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New strategies and biomarkers for the control of visceral leishmaniasis

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Abstract

Effective diagnosis and treatment of visceral leishmaniasis, together with the study of vectors and reservoirs, can lead to a better understanding of the parasite transmission dynamics and the development of more efficient control measures. Recent studies have applied new methodologies and biomarkers, and these have contributed to: the early and rapid diagnosis of the disease; assessment of success of pharmacological treatments; efficient monitoring of immunosuppressed individuals, and to population screening for field trials of vaccine efficacy. This opinion article proposes an update to the diagnostic tools for visceral leishmaniasis and their rational and combined use to establish the real prevalence of infection or of exposure to *Leishmania* in endemic areas.

29 **Unveiling the complexity of visceral leishmaniasis**

30 Leishmaniasis is a vector borne infectious disease caused by parasites of the genus
31 *Leishmania*. Globally distributed, it is a poverty-related and among the deadliest of the
32 neglected tropical diseases (NTDs). Visceral leishmaniasis (VL) caused by *L. donovani*
33 and *L. infantum* is the most severe clinical form. It affects internal organs and is fatal in
34 95% of cases if not successfully treated. On some occasions, after an episode of visceral
35 leishmaniasis caused by *L. donovani*, the patient may develop a post-kala-azar dermal
36 leishmaniasis (PKDL). So far, little is known about the mechanism by which a patient
37 with VL develops PKDL [1].

38 The overall incidence of VL has declined in recent years, mainly because of the
39 elimination efforts carried out in South Asia [2]. However, the incidence of VL has
40 increased alarmingly in the Americas, where the recent report by Pan American Health
41 Organization (PAHO) and World Health Organization (WHO) indicates that VL is
42 expanding geographically: the number of cases has increased by 26.4%, while the fatality
43 rate and number of deaths have grown progressively since 2014 [3]. In addition, epidemic
44 outbreaks have appeared in Europe, the Indian subcontinent, and Eastern Africa [4-7].

45 The **transmission dynamics** (see Glossary) of *Leishmania* are complex and variable;
46 dependent on environmental conditions, the distribution and biology of the vector, the
47 **reservoirs** involved, and on the health, social and economic aspects that affect the human
48 host [2]. In the absence of an effective vaccine, control of VL has been based on the
49 prevention of **sand fly** bites, the elimination of animal reservoirs (if the VL is zoonotic),
50 and the early detection and effective treatment of human cases [8]. Nevertheless, in
51 regions endemic for VL, most infected individuals remain **asymptomatic**. Their possible
52 role as "**parasite carriers**" with capacity to infect sand flies has been suggested and is
53 under active consideration [9]. Individuals who suffered previous VL infections for which
54 treatment was not fully effective can also remain asymptomatic and subsequently relapse
55 later. Additionally, immunosuppressed patients can remain asymptomatic after VL
56 therapy, because they receive **secondary prophylaxis;** however, they can still act as
57 reservoirs, as confirmed by **xenodiagnoses** [10].

58 This complex scenario for the transmission dynamics of VL make it even more difficult
59 to establish effective control measures and highlight the clear need for improved tests
60 able to distinguish between all the different conditions (Box 1).

61 - Diagnostic tests have to provide an immediate, reliable, confirmatory diagnosis
62 of active VL cases independent of a central laboratory. Improved tests are also
63 necessary to assess treatment success, a fundamental measure for predicting and
64 avoiding relapses. This requires a specific test that goes beyond the clinical
65 recovery of the patient and the non-detection of the parasite and confirms cure
66 [11, 12].
67 - New tests are required for screening for *Leishmania* infection in healthy
68 populations. Asymptomatic infection goes unnoticed if not actively sought and is
69 usually determined by a positive serological test, a positive molecular test, a
70 cellular positive test like the rK39- immunochromatographic test (rK39-ICT), the
71 polymerase chain reaction (PCR) or a positive **leishmanin skin test (LST)** [13].
72 The rates of prevalence of asymptomatic infection obtained in different studies
73 vary dependent on the type of test used [14]; thus it is necessary to combine
74 serological, molecular and cellular tests to obtain a total prevalence value [15, 16].

75

76 **New rapid, simple and sensitive tests for the detection of *Leishmania* infection.**

77 Currently, different techniques are used for VL diagnosis, patient follow-up and
78 asymptomatic screening. Serological techniques such as the rapid rK39-
79 **immunochromatographic test** (rK39-ICT) have been validated in different endemic
80 areas of VL and are used with good sensitivity for the detection of active cases, except in
81 East Africa where sensitivity is lower [17-19] (Table 1). This test has also been used for
82 the screening of asymptomatic subjects, with lower positivity rates than the **direct**
83 **agglutination test (DAT)** [15]. Molecular diagnosis of VL using PCR or quantitative
84 PCR (qPCR) is useful and sensitive [20-22] (Table 1); but of limited use in asymptomatic
85 subjects due to their null or low parasitemia. However, its use is restricted to scenarios
86 with specialized personnel and equipment, including a stable cold chain. In
87 epidemiological studies for detection of asymptomatic individuals, LST has been widely
88 used [7, 10, 15]. However, it is being abandoned as it is invasive and could cause antigen
89 sensitization, and there is a lack of leishmanin of proven quality produced under GMP
90 conditions

91 Due to the limitations of rK39 antigen, the recombinant antigen rK28 has been introduced
92 for serological diagnosis [23, 24], and incorporated into a prototype commercial rapid test

93 for use in hospitals, health centers or at point-of-care (PoC). The few studies performed
94 in Sudan and Ethiopia have reported that rK28-ICT may have better efficacy than DAT
95 for the diagnosis of active cases of VL, and increased sensitivity over rK39-ICT [25-27].

96 Regarding molecular diagnosis, the combination of simplicity, rapidity and high
97 sensitivity make Loop-mediated isothermal amplification (LAMP) a more appropriate
98 technique than conventional techniques for the diagnosis of leishmaniasis at PoC [28].
99 Due to the displacement activity of the *Bst*-polymerase, no changes in temperature are
100 required for denaturation of the DNA and hybridization with the primers; thus, the use of
101 the thermal cycler in the incubation is not required, and only a thermoblock or incubator
102 is necessary for the DNA amplification [28]. LAMP together with a quick and easy DNA
103 extraction method, such as boiling (Boil & Spin), provide a suitable combination to
104 transfer the test to the field. Recently, Eiken Chemical Co. and the Foundation for
105 Innovative New Diagnostics (FIND) have developed a kit for the diagnosis of
106 leishmaniasis known as Loopamp ™ *Leishmania* detection. It is designed with an easy-
107 to-use format, that is, all the reagents are lyophilized in the lid of the reaction tube, so the
108 cold chain is not necessary for transport and/or storage. This kit has been evaluated in
109 Spain, Sudan, Brazil and Ethiopia, presenting sensitivities and specificities equivalent to
110 the reference tests in each of the endemic areas [27, 29, 30].

111 In relation to alternative tests to LST or the *in vitro* PBMC proliferation assay, able to
112 measure the cellular immune response against *Leishmania*, the whole blood stimulation
113 assay (WBA) is particularly interesting. This test consists of the *in vitro* stimulation of
114 blood cells with *Leishmania* specific antigens, followed by the detection of **cytokines**
115 and/or **chemokines** in stimulated plasma. The whole blood sample is the finest way to
116 study cell activation and the production of analytes *in vitro*, since it mimics the natural
117 environment. This technique has been used in different fields within medicine, including
118 the evaluation of autoimmune diseases, the control of drug and vaccine efficacy, and
119 immunotoxicity [31-33]. The low volume of blood used and the minimal processing
120 involved, make it a quick, easy and inexpensive tool that can be taken on field studies to
121 detect different microorganisms [34].

122 When testing the cellular response against *Leishmania*, WBA solves the limitations of
123 LST and it is easier and faster than the *in vitro* cell proliferation assay with PBMC (CPA),
124 which has limited field use due to its complexity [16, 35]. The levels of analytes detected
125 in the plasma of WBA are useful as **biomarkers** of infection/exposure and cure/relapse.

126 Therefore, this test can be used with lyophilized SLA antigens, both in epidemiological
127 surveys to evaluate levels of asymptomatic infection, and in making clinical decisions
128 regarding treatment [36-38]. The specific production of cytokines/chemokines could
129 confirm a cure or warn of possible relapses [37, 38], which is of special interest in clinical
130 trials of new drugs and drug combinations against VL. In this context, filter paper (Protein
131 Saver 903 cards) has been proposed as an effective and safe means of storing, conserving
132 and transporting SLA-stimulated plasmas, thus avoiding cold chain storage and transport
133 of samples, reducing sample storage space and transport costs to a reference laboratory
134 [39].

135

136 **Cytokines and chemokines as biomarkers of exposure, disease and cure**

137 The cellular response accompanying the control of *Leishmania* infection is associated
138 with the specific activation/proliferation of $CD4^+$ T lymphocytes, with a predominant Th1
139 profile that essentially synthesizes interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α)
140 and interleukin-2 (IL-2). This in turn activates the leishmanicidal function of
141 macrophages through the production of nitric oxide (NO) [40]. Likewise, the Th1
142 protective response is characterized by a strong activation of $CD8^+$ T cells [41], which
143 eliminate infected macrophages through the release of perforins and granzymes [42]. In
144 addition, Faleiro *et al.* have proposed that the cytokines IL-17 and IL-22 can play
145 complementary functions to the Th1 cytokines to control the growth of the parasite and
146 prevent the development of VL [43]. Recent studies have shown that some chemokines
147 (IL-8/CXCL8, MCP-1/CCL2, MIG/CXCL9 and IP-10/CXCL10) are associated with the
148 Th1 type cellular response [43]. On the contrary, when the parasite is able to evade the
149 specific immune response of the host the disease develops [44]. In VL, an invasion of the
150 spleen and liver, lymph nodes and bone marrow occurs. The cellular immune response in
151 active VL patients is associated with a cellular anergy or a Th-2 type response, with a
152 production of interleukin-10 (IL-10), interleukin-4 (IL-4), interleukin-13 (IL-13),
153 interleukin-5 (IL-5) and transforming growth factor beta (TGF-β), which causes the
154 activation of B lymphocytes and the consequent high production of *Leishmania* specific
155 antibodies, a VL clinical characteristic, which cannot control the infection [43]. Recent
156 studies have found an increase in the production of circulating pro-inflammatory
157 cytokines and chemokines in VL. Elevated levels of IL-1, IL-6, IL-8, IL-12, IL-15, IL-
158 27, IFN-γ, TNF-α, IP -10 and MIG have been reported in the unstimulated serum of

159 subjects with active disease [42, 45]. This supports the concept of a balance between the
160 Th1 and Th2 responses in human VL [42, 43].

161 Along with its simplicity and ease of realization, the WBA test allows the detailed
162 analysis of cytokine and chemokine patterns produced after the stimulation of the
163 circulating *Leishmania*-specific T clones. Different studies have evaluated the expression
164 of the cytokines IFN-γ, TNF-α and IL-10 by WBA in different endemic areas of
165 *Leishmania* [16, 35, 38, 46, 47]. Studies in India showed that IFN-γ is the cytokine
166 expressed in a statistically significant way in both cured VL patients after treatment and
167 asymptomatic individuals. Some active VL patients also had a high expression of IFN-γ
168 [46]. In contrast, WBA performed in blood from active VL patients from Ethiopia and
169 Spain did not produce specific IFN-γ [16, 38]. These differences are mainly due to the
170 time after disease when the assay was performed, IFN-γ being a good biomarker to
171 distinguish between active and cured patients.

172

173 With the aim of expanding and refining analysis of WBA, we carried out multiple
174 screenings by cytokine/chemokine measurement using cytometric bead array. As a result,
175 in addition to IFN-γ, we also propose the cytokine IL-2 and chemokines IP-10, MIG and
176 MCP-1 as biomarkers of infection, exposure and cure in endemic areas of *L. infantum*
177 and *L. donovani* [36, 37, 48]. The behavior of these chemokines has been consistent
178 throughout the studies and their higher expression in WBA with respect to IFN-γ, allows
179 for easier detection, without loss of sensitivity when plasma samples are preserved on
180 filter paper. Further, our group has also been working on the application of WBA in the
181 characterization of immunosuppressed patients, both to assess recovery after treatment
182 and presence of asymptomatic infection [49, 50].

183 As results of these studies, we have been able to produce a diagram showing the
184 differences of expression of a significant number of cytokines and chemokines in the
185 WBA plasma after stimulation with SLA (Figure 1). The figure shows a heat map of the
186 median production of these factors associated with both the Th1 and Th2 responses in
187 different situations of *L. infantum* infection: asymptomatic (Asympt), active disease
188 (AVL), cure (CVL) and negative controls (NC) [16, 37, 48]. As expected, increased
189 production of Th1 type cytokines and chemokines is associated with both cure and

190 asymptomatic status. Th2-type cytokines, such as IL-10, increase their production in
191 patients with active disease [16, 46], while IL-4, IL-5, IL-13 and the cytokine IL-17 are
192 produced in very low concentrations. The importance of these results is that this scheme
193 can be key to design of epidemiological studies or clinical trials in leishmaniasis. It can
194 be particularly useful for post-kala azar dermal leishmaniasis, PKDL, in which the current
195 biomarkers are unsatisfactory and new approaches need to be explored**.** In a recent review
196 Zijlstra (2019) affirms the need for longitudinal studies to describe the dynamics of
197 parasite and immunological biomarkers, in the pathophysiology of PKDL [1].
198

199 **Application of new approaches for the control of leishmaniasis**

200 Any new serological, molecular and cellular approaches to the diagnosis of VL and the
201 follow-up of patients have to be rapid, easy to perform and interpret and with no need for
202 large facilities if they are to allow significant advances in the control of VL.

203 In our experience, the panel of expression in WBA has allowed us to identify the cytokine
204 IL-2 as a preferential biomarker of infection/exposure to *Leishmania* infection [16]. We
205 have used this cytokine in an epidemiological survey in the Madrid region (CAM) with
206 4000 samples, where we could identify areas of high prevalence of asymptomatic *L.*
207 *infantum* infection. We have especially found that in the post-outbreak area of the
208 southwest region of the CAM (Fuenlabrada) there was up to 20.7% asymptomatic
209 infection prevalence (by IL-2 quantification), whilst with serological and PCR techniques
210 the prevalence found was 1.01%, underestimating the asymptomatic population [36].

211 On the other hand, IFN-γ and MIG were selected as possible biomarkers of cure in a pilot
212 study to monitor the pharmacological treatment of VL patients [37]. These factors were
213 produced in 100% of the blood samples from patients with VL after 6 months of
214 successful treatment, the time proposed by the WHO for definition of cure and absence
215 of relapse. Further, despite the high proportion of relapses suffered by co-infected
216 HIV/VL patients, monitoring after treatment using **IGRA** has also confirmed that a
217 positive cell response is associated with the lack of relapse. Under such conditions, it is
218 possible to withdraw secondary prophylaxis, independently of the number of circulating
219 CD4 + T lymphocytes, with no risk of relapse [51]. In the same way, to avoid relapses,
220 WBA may help to determine the time to restart immunosuppressive therapy in those
221 patients treated for VL and needing to return to their previous pharmacological treatment

222 [50]. This approach has been shown to be useful for following up solid organ transplant
223 recipients treated for VL, since it could help to confirm cure and prevent relapse, as well
224 as detect asymptomatic infection prior to the transplant surgery and subsequent
225 immunosuppression.

226 Serological and molecular diagnosis such as the rK28 immunochromatographic test and
227 the *Leishmania* Loopamp detection kit are fast, simple and robust tools and allow the
228 early diagnosis of VL in endemic areas without the need for large blood samples or
229 complex facilities [27]. A recent report has confirmed that progression to clinical VL is
230 associated with a positive serology and PCR for *Leishmania*, but not with the expression
231 of IFN-γ in whole blood stimulation tests [47]. Therefore, the combination of these three
232 new techniques would allow the screening of preclinical patients to identify those who
233 will develop visceral leishmaniasis. Such an approach is particularly useful for
234 immunosuppressed individuals in whom treatment is more likely to fail and risk of relapse
235 is higher than in immunocompetent patients. This would facilitate management of
236 patients and on-site decision-making by the physician, whether to maintain or withdraw
237 secondary prophylaxis or reintroduce immunosuppressive treatment, suspended prior to
238 VL therapy.

239

240 **Concluding Remarks**

241 The rapid and easy use of new biomarkers and diagnostic tests for leishmaniasis can
242 facilitate not only the confirmation of active VL cases but also epidemiological studies
243 and the identification of asymptomatic individuals, allowing the screening of a higher
244 number of individuals in less time. When the three tests are combined, a real value of
245 prevalence of *Leishmania* infection can be obtained. Also, cellular and molecular
246 techniques have proven useful in the monitoring of treatment and in the determination of
247 successful treatment against *Leishmania*. Nevertheless, many important questions remain
248 unsolved (see Outstanding Questions). It is critical to identify biomarkers/hall markers
249 associated with parasite infectiveness to assess the epidemiological risk of the parasite
250 carriers and the relationship with the parasite burden. It is also necessary to establish an
251 algorithm that differentiates between patients that are going to progress to active VL from
252 those that will remain asymptomatic. A similar problem that remains is to identify
253 biomarkers for development of PKDL in treated patients. The recognition of VL-treated

patients going to develop PKDL could help prevent this form of leishmaniasis, which would also have an impact on control, since PKDL patients are considered the main reservoir for *L. donovani*. All new approaches discussed in this article could help in the decision making regarding the state of the treatment, withdrawal of prophylaxis or reintroduction of immunosuppression. With regard to the management of immunosuppressed patients, it is particularly necessary to establish biomarkers to measure the immune response against the parasites and to determine their disappearance. Finally, most of the biomarkers discussed in this article are related with the human host, nevertheless, other relevant biomarkers are those related to virulence of the parasite, to give prior information on severity of the disease and susceptibility of the parasite to chemotherapy.

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474 **Glossary**

475 **Asymptomatic:** Individuals who after being inoculated with *Leishmania* by sand fly bites
476 become positive for any *Leishmania*-specific diagnostic test but do not develop clinical
477 symptoms of VL.

478 **Biomarker:** A biological molecule found in blood, fluids or tissues that is a sign of a
479 normal or abnormal process, or of a condition or disease.

480 **Chemokine:** Specific type of cytokine that induce migration of leukocytes.

481 **Cytokine:** Small signaling protein secreted by cells of the immune system to induce the
482 growth of lymphocytes and to regulate the response to infections.

483 **Direct Agglutination Test (DAT):** Serological test for visceral leishmaniasis that uses
484 whole fixed and stained *Leishmania* promastigotes to measure *Leishmania*-specific serum
485 antibodies. The antibodies produce the agglutination of parasites which forms a pale blue
486 film, while the absence of antibodies allows the promastigotes to settle on the bottom of
487 the well.

488 **IGRA:** Interferon gamma release assay. This term refers to all cell tests based on the
489 detection of the interferon gamma cytokine after lymphocyte stimulation, either in
490 plasma, supernatant or as spots on filter membranes.

491 **Leishmanian Skin Test (LST):** Also called "Montenegro test", is an immunological
492 method that measures delayed-type hypersensitivity caused by previous exposure to the
493 parasite. A suspension of killed promastigotes is injected intradermally and, in the case
494 of a positive reaction, an induration of more than 5 mm in diameter occurs.

495 **Immunochromatographic test (ICT):** Simple cellulose-based strip devices with crude
496 or recombinant leishmanial antigen to detect the presence of *Leishmania*-specific
497 antibodies in sera or blood samples, without the need for specialized and costly
498 equipment.

499 **Parasite carrier:** *Leishmania*-infected individual who is capable of transmitting the
500 parasite to the sand flies that bite them and, therefore, can act as a reservoir

501 **Reservoir:** Person or animal in which an infectious pathogen lives and multiplies, and
502 serves as a source from which other individuals can be infected.

503 **Sand fly:** Colloquial name for any species or genus of phlebotomine diptera.
504 Phlebotomine sand flies occur throughout the tropics and sub-tropics, as well as in
505 temperate zones. Some species are vectors of human and canine leishmaniasis, and
506 phleboviruses such as Toscana virus or Rift Valley fever virus.

507 **Secondary prophylaxis:** Maintenance treatment given to a patient to prevent recurrence
508 of the infection once the patient has been treated and has recovered clinically.

509 **Transmission dynamic:** The term refers to the way in which the different elements
510 involved in the transmission cycle of an infectious disease interact with each other and
511 are affected by other environmental factors.

512 **Xenodiagnosis:** Experimental assay to confirm the infective capacity of an individual.
513 The test is performed by exposing the individual to the bite of uninfected sand flies under
514 controlled conditions. Some days later, sand flies are dissected to check if they are
515 infected, confirming or not the ability of the individual tested to transmit the parasite and
516 act as reservoir.

517

518 **Box 1. Different situations in *Leishmania* infection require different diagnostic**
519 **approaches.**
520 In VL endemic areas there are different conditions besides active cases. The precise
521 characterization of such conditions requires the combination of *Leishmania*-specific
522 parasitological (PAR), serological (SER) and cellular (CEL) tests; further, in order to
523 establish the epidemiological role at the different conditions, xenodiagnostic (XDI) tests
524 are also necessary. Below in **Table I**, we have defined the diagnostic profile of several
525 conditions and the test results. The profiles coincide for some of the conditions, but in
526 those cases it is necessary to consider whether the individuals have active/previous VL or
527 not.

528

529 **Table I. Diagnostic profile of different situations in *Leishmania* infection.**

INDIVIDUALS WITH ACTIVE OR PREVIOUS VL			
Active visceral leishmaniasis patients			
PAR	+ +	The parasite appears in the target organs and peripheral blood	[11, 12, 52]
SER	+ +	High titers of *Leishmania*-specific serum antibodies	[11, 12, 52]
CEL	**-**	No or low cellular response to *Leishmania* antigens	[53]
XDI	+	Transmission to sand flies demonstrated	[54]
Cured patients			
PAR	**-**	Rapid parasite clearance	[11, 53, 54]
SER	+ +	Serum antibody titers remain high for a period	[11, 12, 53, 54]
CEL	+ +	Specific cellular response against *Leishmania* appears	[38, 53, 54]
XDI	**-**	No transmission	[54]
Patients who relapse after treatment			
PAR	+	No clearing of the parasite, molecular tests remain positive or become positive after some time	[50, 51]
SER	+ +	Serology remains positive	[55]
CEL	**-**	No cellular response appears or disappears shortly after treatment	[50, 51]
XDI	**?**	Ability to transmit	Unknown
INDIVIDUALS WITHOUT PREVIOUS VL			
Patients in the prepatent period			
PAR	+	Molecular tests are positive	[47]
SER	+ +	Serum antibody levels are high	[47]
CEL	**-**	The cellular response is negative	[47]
XDI	**?**	Ability to transmit	Unknown
Asymptomatic individuals			
PAR	+/-	Presence of detectable parasites very infrequent	[10, 15, 36, 54]
SER	+/-	Null or low levels of *Leishmania*-specific serum antibodies	[10, 15, 36, 54]
CEL	+	Frequent cellular response	[15, 36, 54]
XDI	**?**	Ability to transmit - null / under tests	[54]

530

Table 1. Diagnostic techniques for the identification of active visceral leishmaniasis in different endemic areas of *Leishmania* spp.

Technique	Country of study population	Time spent	Skill level required	Antibody/ antigen employee	Sensitivity (%)	Specificity (%)	Ref.
PCR	Indian Subcontinent	Hours	High	18s rRNA	70-100	85-99	[55, 56]
	East Africa			18s rRNA	93.8-96	96-100	[57]
	Brazil			kDNA	100	95	[58]
	Mediterranea n basin			18s rRNA	94.5	100	[59]
IFAT	Indian Subcontinent East Africa	Hours	High	Promastigote *Ld*	86.5	99.9	[60]
				Promastigote *Li*	80.3	90.5	[61]
	Brazil			Promastigote *Ld*	100	98.5	[62]
	Mediterranea n basin			Promastigote *Lc*	80-100	83.3-100	[63, 64]
	Indian Subcontinent			Promastigote *Li*	79.4	99.2	[18]
ELISA	Indian Subcontinent	Hours	Medium	Promastigote *Ld* (p-ELISA)	76.9-92.3	93.1	[60, 65]
				SLA *Li*	83.6	90.5	[61]
	East África			SLA *Ld*	78.1-87	90.3-100	[65]
	Brazil			SLA *Li*	76-89.9	81	[64]
ICT	Indian Subcontinent	Minutes	Low	rK39	96.2-100	71-100	[60, 66, 67]
	East Africa				67.6-90.5	27.8-92	[62]
	Brazil				84.7-100	82-96.8	[19, 68, 69]
	Mediterranea n basin				71.4-78	100	[18]
DAT	Indian Subcontinent	Days	Medium	Liof ag *Ld*	92.3-100	96.6-100	[66, 70, 71]
	East Africa				88.1-100	78.2-100	[27, 70]
	Brazil			Liof ag *Ld*	96.6-100	53.3-100	[19, 72]
				Liof ag *Li*	96.2-99.5	88,6-97.5	[64]
	Indian Subcontinent			Liof ag *Ld*	86.5-88.5	85.9	[18, 70]

DAT, direct agglutination test; ELISA, enzyme linked immunosorbent assay; ICT, immunochromatographic assay; IFAT, indirect fluorescent antibody test; kDNA, kinetoplast DNA; *Lc*, *Leishmania chagasi*; *Ld*, *Leishmania donovani*; *Li*, *Leishmania infantum*; Liof ag, lyophilized antigen; PCR, polymerase chain reaction; SLA, soluble *Leishmania* antigen.

537 **Figure Legends.**

538 **Figure 1. Cytokine and chemokine expression in visceral leishmaniasis**. Heat map
539 represents the median of cytokines and chemokines after stimulation of whole blood with
540 soluble *Leishmania* antigen (SLA) with samples from healthy negative control subjects
541 (NC), patients with active visceral leishmaniasis (AVL), cured VL patients (CVL) and
542 individuals with asymptomatic infection (Asympt) [16, 36, 37, 39, 48]. The variations of
543 the heat map in the intensity of the color correspond to the levels of secretion. The data
544 were transformed to $\log_{10}$. The blank corresponds to null or very low secretion levels.
545 Each increment represents an increase of the logarithmic unit in the level of secretion.

546