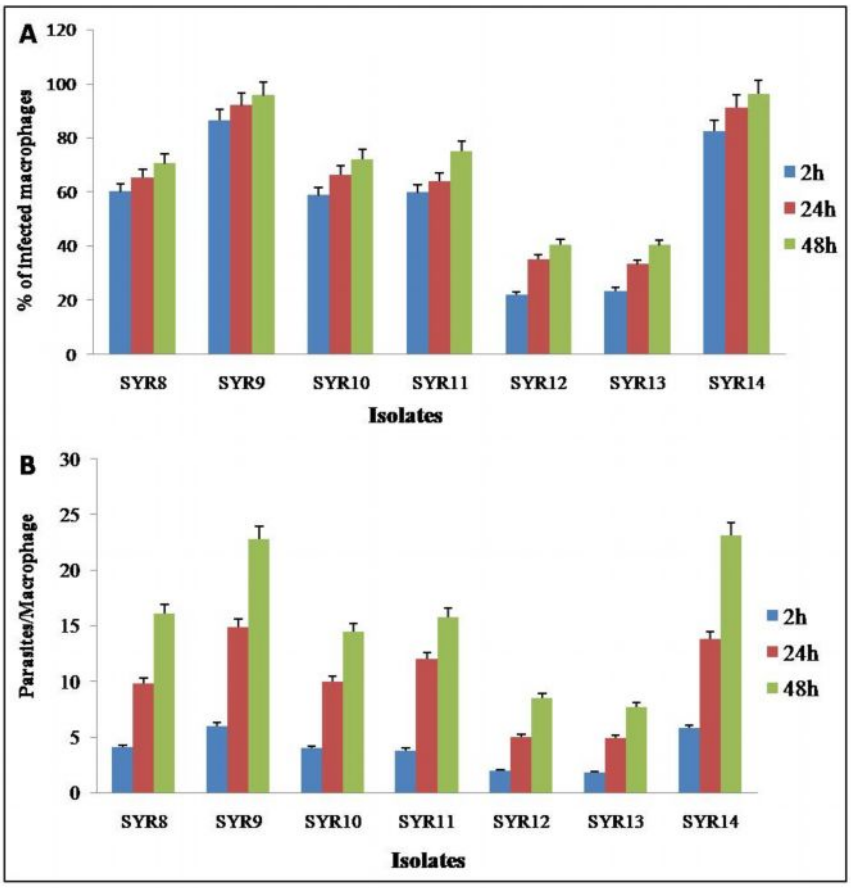

Figure (1). *In vitro* differential infectivity of *L. tropica* isolates. Macrophages were infected with promastigotes of each isolate in a ratio of 5:1 (parasites: macrophage). At the indicated time, the fraction of infected macrophages (A) and the mean number of intracellular parasites per macrophage (B) were determined. Results are reported as the mean ± the standard deviation of three different experiences.

Promastigote Growth Rates in Vitro

Since changes in the kinetics of in vitro parasite growth are classically associated with changes in parasite virulence, *L. tropica* isolates displaying different levels of virulence in the experimental macrophages infection were used for *in vitro* growth experiments: (i) two high virulence isolates (Syr-9 and Syr-14); (ii) three isolates with intermediate virulence (Syr-8, Syr-10 and Syr-11); and (iii) two low virulence isolates (Syr-12 and

Syr-13). Promastigote growth rates were measured over 7 days of culture in liquid medium. Starting with an inoculum of 2×10^6 promastigotes/ml obtained after first passage, different growth curves were obtained (Fig. 2). Interestingly, the strains highly pathogenic in *in vitro* infection, Syr-9 and Syr-14, exhibited the most rapid growth, reaching a peak density of ~32×10^6 promastigotes/ml on day 5 of culture. In contrast, the less virulent isolates, Syr-12 and Syr-13, grew very slowly and reached a maximum cell density of almost 14×10^6 promastigotes/ml on day 6. The Syr-8, Syr-10 and Syr-11, which had an intermediate pathogenicity, also showed a growth rate which was intermediate between those expressed by the high or the low virulence strains (Fig. 2). The growth characteristics of these strains were reproducible over two different experiments.

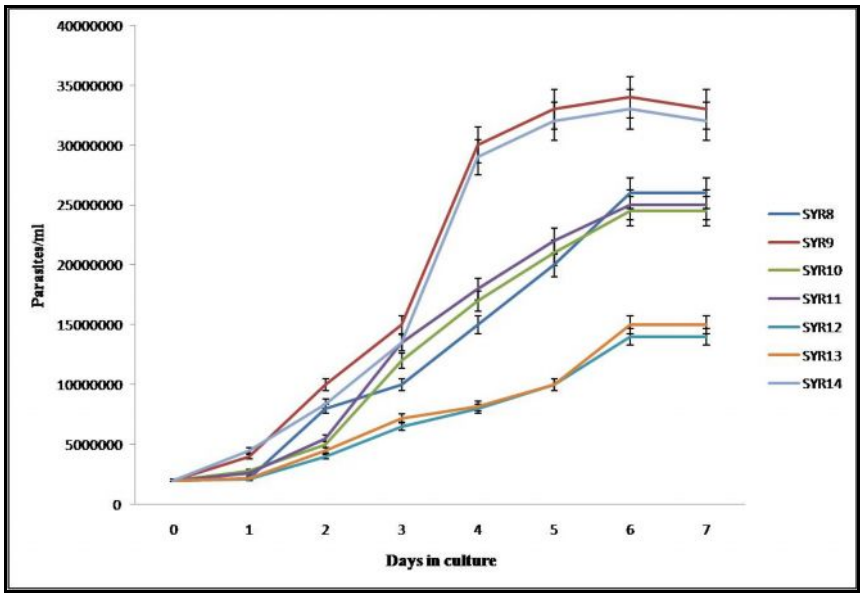

Figure (2). Growth kinetics of seven *L. major* isolates: Syr-9 and Syr-14 (high virulence), Syr-8, Syr-10 and Syr-11 (intermediate virulence), and Syr-12 and Syr-13 (low virulence). Cultures were initiated in liquid medium at 2×10^6 Promastigotes/ml. Results are reported as the mean ± the standard deviation of two samples derived from three separate experiments.

Evaluation of no Production in Macrophages Infected with Different *L. Tropica* Isolates

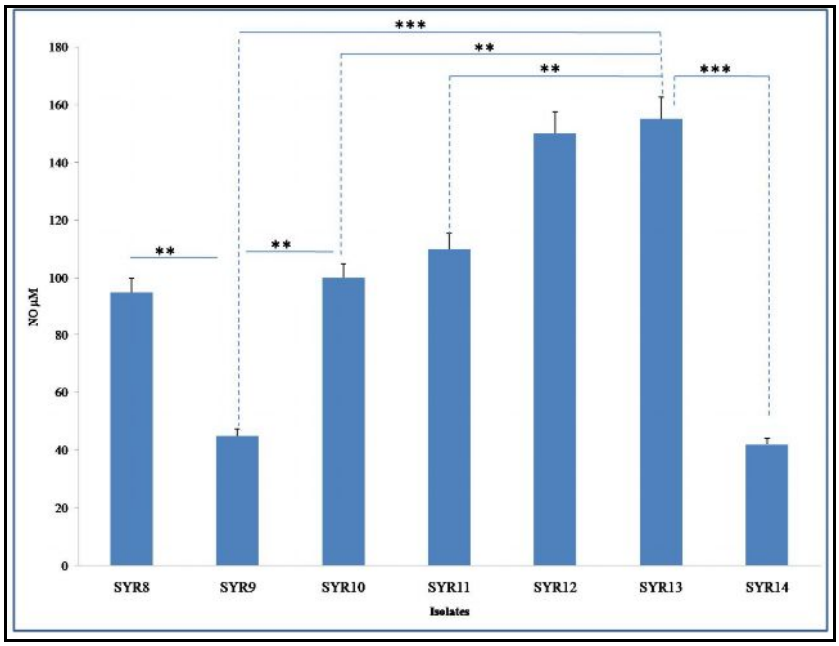

Figure (3). NO production by infected macrophages with *L. tropica* isolates. Cells were primed with rHu-IFN-γ and infected with metacyclic promastigotes from each strain. The culture mediums collected after 48 h were assayed by Griess kit for NO. Results are reported as the mean ± the standard deviation of three different experiences.

NO production was comparatively analyzed for IFN-γ primed macrophages infected with different *L. tropica* isolates. Experiments were performed 48 h after infection using a Griess reagent kit. Differences in NO (Fig. 3) levels were found. Interestingly, the most virulent strains, Syr-9 and Syr-14, induced the lowest NO level compared with the other isolates. In contrast, Syr-12 and Syr-13 isolate induced the highest level of NO production compared with the other isolates. The Syr-8, Syr-10 and Syr-11, which had an intermediate virulence, also showed a NO production which was intermediate between those expressed by Syr-9 and Syr-14 or Syr-12 and Syr-13 strains.

Discussion:

The distinct clinical outcomes evidenced by Leishmania sp. infections are dependent of the interactions established between the parasite and the host, reflecting parasite virulence, genetic background of the host and their immunological condition. The present study has tried to analyze the variability in the natural virulence of *L. tropica* strains isolated from human lesions. The pathogenicity in macrophage infection was compared and studied some biologic properties of seven *L. tropica* isolates collected from patients in Syria.

Our results show that the seven strains expressed a significant variability in the pathogenicity induced in macrophages. More importantly, these differences could be reproduced in independent experiments and could be demonstrated not only with promastigotes but also with amastigotes (data not shown). The latter result rules out the possibility that the detected differences might merely reflect variable levels of *in vitro* metacyclogenesis reached by the different strains. Some isolates were able to induce high level of infection. However, several isolates induced only very small rate of infection. Our finding is in agreement with others findings wherein pathogenic heterogeneity within different *L. major*, *L. infantum*, *L. braziliensis* and *L. amazonensis*, isolates was also observed[13-16].

Our data allowed us to classify the seven strains into three virulence levels: high (Syr-9 and Syr-14), intermediate (Syr-8, Syr-10 and Syr-11), or low (Syr-12 and Syr-13).

Similarly, the kinetics of growth in liquid medium of the seven strains was strikingly different. The higher the virulence, the faster the in vitro growth. Thus, the parasite density reached by strains Syr-9 and Syr-14 after 3 days of culture was higher than that reached by the least virulent strains (Syr-12 and Syr-13) after 6 days of culture. The strains (Syr-8, Syr-10 and Syr-11) with intermediate virulence showed also an intermediate kinetics of growth. Moreover, the plateau phase reached significantly higher levels with the virulent strain compared to the two others. Furthermore, higher parasite infectivity seems to be associated to faster growth levels of promastigotes in culture and a more efficient macrophage infection. Several studies also demonstrated that more pathogenic *L. major* strains and *L. infantum* MON-1 strains presented rapid growth in culture medium [13, 14].

Our results also show that isolates with higher virulence tend to generate less NO than low-virulence strains when macrophages are stimulated with INF-γ and infected with leishmania promastigotes. These data suggest that the former strains tend to induce a stronger TH2 response than the latter. Similar results were previously reported with *Leishmania* clones isolated *in vitro* and *in vivo*[14-17].

Former study has tried to analyze the variability in the natural virulence of 19 *L. major* strains isolated in the field from human lesions, the reservoir, or the vector. They showed that the 19 strains expressed a significant variability in the pathogenicity induced in BALB/c mice. Interestingly, the high-virulence strain was characterized by a significantly higher ability to infect murine bone marrow macrophages, especially during the first 2 h after infection, and to survive at higher density within the infected macrophages. Moreover, strains with the highest virulence tend to generate more IL-4 and less IFN-γ in vitro at week 5 post-infection as well as higher levels of early IL-4 mRNA in the lymph node draining the inoculation site at 16 h post-infection[13].

Another study has evaluated the variability of virulence in *L. infantum* MON-1 strains through the assessment of their in vitro growth and infectivity and host immunopathogenicity. Three of the six strains evaluated exhibited a better adaptation to culture medium, with higher replication levels and with an increased number of promastigotes. IMT 151 and IMT 311 strains caused infection in macrophages during the first 24 h, being able to replicate within the cells and reach higher amastigote densities at 48 h after in vitro infection[14].

Our data present further evidence for the fact that *L. tropica* strains can have intrinsic differences in their ability to induce infection at least during their initial interactions with the phagocytes.

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