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Assessment of the infectivity potential of *Leishmania infantum*, using flow cytometry

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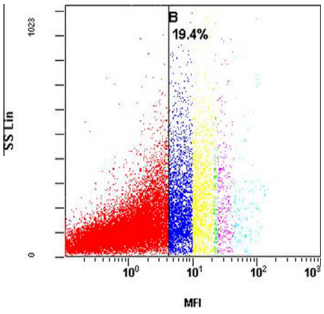
HIGHLIGHTS

- A method is proposed to estimate *Leishmania* amastigote infectivity potential.
- The *in vitro* method is easy, fast and inexpensive.
- It estimates the number of parasites per THP-1 infected cell.
- A "standard reference curve" was developed by flow cytometry.

GRAPHICAL ABSTRACT



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ABSTRACT

The protozoan parasite *Leishmania infantum* causes leishmaniases, a sandfly-borne disease of humans and dogs, in all countries of the Mediterranean basin. The promastigote, infective stage of the parasite, once inoculated to the mammalian host by the vector, is ingested by macrophages. *Leishmania* lives within the lysosome of the phagocytic immune cells inactivating the enzymes contained. The ability of an isolate to survive within the macrophage and its rate of multiplication in this environment is an important factor determining the infectivity potential of the isolate and the manifestation of the disease. This capacity of the parasite is measured as the percentage of infected cells and the mean value of parasites per cell. The infectivity potential, of clinical isolates of *L. infantum* infecting THP-1 cells *in vitro*, was studied by flow cytometry and light microscopy. The percentages of cells in a sample containing a specific number of parasites, as recorded by light microscopy, were used in flow cytometry to manually gate the mean fluorescence intensity which corresponded to the percentage of cells with that number of parasites. The gating obtained, was then used as a "standard reference curve" to evaluate results by flow cytometry compared to those obtained by light microscopy. The results, of the overall percentage of infected cells and the number of parasites per cell in the culture, matched in the two methods. So, flow cytometry can be used as a rapid, cost effective, easy and reproducible method to study the infectivity potential of isolates, either in biological, epidemiological, or clinical tests, particularly for the assessment of drug efficiency trials.

1. Introduction

Leishmania spp. (family Trypanosomatidae), protozoan parasites responsible for the leishmaniases, constitute a major public health problem in 98 countries (Alvar et al., 2012). It is a dimorphic

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organism alternating between the promastigote and the amastigote stage. The promastigote lives extracellularly in its vector, the phlebotomine sandfly (Diptera, Psychodidae), and the amastigote resides within the phagolysosome of mononuclear macrophages of mammalian hosts (Dedet et al., 1999). *Leishmania* phagocytosis is a multi-step phenomenon and has been studied in detail in promastigotes of various species (Chang et al., 1990; Brittingham et al., 1999; Handman, 1999). The onset of disease depends chiefly on the successful inoculation of promastigotes to the host by the vector, their ability to infect macrophages and survive the phagocytic processes (Paul, 1993) but also on the immunological profile of the host. Inside the macrophages, promastigotes transform into amastigotes and start their replication. Therein, the amastigotes produced are responsible for maintaining and disseminating the infection. The proliferation potential of a strain, together with host factors, is therefore important for the outcome of the disease.

L. infantum causes visceral (VL), cutaneous (CL) and canine (CanL) leishmaniases in the Mediterranean basin where it is endemic (WHO, 2010). This parasite species is an opportunistic parasite and the importance of VL (which can be lethal if not treated), is not fully appreciated, since asymptomatic infections occur in the 2–36% of the human population in endemic areas (Kyriakou et al., 2003; Michel et al., 2011). However, if asymptomatic people become infected by the human immunodeficiency virus (HIV), they will develop the disease and, in addition, may be able to transmit the parasite to sand flies, thus changing the epidemiology of leishmaniases (Alvar et al., 2008). Also, it has been shown that other forms of immunosuppression, like organ transplantation, psychological stress and malnutrition may allow this opportunistic parasite to cause disease (Christodoulou et al., 2012).

The population growth rate of *Leishmania* in the mammalian host is different in each strain and depends on the host immune system and on the isolate's capability. The infectivity potential of an isolate should be given, not only as the percentage of infected cells but also as the mean value of parasites produced per infected cell. Since the proliferation capacity of a strain inside the host plays a key role in its infectivity potential, our ability to quantify an isolate's replication rate, *in vitro*, is useful in comparing strain characteristics, in competition studies associated with co-infections and most importantly, in assays for testing drug efficacy at a time that *Leishmania* drug resistance is becoming a growing concern.

In laboratory studies of *Leishmania*, the THP-1 cell line, human acute monocytic leukemia cells, is considered a good macrophage-amastigote model (Ogunkolade et al., 1990). The post infection status of these cells can easily be determined using light microscopy (LM) after Giemsa staining (Gebre-Hiwot et al., 1992). On the other hand, flow cytometry (FC) is a reliable tool for scoring cells, infected and non-infected with *Leishmania*, after immunofluorescence staining (Abdullah et al., 1998). With FC, the analysis of multiple surface and intracellular markers at the level of a single cell can be achieved thus, the identification of heterogenous components in a cell population can be analyzed in a big number of cells, and samples, rapidly and credibly. If only one parameter needs to be visualized, univariate histograms can be used for the display of flow cytometric data. The pattern of expression of the marker can be discriminated and quantitatively expressed as the frequency/percentage of cells possessing the same characteristics and fluorescence intensity for that marker. Most software packages provide tools for the overlay of histograms/gates from multiple samples, allowing a rapid comparison of the parameter of interest (Lugli et al., 2009).

Di Giorgio et al. (2000) used FC to quantify *Leishmania* infection in human monocyte-derived macrophages as well as nonadherent mouse peritoneal macrophages after labeling the intracellular amastigotes with monoclonal antibodies. They separated amastigote – containing cells from noninfected cells, a method used previously by Abdullah et al. (1998) and Guinet et al. (2000). Nevertheless, in these studies, the quantification of *Leishmania* infection in cultured cells was assessed as the overall percentage of infected cells in a sample.

The aim of this work was to test if FC can be used to evaluate the infectivity potential of *L. infantum* amastigotes, in THP-1 human macrophages, *in vitro*; that is, in one assay to measure the percentage of infected cells and the mean number of amastigotes per cell in the sample. If a "standard reference curve" could be developed, by relating the mean fluorescence intensity of the cells to the number of parasites per cell obtained by LM, this could be applied to determine the number of parasites in infected macrophages.

2. Materials and methods

2.1. Parasites

Three *L. infantum* strains isolated from infected dogs during an epidemiological survey conducted in Greece (Ntais et al., 2013), were used in the experiments (codes GD1, GD2 and GD3). They had been kept at −80 °C after a maximum of three passages. Freshly thawed parasites were maintained in supplemented RPMI 1640 (Gibco–BRL, UK) culture medium at 26 ± 1 °C and the promastigotes' condition was checked every 2 days (Lemesre et al., 1988; Kamau et al., 2000).

2.2. THP-1 cell line: culture and infection

Freshly thawed cell cultures of the human monocytic cell line, THP-1 (Sigma–Aldrich, Inc., St Louis, MO, USA) were maintained in supplemented RPMI 1640 culture medium at 37 °C, 5% CO_2 and 80% humidity (Lightner et al., 1983; Lemesre et al., 1988). They were infected with exponential phase *L. infantum* promastigotes of each of the three isolates at a ratio of 5 parasites to 1 host cell, in triplicates. They were then incubated overnight at 37 °C, 5% CO_2 and 80% humidity. The cultures were used for infection if dead cells were less than 5%, using the trypan blue exclusion assay (Warren, 1997).

2.3. Light microscopy

Following overnight incubation, cytospin preparations were made using 100 μl from each infected cell culture, in triplicates. The slides were left to dry at room temperature before staining with Diff-Quick (Polysciences Europe GmbH, Eppelheim, Germany). The percentage of infected macrophages was determined microscopically at 1000× magnification, performing three counts of 100 cells for each cytospin preparation to obtain the average. The infected THP-1 cells were assessed for: overall percentage of infected cells, the percentage of cells infected with 0-1-2-3-4-5-6-7+ amastigotes, and the mean number of parasites per cell, for each sample, was estimated.

2.4. Immunofluorescence staining for FC

Infected THP-1 cells were harvested, after centrifugation at 1200 rpm for 5 min and resuspended in 1 ml of acetone, for 10 min. After washing with 1 ml PBS, 7.2 pH, they were incubated at 37 °C for 30 min. Following this, 20 μl of positive, against *Leishmania*, dog serum (IFAT titer 1/1600) were added. The cells were then washed with 1 ml PBS, 7.2 pH and 10 μl anti-dog IgG [FITC labeled rabbit anti-dog IgG antibodies (Sigma–Aldrich Inc., St Louis, MO, USA) (dilution 1:200)] and 50 μl Blue Evans were added. The cells were incubated at 37 °C, in the dark, for 30 min, washed twice