


# Comparison of laboratory methods for diagnosis of human visceral leishmaniasis in the Center-West Region of São Paulo State, Brazil

Livia Carla Ramos<sup>1</sup>, Kethlyn Magliani de Figueiredo Reihner<sup>1</sup>, Luciana da Silva Ruiz<sup>1</sup>, Laís Anversa<sup>1</sup>, Sâmea Fernandes Joaquim<sup>2</sup>, José Eduardo Tolezano<sup>3</sup>, Diego Borin Nóbrega<sup>4</sup> and Virgínia Bodelão Richini-Pereira<sup>1\*</sup> 

<sup>1</sup>Centro de Laboratório Regional II Bauru, Instituto Adolfo Lutz, Rua Rubens Arruda, Q6, s/n, Centro, 17015-110, Bauru, São Paulo, Brasil. <sup>2</sup>Departamento de Produção Animal e Medicina Veterinária Preventiva, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, São Paulo, Brasil. <sup>3</sup>Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, São Paulo, São Paulo, Brasil. <sup>4</sup>Faculty of Veterinary Medicine, University of Calgary, Calgary, Canada. \*Author for correspondence. E-mail: virichini@yahoo.com.br

**ABSTRACT.** Visceral leishmaniasis (VL) is a neglected disease and represents an important public health problem. The present study aimed to compare the conventional laboratory methods with the molecular method for VL diagnosis in human blood samples. Direct parasitological test of bone marrow aspirate (direct parasitological test), rapid immunochromatographic test (RIT), indirect immunofluorescent antibody test (IFAT) and polymerase chain reaction (PCR) were used to evaluate 26 human samples with clinical suspicion of VL. The positivity rates obtained for direct parasitological test, RIT, IFAT and PCR were 80.8%, 80.8%, 73.1% and 84.6%, respectively. PCR had greater positivity and agreement with the other methodologies. Therefore, the use of PCR in the diagnostic routine should be encouraged to complement the serological results.

**Keywords:** visceral leishmaniasis; diagnosis; polymerase chain reaction.

Received on March 16, 2022  
 Accepted on September 29, 2022

## Introduction

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the genus *Leishmania* and represents a group of distinct diseases, including visceral leishmaniasis (VL) and American tegumentary leishmaniasis (ATL) (Alvar et al., 2012). In 2018, more than 95% new cases of VL reported to the World Health Organization occurred in 10 countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan (WHO, 2020). In Brazil, the major species involved in VL is *Leishmania infantum* (syn. *chagasi*) (Brasil, 2006).

Visceral leishmaniasis is a neglected disease and represents an important public health problem. The Brazilian program for VL control recommends clinical and laboratory diagnosis, treatment of infected individuals, identification and control of domestic reservoirs, control of vectors, environmental management and health education services (Brasil, 2006). Nevertheless, VL is endemic, leading to autochthonous cases in 25% municipalities in 21 of 26 Brazilian states. According to the last update in 2021, Brazil notified 50,270 cases from 2006 to 2020, accounting for a mean of 3,351 cases per year (WHO, 2021).

Diagnosis of leishmaniasis is obtained by an association among clinical, epidemiological and laboratory information. Conventional laboratory methods have been used, including parasitological and serological tests; however, they have limitations, while molecular methods have increasingly been explored and adopted as a complement and/or an accurate diagnostic alternative (de Paiva-Cavalcanti et al., 2015; Ghasemian et al., 2016). Polymerase chain reaction (PCR) is a highly sensitive and specific methodology which can be adopted for both diagnosis and treatment follow-up, constituting a useful diagnostic tool for low parasitic load, HIV co-infection and relapsed and asymptomatic cases (Reithinger & Dujardin, 2007; Fraga et al., 2010; Lindoso, Cunha, Queiroz & Moreira, 2016; Hossain et al., 2017).

The region of Bauru, located in the Center-West part of São Paulo State, Brazil, is endemic for VL and is considered a regional pole that plays an important role in the dissemination and expansion of this disease

(Cardim et al., 2013); such high incidence indicates the need of studies comparing the different diagnostic methods and evaluating the possible inclusion of other methodologies. Thus, the current study aimed to compare the conventional methods used for VL diagnosis in human blood samples with the molecular method.

## Material and methods

### Ethical statement

The present study was approved by the Research Ethics Committee of Adolfo Lutz Institute No. 1.131.447.

### Area of study

Adolfo Lutz Institute (IAL) is a National Laboratory on Public Health, which focuses on diagnostic, sanitary and epidemiological surveillance. The Institute, in Bauru region, operates in 38 cities in the Center-west part of São Paulo State and attends several Programs such as Visceral Leishmaniasis Surveillance and Control Program (VLSCP). The analyzed human samples derived from cities covered by the Institute.

### Samples

Visceral leishmaniasis is a mandatory notifiable disease. In Brazil, every clinical suspicion of VL requires the control program to immediately notify this patient through a specific form of the Brazilian Notifiable Diseases Information System. The local Surveillance Department collects all clinical and epidemiological data to investigate the case. During this process, additional information regarding diagnosis, treatment and results are included. For diagnosis, the human sample is forwarded to and analyzed at the IAL, based on a previous suggestive clinical condition of VL. Twenty-six human cases with clinical suspicion of VL were selected to perform routine laboratory methods, direct parasitological test of bone marrow aspirate, rapid immunochromatographic test (RIT) and indirect immunofluorescent antibody test (IFAT), as well as complementary methodology, polymerase chain reaction (PCR). The study did not include patients with relapsed and/or HIV co-infection, but only cases with suspected primary infection.

### Direct parasitological test

Direct parasitological test was performed for bone marrow aspiration puncture swabs colored with Giemsa. Slides were visualized with 100x immersion oil objective under an optical microscope to confirm the presence of amastigote forms in the tissue.

#### Rapid immunochromatographic test (RIT)

The RIT adopted for human serum samples was IT LEISH® (Biorad Laboratories, Marnes-la-Coquette, France), which uses recombinant K39 antigen to detect antibodies to *Leishmania* spp. (Assis et al., 2008). The tests were performed according to the manufacturer's recommendations.

#### Indirect immunofluorescent antibody test (IFAT)

To perform IFAT, the human Leishmaniasis IFAT Kit (Bio-Manguinhos, Rio de Janeiro, RJ, Brazil) was used to detect antibodies to *Leishmania* spp. in human serum with clinical suspicion of VL, according to the manufacturer's recommendations (Brasil, 2006). Slides were visualized under a fluorescence microscope (Olympus BX51, JPY). According to the Brazilian program for VL control, positive titration equal or superior to 1:80 and 1:40 titers is considered indeterminate, and the test should be repeated after 30 days (Brasil, 2006).

## Molecular analyzes

### DNA extraction and quantification

DNA extractions from blood samples were performed with the Illustra blood genomic Prep Mini SpinKit (GE Healthcare, Pittsburgh, USA), as instructed by the manufacturer. Quantification was evaluated in a spectrophotometer (Epoch-Biotek, USA).

### Polymerase chain reaction (PCR)

The target sequence used to analyze the human blood samples was for the ITS-1 (Internal Transcribed Spacer) region of ribosomal DNA, while LITSR (5'CTGGATCATTTTCCGATG3') and L5.8S (5'TGATACCACTTATCGCACTT3') were used for the screening of Trypanosomatidae family (El Tai, Osman, El Fari, Presber &Schönian, 2000). PCR included, as positive controls, the reference DNA strains *L. infantum*

(MHOM / BZ / 1982) and *L. major* (MHOM / BZ / 1982) from the Leishmanias Collection of Oswaldo Cruz Institute (CLIOC FIOCRUZ) and, as a negative control, ultrapure sterile water. Reactions were carried out under the following conditions: each 0.2 mL microtube received reaction buffer 10 mM Tris HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 10 pM each primer, 0.2 units Taq Platinum DNA polymerase (Invitrogen) and 10 ng genomic DNA. Thus, each tube had 11 µL MIX-PCR and 1 µL DNA extraction product. Incubation was performed in a gradient thermal cycler (Life Technologies®, Carlsbad, USA).

### Electrophoresis on agarose gel

An 8-µl aliquot of each amplified product was added of 2 µl run buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 70% Milli-Q water) and homogenized. The adopted molecular marker was 4 µl 100 bp ladder (Invitrogen, USA). The amplified material was visualized by electrophoretic run on 1.5% agarose gel added of 0.1 µl/mL SYBR Safe DNA gel stain (Invitrogen, USA). The electrophoretic run was performed in a horizontal vessel containing 1X TBE (89 mM Tris-HCl, 89 mM boric acid and 20 mM EDTA), at 65V, for 60 minutes. After the end of the run, the amplified bands were visualized under ultraviolet light (296 nm) and recorded with a transilluminator (Syngene, USA); the image was captured by the digital documentation system.

### Statistical analysis

Kappa (k) coefficient of agreement and its respective intervals with 95% confidence were calculated with R version 3.4.1. Kappa coefficients were interpreted according to Landis and Koch (1977): 1.00-0.81 excellent, 0.80-0.61 good, 0.60-0.41 moderate, 0.40-0.21 weak, and 0.20-0.00 negligible agreement.

## Results and discussion

Of the 26 samples analyzed, 21 (80.8%) were positive by direct parasitological test of bone marrow aspirate, 21 (80.8%) were positive by RIT, 19 (73.1%) were positive by IFAT with titration equal to 80, six (23.1%) were indeterminate by IFAT with titration equal to 40, and 22 (84.6%) were positive by PCR. The level of agreement between methodologies performed for VL diagnosis was good and excellent for direct parasitological test, RIT and PCR. However, IFAT showed less positivity and moderate to weak agreement with the other methodologies, possibly due to its indeterminate results (Table 1).

**Table 1.** Kappa coefficient of agreement with 95% confidence interval, according to the laboratory methods performed for the diagnosis of human visceral leishmaniasis.

Laboratory methods	Kappa agreement (CI 95%)	p-value
RIT <sup>a</sup> versus direct parasitological test <sup>b</sup>	0.752 (0.427 - 1.000)	< 0.001
RIT versus IFAT <sup>c</sup>	0.426 (-0.461 - 1.000)	< 0.001
RIT versus PCR <sup>d</sup>	0.866 (0.611 - 1.000)	< 0.001
Direct parasitological test versus IFAT	0.426 (-0.461 - 1.000)	< 0.001
Direct parasitological test versus PCR	0.866 (0.611 - 1.000)	< 0.001
IFAT versus PCR	0.386 (0.156 - 0.685)	< 0.001

<sup>a</sup> RIT = rapid immunochromatographic test; <sup>b</sup> direct parasitological test = direct parasitological test in bone marrow aspiration puncture; <sup>c</sup> IFAT = indirect immunofluorescent antibody test; <sup>d</sup> PCR = polymerase chain reaction.

Parasitological examination is considered a "gold standard" for the diagnosis of leishmaniasis, although lower and more variable sensitivity has been described depending on the used sample (Srivastava, Dayama, Mehrotra & Sundar, 2011; Muñoz et al., 2016). In the current study, this method allowed the identification of 80.8% individuals with VL. Several studies have demonstrated that the sensitivity of molecular methods is higher than that of parasitological examination. In addition, the molecular method has been used for patients with negative results by the parasitological test (Osman et al., 1997; Romero et al., 2001; Antinori et al., 2007; Fraga et al., 2010). Piarroux et al. (1994) analyzed 73 peripheral blood samples from patients with suspected VL and found that PCR had a high sensitivity (82%), compared to parasitological test (55%) and culture (55%).

Among the serological tests performed in humans, RIT (rK39) allowed identification of 80.8% individuals with VL. Recombinant K39 antigen is described as a very useful tool for VL diagnosis, but its performance may vary according to the applied region due to the parasite diversity and the individuals' different antibody concentrations, age, nutritional status and immunological response (Cunningham et al., 2012). In Brazil, its sensitivity has been described between 84.7 and 93% (Guimarães, Lemos, Corey, & Dietze, 2003; Assis et al., 2008; Cunningham et al., 2012). However, according to da Silva et al. (2018), there is a decrease in sensitivity

from 95.45% to 61.29% for patients co-infected with HIV. Ozerdem, Eroglu, Genc, Demirkazik & Koltas (2009) compared PCR, RIT (rK39) and parasitological tests in 50 peripheral blood samples from patients with suspected VL in Turkey and demonstrated that PCR is the most sensitive technique.

IFAT obtained the lowest positivity and agreement with the other methodologies, possibly due to the presence of indeterminate results. Its performance is associated with the disease time, clinical form, involved species, and used antigenic preparation (Brasil, 2006; Assis et al., 2008; Barroso-Freitas et al., 2009). The major disadvantage of IFAT is the possibility of cross-reactions with other diseases, especially Chagas disease (Brasil, 2006; Pedras, de Gouvê Viana, de Oliveira & Rabello, 2008). Several authors have suggested that it should be replaced and/or complemented with other serological tests or molecular methods, which are considered more sensitive and specific (Neitzke-Abreu et al., 2013; Machado de Assis, Azeredo-da-Silva, Werneck & Rabello, 2016).

In the current study, the high positivity of PCR to detect the genus *Leishmania* was demonstrated in 84.6% samples from patients with clinical signs of VL. Another study reported 91.3% positivity for PCR and real-time PCR in 100 peripheral blood samples from patients with clinical signs of VL (da Costa Lima et al., 2013). In addition, Fraga et al. (2010) proved PCR is suitable for diagnosing VL in children since it is sensitive and safe and involves less invasive tissue collection, compared to other tests.

Several authors have demonstrated that early diagnosis of VL is decisive in reducing morbidity and mortality rates (da Costa Lima et al., 2013; Martins-Melo, Lima, Ramos Jr, Alencar & Heukelbach, 2014). Therefore, all efforts must be made to improve the diagnosis of such an important public health disease, including the adoption of more accurate and affordable methods, since PCR, due to its costs, is not generally available in resource-limited settings.

## Conclusion

In the present study, PCR obtained greater positivity and agreement with the other analyzed methodologies. Therefore, the use of PCR in routine diagnosis of human VL should be encouraged to complement serological results.

## Acknowledgements

We express our deepest thanks to Milena Zambon Garcia and Maria José Gomes Marques, for laboratory tests. This study was supported by São Paulo State Research Foundation (FAPESP), grant number 2012/21145-4.

## References

- Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., ... WHO Leishmaniasis Control Team. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*, 7(5). DOI: <https://doi.org/10.1371/journal.pone.0035671>
- Antinori, S., Calattini, S., Longhi, E., Bestetti, G., Piolini, R., Magni, C., ... Corbellino, M. (2007). Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 44(12), 1602-1610. DOI: <https://doi.org/10.1086/518167>
- Assis, T. S. M., Braga, A. S. C., Pedras, M. J., Barral, A. M. P., Siqueira, I. C., Costa, C. H. N., ... Rabello, A. (2008). Validação do teste imunocromatográfico rápido IT-LEISH® para o diagnóstico da leishmaniose visceral humana. *Epidemiologia e Serviços de Saúde*, 17(2), 107-116. DOI: <http://dx.doi.org/10.5123/S1679-49742008000200004>
- Barroso-Freitas, A. P., Passos, S. R., Mouta-Confort, E., Madeira, M. F., Schubach, A. O., Santos, G. P., ... Marzochi, K. B. (2009). Accuracy of an ELISA and indirect immunofluorescence for the laboratory diagnosis of American tegumentary leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 103(4), 383-389. DOI: <https://doi.org/10.1016/j.trstmh.2008.12.019>
- Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica (2006). *Manual de vigilância e controle da leishmaniose visceral / Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica*. Brasília, DF: Ministério da Saúde.

- Cardim, M. F. M., Rodas, L. A. C., Dibo, M. R., Guirado, M. M., Oliveira, A. M., & Chiaravalloti Neto, F. (2013). Introdução e expansão da leishmaniose visceral americana em humanos no Estado de São Paulo, 1999-2011. *Revista de Saúde Pública*, 47(4), 691-700. DOI: <https://doi.org/10.1590/S0034-8910.2013047004454>
- Cunningham, J., Hasker, E., Das, P., El Safi, S., Goto, H., Mondal, D., ... WHO/TDR Visceral Leishmaniasis Laboratory Network. (2012). A global comparative evaluation of commercial immunochromatographic rapid diagnostic tests for visceral leishmaniasis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 55(10), 1312-1319. DOI: <https://doi.org/10.1093/cid/cis716>
- da Costa Lima, M. S., Junior, Zorzenon, D., Dorval, M., Pontes, E., Oshiro, E. T., Cunha, R., ... Matos, M. (2013). Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood. *Asian Pacific Journal of Tropical Disease*, 3(1), 10-15. DOI: [https://doi.org/10.1016/S2222-1808\(13\)60003-1](https://doi.org/10.1016/S2222-1808(13)60003-1)
- da Silva, M. R. B., Brandão, N. A. A., Colovati, M., de Sousa, M. M. P., de Lima, L. C., Dorta, M. L., ... de Oliveira, M. A. P. (2018). Performance of two immunochromatographic tests for diagnosis of visceral leishmaniasis in patients coinfecting with HIV. *Parasitology Research*, 117(2), 419-427. DOI: <https://doi.org/10.1007/s00436-017-5716-3>
- de Paiva-Cavalcanti, M., de Moraes, R. C., Pessoa-E-Silva, R., Trajano-Silva, L. A., Gonçalves-de-Albuquerque, S., Tavares, D., Brelaz-de-Castro, M. C., ... Pereira, V. R. (2015). Leishmaniasis diagnosis: an update on the use of immunological and molecular tools. *Cell & Bioscience*, 5(31). DOI: <https://doi.org/10.1186/s13578-015-0021-2>
- El Tai, N. O., Osman, O. F., El Fari, M., Presber, W., & Schöni, G. (2000). Genetic heterogeneity of ribosomal internal transcribed space in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 94(5), 575-579. DOI: [https://doi.org/10.1016/S0035-9203\(00\)90093-2](https://doi.org/10.1016/S0035-9203(00)90093-2)
- Fraga, T. L., Brustoloni, Y. M., Lima, R. B., Dorval, M. E., Oshiro, E. T., Oliveira, J., ... Pirmez, C. (2010). Polymerase chain reaction of peripheral blood as a tool for the diagnosis of visceral leishmaniasis in children. *Memórias do Instituto Oswaldo Cruz*, 105(3), 310-313. DOI: <https://doi.org/10.1590/S0074-02762010000300011>
- Ghasemian, M., Gharavi, M. J., Akhlaghi, L., Mohebbi, M., Meamar, A. R., Aryan, E., ... Ghayour, Z. (2016). SYBR green-based detection of *Leishmania infantum* DNA using peripheral blood samples. *Journal of Parasitic Diseases: Official Organ of the Indian Society for Parasitology*, 40(1), 81-87. DOI: <https://doi.org/10.1007/s12639-014-0452-4>
- Guimarães, C. S. F., Lemos, E. M., Corey, R., & Dietze, R. (2003) Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *The American Journal of Tropical Medicine and Hygiene*, 68(3), 321-324. DOI: <https://doi.org/10.4269/ajtmh.2003.68.321>
- Hossain, F., Ghosh, P., Khan, M. A. A., Duthie, M. S., Vallur, A. C., Picone, A., ... Mondal, D. (2017). Real-time PCR in detection and quantitation of *Leishmania donovani* for the diagnosis of Visceral Leishmaniasis patients and the monitoring of their response to treatment. *PLoS One*, 12(9). DOI: <https://doi.org/10.1371/journal.pone.0185606>
- Landis, J. R., & Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics*, 33(1), 159-174.
- Lindoso, J. A., Cunha, M. A., Queiroz, I. T., & Moreira, C. H. (2016). Leishmaniasis-HIV coinfection: current challenges. *HIV/AIDS (Auckland, N.Z.)*, 8, 147-156. DOI: <https://doi.org/10.2147/hiv.s93789>
- Machado de Assis, T. S., Azeredo-da-Silva, A. L., Werneck, G. L., & Rabello, A. (2016). Cost-effectiveness analysis of diagnostic tests for human visceral leishmaniasis in Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 110(8), 464-471. DOI: <https://doi.org/10.1093/trstmh/trw050>
- Martins-Melo, F. R., Lima, M., Ramos, A. N., Jr, Alencar, C. H., & Heukelbach, J. (2014). Mortality and case fatality due to visceral leishmaniasis in Brazil: a nationwide analysis of epidemiology, trends and spatial patterns. *PloS One*, 9(4). DOI: <https://doi.org/10.1371/journal.pone.0093770>
- Muñoz, E. B., Santander, S., Rojas-Silva, P., Cardenas, P. A., Fornasini, M., Cifuentes, S. C., ... Baldeón, M. E. (2016). Diagnostic efficacy of molecular techniques for detection and identification of *Leishmania*

- species in human whole blood and 733 skin samples from Ecuador. *The American Journal of Tropical Medicine and Hygiene*, 95(4), 803-805. DOI: <https://doi.org/10.4269/ajtmh.16-0385>
- Neitzke-Abreu, H. C., Venazzi, M. S., Bernal, M. V., Reinhold-Castro, K. R., Vagetti, F., Mota, C. A., ... Lonardoni, M. V. (2013). Detection of DNA from *Leishmania (Viannia)*: accuracy of polymerase chain reaction for the diagnosis of cutaneous leishmaniasis. *PLoS One*, 8(7). DOI: <https://doi.org/10.1371/journal.pone.0062473>
- Osman, O. F., Oskam, L., Zijlstra, E. E., Kroon, N. C., Schoone, G. J., Khalil, E. T., ... Kager, P. A. (1997). Evaluation of PCR for diagnosis of visceral leishmaniasis. *Journal of Clinical Microbiology*, 35(10), 2454-2457. DOI: <https://doi.org/10.1128/JCM.35.10.2454-2457.1997>
- Ozerdem, D., Eroglu, F., Genc, A., Demirkazik, M., & Koltas, I. S. (2009) Comparison of microscopic examination, rK39, and PCR for visceral leishmaniasis diagnosis in Turkey. *Parasitology Research*, 106(1), 197-200. DOI: <https://doi.org/10.1007/s00436-009-1650-3>
- Pedras, M. J., de Gouvêa Viana, L., de Oliveira, E. J., & Rabello, A. (2008). Comparative evaluation of direct agglutination test, rK39 and soluble antigen ELISA and IFAT for the diagnosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(2), 172-178. DOI: <https://doi.org/10.1016/j.trstmh.2007.11.004>
- Piarroux, R., Gambarelli, F., Dumon, H., Fontes, M., Dunan, S., Mary, C., ... Quilici, M. (1994). Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral Leishmaniasis in immunocompromised patients. *Journal of Clinical Microbiology*, 32(3), 746-749. DOI: <https://doi.org/10.1128/jcm.32.3.746-749.1994>
- Reithinger, R., & Dujardin, J. C. (2007). Molecular diagnosis of leishmaniasis: current status and future applications. *Journal of Clinical Microbiology*, 45(1), 21-25. DOI: <https://doi.org/10.1128/jcm.02029-06>
- Romero, G. A., Guerra, M. V., Paes, M. G., Cupolillo, E., Bentin, T. C., Macedo, V. O., & Fernandes, O. (2001). Sensitivity of the polymerase chain reaction for the diagnosis of cutaneous leishmaniasis due to *Leishmania (Viannia) guyanensis*. *Acta tropica*, 79(3), 225-229. DOI: [https://doi.org/10.1016/S0001-706X\(01\)00140-1](https://doi.org/10.1016/S0001-706X(01)00140-1)
- Srivastava, P., Dayama, A., Mehrotra, S., & Sundar S. (2011). Diagnosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 105(1), 1-6. DOI: <https://doi.org/10.1016/j.trstmh.2010.09.006>
- World Health Organization [WHO]. (2020). *Leishmaniasis*. Retrieved from [https://www.who.int/health-topics/leishmaniasis#tab=tab\\_1](https://www.who.int/health-topics/leishmaniasis#tab=tab_1).
- World Health Organization [WHO]. (2021). *Leishmaniasis, number of cases of visceral leishmaniasis reported*. Retrieved from [http://apps.who.int/neglected\\_diseases/ntddata/leishmaniasis/leishmaniasis.html](http://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html)