Short Report: Quantiferon-*Leishmania* as an Epidemiological Tool for Evaluating the Exposure to *Leishmania* Infection

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Abstract. The aim of the present preliminary study was to investigate the potential of measurement of IFN- γ secretion by T cells into blood plasma using QuantiFERON assay with leishmanial antigens to determine the presence of *Leishmania* infection. Blood samples from cured visceral ($N = 18$), and cutaneous ($N = 20$) leishmaniasis cases, and 20 healthy controls were tested. The IFN- γ responses to *Leishmania major* H2B and *Leishmania infantum* H2B antigens were detected from the majority of treated old visceral leishmaniasis cases, but not from controls. Future studies using larger groups will be required to establish the true potential of the assay for epidemiological screening of leishmaniasis.

The leishmanin skin test (LST) measures delayed-type hypersensitivity to *Leishmania* and is frequently used for clinical diagnosis, and as an epidemiologic tool on the prevalence of exposure to *Leishmania* parasites to characterize populations in endemic areas.^{1–3} Injection of parasite antigen can have side effects and cause social problems in different parts of the world. Other problems with the LST include scarcity of good manufacturing practice grade antigen and the cumbersome reading procedure.⁴ Thus, there is a need for a simple and rapid tool, such as an *in vitro* T-lymphocyte assay based on *Leishmania* antigen, to facilitate a better understanding of the epidemiological situation in leishmaniasis endemic areas.

The *in vitro* measurement of cell-mediated immune responses to specific antigens can be an alternative method suitable for epidemiological studies where detection of infections by intracellular pathogens, such as *Leishmania* spp., is required. QuantiFERON-TB Gold In-Tube, approved by the United States Food and Drug Administration (USFDA), is a diagnostic test system for tuberculosis using enzyme-linked immunosorbent assay (ELISA) to measure the amount of IFN- γ produced after overnight incubation of whole blood cells with specific mycobacterial antigens.⁵ Thus, the aim of this preliminary study was to investigate the potential of a similar QuantiFERON (QFN) assay using leishmanial antigens and whole blood from people with a history of cured cutaneous or visceral leishmaniasis to monitor previous exposure to *Leishmania* infection. The new non-invasive diagnostic tool measures IFN- γ secretion by T cells in response to parasite antigen.

Peripheral whole blood samples were collected from 38 previously diagnosed and successfully treated leishmaniasis patients (18 visceral leishmaniasis [VL] cases caused by *Leishmania infantum* MON-1 and 20 cutaneous leishmaniasis [CL] cases caused by *Leishmania tropica*, healed with scarring). Twenty healthy controls from non-endemic areas were also included in the study. All patients and healthy volunteers gave their written informed consents to participate in this study. Initial screening of 16 potential antigens identified two candidate molecules (Histone H2B and PSA-2) that produced requisite sensitivity and specificity. There is significant amino acid sequence homology among *Leishmania* spp. for both these proteins. The leishmanial antigen panel used for QFN test-

ing was as follows: *Leishmania major* H2B, *L. infantum* H2B, *L. major* PSA-2, *L. infantum* PSA-2, and a mixture of all antigens. Each antigen consisted of a pool of 10 peptides (22–28mer) covering the amino acid sequence of H2B or predicted CD4 T cell epitopes of PSA-2. Whole *L. infantum* lysate, sterile phosphate-buffered saline (PBS) (no-antigen), and phytohemagglutinin (PHA) (positive mitogen) were also used as controls. Blood samples (9–10 mL) from patients and healthy controls were collected in tubes containing heparin and were kept at 17–27°C during transportation. One mL aliquots of mixed heparinized whole blood were incubated in plasma separating gel filled tubes with 100 μ L leishmanial antigens, sterile PBS or PHA. After thoroughly mixing, tubes were placed upright in a 37°C incubator for 20 hrs without humidity and CO₂. After incubation and centrifugation of tubes at 3,000 g for 15 min, individual plasma samples were harvested from above the gel and stored at 2–8°C for up to 8 weeks before ELISA measurement of IFN- γ . The QFN-ELISA assay was performed using CMI kit (Cellestis Ltd., Vic, Australia) with a 4-point standard curve of known amounts of IFN- γ in international units per mL (IU/mL) to determine the amount of IFN- γ produced in response to leishmanial antigens. Results were calculated and interpreted according to the manufacturer's instructions. Calculations were performed using software provided by the kit manufacturer (Analysis Software v1.51, Cellestis Ltd.). Receiver operating characteristic (ROC) analysis, using 50 blood samples from volunteers in non-endemic areas (Turkey and Australia) and 18 cured VL cases from Turkey, was performed to determine the cut-off for the QFN assay using the H2B (*L. infantum*) peptides. Because the 0.2 IU/mL cut-off was shown to give the highest specificity and sensitivity, volunteers with IFN- γ levels greater than 0.2 IU/mL were deemed positive.

Subjects with a previous history of VL and CL were mainly recruited from western and southeastern Turkey, respectively. The period of time between the original patient diagnosis and cure, and execution of the QFN assay, varied from 3 to 17 years for the VL cases, and from 3 to 11 years for the CL cases. Fourteen CL cases out of 20 had single lesions, whereas six cases had more than one lesion. The IFN- γ responses and reactivities to leishmanial antigens are shown in Figure 1 and Table 1. The sensitivity and specificity of QFN using H2B antigens for VL cases were 83% and 100%, respectively, and were superior to PSA-2. The QFN using H2B antigens detected only 40% of CL patients. Interestingly, leishmanial lysate failed to generate IFN- γ responses in CL patients. The IFN- γ response

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FIGURE 1. IFN- γ responses to leishmanial antigens used in the study (Mean $\pm$ SD). VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; Control, healthy volunteers coming from non-endemic areas.

to mitogen (PHA) was strong in all samples from all participants. The mean IFN productions to mitogen (PHA) were strong as 60.58, 66.13 and 26.40 IU/mL in samples from old CL, VL cases and controls, respectively.

The leishmanin skin test has been widely used as an epidemiologic tool, but using not good manufacturing practice (GMP) products in most parts of the endemic areas as an antigen, influence of subjectivity on reaction size measurement, need to return in 3 days to read the result, and the unwillingness of individuals to accept the test limited the use of LST. The *Quantiferon-TB* test was developed to overcome some of the limitations of the tuberculin skin test (TST) for tuberculosis.⁵⁻⁸ Although TST and LST both measure delayed-type hypersensitivity on the forearm, QFN actually measures *in vitro* antigen-specific IFN- γ production. The IFN- γ is one of the key cytokines involved in the cell-mediated immune response against both pathogens.

Thus, a QFN test for leishmaniasis could provide a rapid and convenient alternative test to measure specific T-cell-mediated immune responses and IFN- γ production of people living in endemic areas to *Leishmania*. Whole blood incubated for 16–24 hrs with parasite antigens should cause IFN- γ secretion from previously primed memory T cells in people previously exposed to the leishmaniasis, but not from naive T cells of healthy volunteers. This study shows that use of *L. major* or *L. infantum* H2B as antigens stimulated the highest IFN- γ production from subjects with a previous history of VL, whereas people who had CL caused by *L. tropica* in the past produced less IFN- γ in a response to these antigens. The reason for the cells from old CL cases not responding to lysate might be caused by either movement of these cases out of the endemic region and not being exposed to parasite, losing memory

T cells or isolated lesion sites, such as mostly on the face and low parasite burden. It is possible that these antigens may induce T-cell proliferation, which was not preferred to be included in this study because of being a less friendly procedure, but they may not cause IFN- γ production.

The combination of all four antigens yielded maximal responses and a number of positives. The H2B molecules were superior to lysate for use as an epidemiological screening marker in leishmaniasis endemic/sporadic areas. The H2B antigens did not produce IFN- γ responses from healthy volunteers. Even though studies have shown lifelong T cell responses to leishmanial antigens measured with *in vitro* tests, others have pointed to the possibility of loss of positivity with time.^{2,9} Two of the three VL cases with negative H2B IFN- γ responses had disease in 1992 and 1997, respectively, and hence their response may have waned with time.

This new test can be used for epidemiological studies as an alternative to the LST presently unavailable as a GMP product in most endemic regions, including Europe. Because of increased IL-10 secretion during the acute phase VL might inhibit IFN- γ production; we are not expecting this test to be useful in diagnosing this disease, though additional studies are needed. For people with no history of symptomatic VL, high IFN- γ production results using QFN may be presumed to indicate prior asymptomatic leishmaniasis infection or exposed to *Leishmania* parasite without developing infection. In such asymptomatic individuals with leishmaniasis, the infection might even reactivate and cause active disease if such people become immunosuppressed later in life. Even though commercial *in vitro* tests are unlikely to be affordable in most markets, if IFN- γ production in QFN assay could give the information about cumulative leishmanial exposure experienced by the community, who remained negative, it might indicate the susceptible segment of the population, which could be suitable candidates for the future vaccine trials or prophylactic procedures for those in endemic areas. This work is a preliminary study aimed to examine the potential usefulness of the technique for *Leishmania* screening and more work needs to be carried out to explain the dynamic kinetics related to different clinical forms of leishmaniasis.

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TABLE 1
Proportion of volunteers positive to leishmanial antigens by QuantiFERON assay

Antigen	VL*	CL	Control
<i>L. major</i> PSA2	4/18	4/20	1/20
<i>L. infantum</i> PSA2	6/18	8/20	3/20
<i>L. major</i> H2B	15/18	8/20	0/20
<i>L. infantum</i> H2B	14/18	5/20	0/20
All	15/18	10/20	3/20
Lysate	11/18	0/20	0/20

*VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; Control, healthy volunteers coming from non-endemic areas.

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