Journal of Immunological Methods 469 (2019) 18–25

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Performance evaluation of anti-fixed *Leishmania infantum* promastigotes immunoglobulin G (IgG) detected by flow cytometry as a diagnostic tool for visceral Leishmaniasis☆

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ARTICLE INFO

Keywords:
Flow cytometry
IgG
Serodiagnosis
Leishmania infantum
Visceral leishmaniasis

ABSTRACT

Visceral Leishmaniasis (VL) is a severe disease, caused by the protozoans *Leishmania infantum* and *L. donovani* that is widely diagnosed using serological tools. These, however, have limitations in performance that limit their use for the correct identification of the cases. This study aimed to evaluate the performance of flow cytometry with fixed parasites for VL diagnosis, comparing it with four other serological tests. Samples from two endemic VL regions in Brazil, diagnosed by direct examination (DG1) and by at least two or one standard serological test (DG2 and DG3, respectively), as well as patients with chronic Chagas' disease (CG1) and healthy controls (CG2) were used in this study. The flow cytometry results were expressed as levels of IgG reactivity, based on the percentage of positive fluorescent parasites (PPFP). Using a 1:4096 serum dilution, a ROC curve analysis of the serum titration on flow cytometry has indicated a PPFP of 2% as the cutoff point to segregate positive and negative results. In the present study, flow cytometry had the best performance for DG1 (sensitivity of 96%) while rK39 (imunocromagraphic rapid test) and DAT (Direct agglutination test) were also associated with high sensitivity and specificity. The substantial agreement and kappa indexes observed suggested similar performances between these two tests and flow cytometry. IFAT (Immunofluorescent antibody test) and ELISA (Enzyme-linked immunosorbent assay) had lower performances and the lower values of agreement with flow cytometry. Together, these findings suggest that although adjustments are needed in order to reduce cross reactivity with other trypanosomatids, flow cytometry has the potential to be a safe serological alternative for the diagnosis of VL.

1. Introduction

Visceral leishmaniasis (VL) is a neglected, highly lethal disease, which is endemic in 76 countries and found in 12 American countries, with Brazil having the highest endemicity and responsible for 96% of the cases reported from the Americas. In this continent, the disease is caused by the protozoa *Leishmania infantum*, part of the *Leishmania donovani* complex (Organização Pan-Americana da Saúde., 2018).

The diagnosis of VL is based on clinical signs and symptoms of patients and is confirmed by laboratory tests (Chappuis et al., 2007). The difficulty in diagnosing patients delays the treatment and, therefore, increases the lethality of the disease (Coura-vital et al., 2014). Despite the availability of several tests, none can diagnose all cases and their effectiveness varies among different geographic regions (Boelaert et al.,

Abbreviations: IFAT, Immunofluorescent antibody test; DAT, Direct agglutination test; DG, Diagnostic group; CG, Control group; FITC, Fluorescein isothiocyanate; PPFP, Percentage of positive fluorescent parasites; CI, Confidence interval; PPV, Positive predictive value; NPV, Negative predictive value; AC, Accuracy; VL, Visceral Leishmaniasis; CV, Coeficient of Variation

☆ Journal of Immunological Methods

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https://doi.org/10.1016/j.jim.2019.02.009
Received 28 June 2018; Received in revised form 8 February 2019; Accepted 22 February 2019
Available online 25 February 2019
0022-1759/

2008; Cunningham et al., 2012; Abass et al., 2015). *Leishmania* identification in bone marrow aspirates is the gold standard for VL diagnosis, but its routine execution is hampered by the need for qualified professionals and an appropriate collection environment (Maia et al., 2012). Serological tests are widely used for the diagnosis of VL, but they have limitations, such as a failure to discriminate between recent and late infections, which make it impossible to use them as cure criteria. In addition, they may cross-react with other parasites (Boelaert et al., 2004; Sakkas et al., 2016).

In this context, studies with flow cytometry have shown its capacity to diagnose VL (Andrade et al., 2007) and Chagas disease (Vitelli-Avelar et al., 2007). Flow cytometry has high sensitivity and specificity values, and could be used as a diagnosis and cure criterion of Chagas disease (Martins-Filho et al., 1995; Matos et al., 2011); cutaneous leishmaniasis (Rocha et al., 2002; Pereira et al., 2012); VL (Lemos et al., 2007; Garcia et al., 2009), and also for post-vaccine control of canine visceral leishmaniasis (Andrade et al., 2007; Ker et al., 2013).

The aims of this study were to evaluate the performance of flow cytometry and its comparison with four other serological tests for the diagnosis of visceral leishmaniasis. Additionally we have also evaluated the performance of flow cytometry to detect cross-reactivity in patients with chronic Chagas' disease.

2. Materials and methods

2.1. Serum samples and study population

The population was defined for convenience of sample size, with the serum samples from two VL endemic areas from northeastern Brazil (Pernambuco and Piauí) divided into three diagnostic (DG) and two control groups (CG). DG1, 2 and 3 were defined using the parasite identification in bone marrow aspirate and four serological tests (DAT, ELISA, rK39 rapid test and IFAT). The groups were: DG1-51 patients with positive VL according to the presence of *Leishmania* parasites in aspirates of bone marrow; DG2-73 patients with positive VL confirmed by at least two serological tests; DG3-82 patients with positive VL confirmed by at least one serological test; CG1-15 patients with chronic Chagas' disease; CG2-18 healthy individuals with no history of VL from non-endemic areas. All serum samples were collected in vacutainer tubes (BD Biosciences), processed by centrifugation (1000 *g*, 10 min, room temperature), inactivated by heating for 30 min at 56 °C and centrifuged at 4 °C, 1000 *g* for five minutes. After centrifugation, the supernatant was aliquoted and kept at −20 °C until further use. All human serum was collected after approval of their use by the appropriate ethics committees from the Federal University of Piauí (0116/2005) and from IAM-FIOCRUZ (CAEE 51603115.7.0000.5190).

2.2. Parasite preparation

The promastigote forms of *L. infantum* strain MHOM/BR/70/BH46 were cultured at 26 °C *in vitro* in Schneider's medium associated with Novy-MacNeal-Nicolle (NNN) medium. Parasites were subjected to expansion and centrifuged at low speed (400 *g*, 10 min, room temperature). For the ELISA assays, the pelleted promastigote cells were washed three times with a phosphate-buffered saline (PBS), pH 7.2, 4 °C, followed by resuspension in lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) supplemented with 1 mM of the protease inhibitors N-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF). Lysis was performed by cycles of freeze-thawing, followed by centrifugation at 10.000 *g* for 15 min at 4 °C. The supernatant was collected and the protein content quantified by the Bradford assay following standard procedures. The crude soluble antigen was stored at −80 °C until further use. For the immunofluorescent antibody test (IFAT), the promastigote pellet was washed once with PBS followed by resuspension in 2% formaldehyde (Merck Millipore Darmstadt, Germany) and storage at 4 °C until use in the assays. For flow cytometry the same promastigote pellet was washed three times with PBS containing 10% Fetal Bovine Serum (FBS), at 4 °C, followed by resuspension for fixation in 1% paraformaldehyde and incubation overnight. The fixed cells were then washed again with PBS plus 10% FBS and the final resuspension adjusted to 5×10^6 promastigotes/mL, then used for the flow cytometry assay.

2.3. Direct parasite identification

Bone marrow (1 ml) aspirates for parasitological analysis were obtained in order to detect *Leishmania* parasites, preparing the smears by slide apposition. The slides were stained with a panoptic stain (Ranylab, Barbacena, Brazil) and evaluated under a light microscope (100×). At least three bone marrow smears were evaluated for each patient (Da Silva et al., 2005).

2.4. rK39 rapid test (IT LEISH)

The IT LEISH rapid test protocol was performed according to the manufacturer's instructions (Bio Rad Laboratories; Marnes-la-Coquette, France) using 10 µl of serum. Anti-*Leishmania*-specific antibodies are captured by a conjugate (protein A and colloidal gold) which reacts with the membrane-coated antigen (k39). After five minutes, a red line appears above indicating the presence of the IgG (control), which validates the kit. The appearance of another red line below indicates the presence of anti-rK39 IgG, representing a positive result.

2.5. Direct agglutination test (DAT)

DAT was carried out according to the manufacturer's instructions (Royal Tropical Institute, Amsterdam, NL). The test was performed on 96-well V-bottom plates (Greiner Bio One, Germany) with serial serum dilutions ranging from 1:50 to 1:51200. The results were read after overnight incubation at room temperature. The DAT titer is indicated by the highest dilution in which the agglutination is visible. Sera with titers from 1:6400 were considered positive (El Harith et al., 1988).

2.6. Immunofluorescent antibody test (IFAT)

The IFAT test was performed with an in house protocol developed by Immunology of Infectious and Parasitic Diseases Research Group at Instituto Aggeu Magalhães - FIOCRUZ, PE, using 10 µl of the antigenic suspension of *L. infantum* promastigotes applied to the region delimited on the IFAT slides (PERFECTLAB, São Paulo, Brazil) and leaving it to rest for two hours at 37 °C. Subsequently, the slides were coated with 10 µl of the patients' serum samples in titers ranging from 1:20 to 1:320 in PBS, pH 7.2. Two control sera (positive and negative) were incubated in a humid chamber for 30 min at 37 °C. After incubation, the excess of serum was removed from the slides by washing them three times through immersion in PBS, pH 7.2, in intervals of 10 min. Anti-human

IgG conjugated to fluorescein isothiocyanate-FITC (Sigma Chemical Corp., St. Louis, MO) prepared in Evans blue (40 mg) in PBS buffer solution (previously diluted at 1:10 ratio in the same buffer) was added to the slides in a 1:50 dilution, incubating them under the same conditions as mentioned before. After the reaction, the slides were washed three times for 10 min in PBS and left at room temperature. The assembly was made with buffered glycerin pH 8.5 and the slides then observed under a fluorescence microscope, with a 100× objective. Sera were considered positive from the 1:40 dilution.

2.7. ELISA

The ELISA test was performed as described by Oliveira et al. (2011). Briefly, 96-well plates (Greiner Bio One, Germany) were sensitized with 600 ng per well of crude *L. infantum* antigen and kept at 4 °C overnight. Plates were aspirated, blocked for one hour at 37 °C with 2% non-fat dried milk in PBS containing 0.05% Tween 20 (PBS-T) and washed four times with PBS-T. The sera were diluted at 1:900 in PBS-T containing 10% of non-fat dried milk and added to the wells in triplicates, and incubated for one hour at 37 °C. After three washes with PBS-T, the peroxidase-conjugated anti-IgG (Calbiochem, EMD Millipore, Billerica, MA) diluted at 1:2000 was added and the plates incubated for one more hour at 37 °C. Plates were then washed three times and incubated with *o*-phenylenediamine (OPD) and H_2O_2. The reaction was quenched by adding 2 M H_2SO_4 (50 µl/well) and the plates read at 490 nm (Spectra Max 190, Molecular Devices, Sunnyvale, USA or MRX II, Dynex Technologies, Chantilly, USA). Positive and negative control sera were present on each plate in order to standardize readings and variations. The cutoff point between non-reagent and reagent readings was calculated as the mean of the negative controls plus two standard deviations.

2.8. Flow cytometry

The flow cytometry assay to detect antibodies against promastigote forms of *L. infantum* was performed according to Rocha et al. (2002). Briefly, the parasite suspension (2.5×10^5/well) was incubated in 96-well, U-bottom plates at 37 °C for 30 min in the presence of different serum dilutions (1:64 to 1:8192). After incubation, the parasites were washed twice with 150 µl of PBS, pH 7.2, plus 10% FBS and then incubated at 37 °C for 30 min, protected from light, with the anti-human IgG conjugated to fluorescein isothiocyanate-FITC (Sigma Chemical Corp, St. Louis, MO) diluted 1:200 in PBS- 10% FBS. After another wash, FITC labeled parasites were fixed with 200 µl of 1% paraformaldehyde and kept from light for 30 min at 4 °C until submitted to reading on the FACScalibur flow cytometer (Becton Dickinson San Jose, CA), using the software “Cell Quest Pro”. The labeled parasites were submitted to the cytometer's acquisition with 10.000 events per sample. Promastigotes were identified based on their specific frontal (FSC) and lateral (SSC) light scattering properties. After FSC and SSC gain adjustments, the parasites assumed a characteristic distribution of these parameters and their fluorescence was evaluated. For each assay, in addition to the FITC-conjugated internal control, unlabeled controls in quadruplicates as well as negative and positive controls were included to validate the assay. For these controls, instead of using a single representative negative or positive sample, we opted to prepare a pool of true negative or positive sera and used an aliquot of either as controls.

2.9. Intra- and inter-assay variations

To investigate intra-assay variation, triplicates of five different control samples were run in one assay. Inter-assay variation was evaluated comparing 27 high controls, 5 medium controls, and 5 low controls that were run in three independent assays. The Coefficient of Variation (CV) for the intra- and inter-assay variations was calculated using the formula:

$$CV = \frac{\text{Standard deviation}}{\text{mean}} \text{ x } 100.$$

2.10. Statistics

Statistical analyses were performed using GraphPad Prism version 7.0 and the ROC curve, by Medcalc Software version 15.8. Each test performance was assessed by the following statistical indexes: Sensitivity = [true positives ÷ (true positives + false negatives)] × 100; specificity = [true negatives ÷ (true negatives + false positives)] × 100; positive predictive value — PPV = [true positives ÷ total positives] × 100; negative predictive value — NPV = [true negatives ÷ total negatives] × 100 and accuracy = [(true positives + true negatives) ÷ (true positives + true negatives + false positives + false negatives)] (Eusebi, 2013). Student's t-test was used for determining the differences between groups. The level of agreement for each of the tests (ELISA, IFI, DAT, rK39 rapid test) with flow cytometry were determined by percent agreement and the kappa index (κ) with 95% confidence interval and p values < 0.05 were considered significant.

3. Results

3.1. Optimization of flow cytometry parameters for the identification of IgG anti-promastigote forms of L. infantum

To establish the flow cytometry assays and to define the IgG reactivity data against *L. infantum* with the best performance indexes, promastigotes incubated with FITC (fluorescein isothiocyanate) conjugated anti-human IgG were evaluated on the flow cytometer in the absence of human sera (internal control) and after incubation with VL positive or negative sera, with representative results shown in Fig. 1. After promastigote identification (Fig. 1A), the relative FITC fluorescence intensity of each event was analyzed. A marker was set on the representation of the FITC-conjugated internal control (Fig. 1B) and used in all data analyzes reported here to determine for each sample the Percentage of Positive Fluorescent Parasites (PPFP). Fig. 1C and D illustrate the results for the fluorescence levels for a negative and positive control sera, respectively, with the derived PPFP values.

Next, to assess the optimal dilutions to be used with the sera in order to best define positive and negative samples, we plotted the average PPFP values obtained from five defined sera groups: DG1 - positive VL confirmed by *Leishmania* visualization; DG2 - positive VL confirmed by at least two serological tests; DG3 - positive VL confirmed by at least one serological test; CG1 - Chagas' disease; CG2 - healthy control sera. Fig. 2 then compares the average seroreactivity values for IgG for the different groups, expressed in PPFP, with different serum dilutions (titration curve ranging from 1:64 to 1:8192). From the graph shown, the serum titration of greatest difference in reactivity considering only

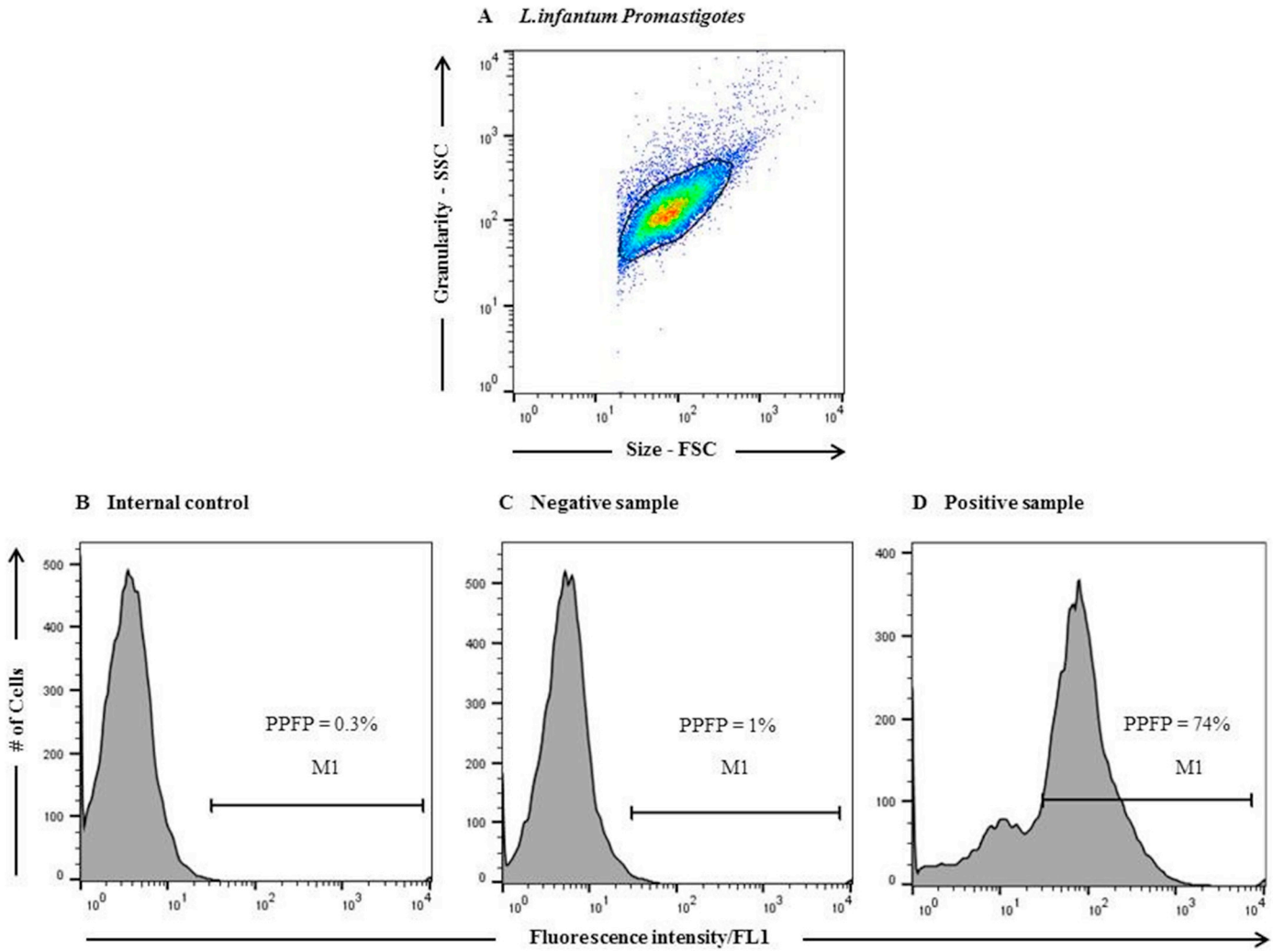

Fig. 1. Representative flow cytometry serology charts used to analyze IgG's reactivity anti-fixed *L. infantum* promastigotes in human serum samples. Promastigotes were selected on a dot plot based on their FSC (size) *versus* SSC (granularity). Fixed parasites were found to assume a homogeneous distribution (A). The relative FL1/FITC fluorescence intensity was quantified in the absence of human serum but in the presence of the FITC-conjugated anti-human IgG (internal control). This condition leads to the establishment of a maximum value of reactivity and determination of the marker M1 (positive region) (B). IgG's reactivity is provided in histograms for each serum samples as PPFP, which represents the frequency of parasite shift towards a higher fluorescence intensity, across M1. This marker was maintained to determine the reactivity in all data analyses performed in sera from both negative (C) and positive (D) samples for VL.

the positive samples and the healthy controls was 1:512. This dilution however is associated with high levels of cross-reactivity and false positive results with the sera from Chagas' disease individuals. Thus, we selected the dilution of 1:4096, as it showed a substantial reduction of the false positive results with minor decreases in fluorescence levels for the true positive samples.

3.2. Evaluating the performance of flow cytometry for VL diagnosis

The individual analysis through flow cytometry of all sera samples belonging to the five groups defined here using the 1:4096 dilution is shown in Fig. 3. Regarding the negative references (CG1 + CG2), flow cytometry has given false positive results in five samples from individuals with Chagas disease, while nearly all of those who were confirmed through parasite visualization (DG1) were identified as positive. Intermediate values were observed for the other groups. Thus, the seropositivity for VL was 96% for DG1, 92% for DG2, 85% for DG3. ROC curves were then drawn individually evaluating the performance by flow cytometry of the three groups of positive sera (DG1, DG2 and DG3) in relation to the two sets of VL negative samples grouped together (CG1 + CG2). The curves (Fig. 4) show that, for the selected serum dilution, the values for the area under the curve were 0.9 (95%, with a confidence interval [CI] between 0.90 and 0.99) for the DG1 group, 0.92 (95%, CI = 0.85–0.97) for DG2 and 0.9 (95%. CI = 0.83–0.95) for DG3. The ROC curves indicated that the 2% PPFP value would be the appropriate cutoff point for separating the VL groups from the negative individuals.

To evaluate potential analytical errors, we also performed intra- and inter-assays to determine the coefficients of variation (CVs), parameters widely used to quantify precision of biological measures. These were based on the values of PPFP to select high, medium, and low serum to use as *in house* controls. The intra-assay CVs ranged from 4% to 13%

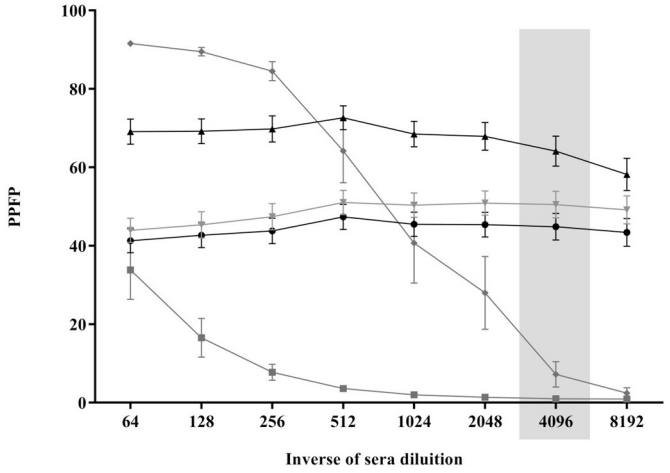

Fig. 2. IgG antibody titration curve detected by flow cytometry of Anti-fixed *Leishmania infantum* promastigotes stratified by groups. DG1 (▲) (n = 51, DG2 (▼) n = 73, DG3 (●) n = 82, CG1 (◆) n = 15, CG2 (■) n = 18. The gray rectangle corresponds to the titration of 1:4096 which was the region of greatest separation among the VL positive groups analyzed and both sets of controls. PPFP = Percentage of Positive Fluorescent Parasites. Bars show the 95% confidence intervals.

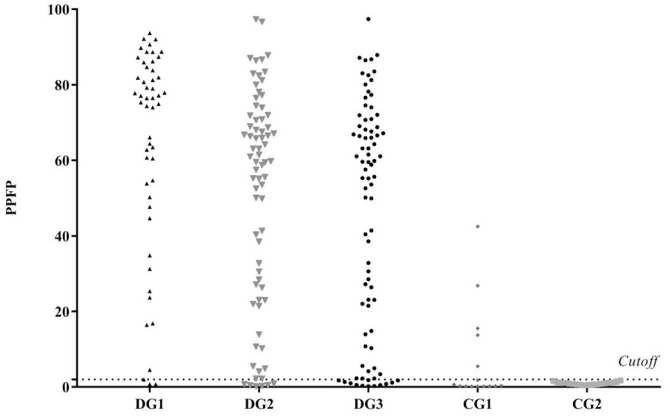

Fig. 3. Distribution of IgG reactivity of promastigote forms of *L. infantum* stratified by groups. DG1 (▲) n = 51, DG2 (▼) n = 73, DG3 (●) n = 82, CG1 (◆) n = 15, CG2 (■) n = 18. All groups are at the 1:4096 dilution and samples above the cutoff 2% PPFP (Percentage of Positive Fluorescent Parasites) are considered positive.

while inter-assay CVs ranged between 8% to 13% (Table 1), fitting within the range of variation considered acceptable, ≤ 20%. No statistically significant difference between the means of the controls ($P > 0.05$).

3.3. Comparison between flow cytometry and serological tests performances on the diagnosis of VL

Based on the previous results, we used the values expressed with the 1:4096 dilution for a comparison of flow cytometry with other currently used serological tests: DAT, ELISA and IFAT, all based on whole parasite cells or extracts; and the rapid test based on the recombinant rK39 antigen. Flow cytometry had the highest diagnostic performance with the DG1 group when compared to the other serological tests, with

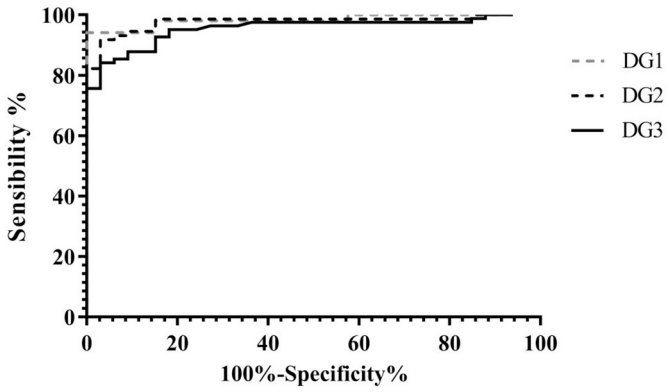

Fig. 4. ROC curve by performance indexes, sensitivity and specificity of flow cytometry for all groups analyzed. The ROC curve analysis was applied to establish the best cutoff point to discriminate PPFP values from positive and negative samples and indicate the area under the curve (AUC = global accuracy). ROC curve of samples at dilution 1:4096 indicating a cutoff point of 2%.

sensitivity of 96%, a positive predictive value of 91% and accuracy of 92% (Table 2). The rK39 rapid test was the best test in diagnosing the cases for group DG2, while for DG3 the best sensitivity was observed for DAT, with the rK39 rapid test having a better positive predictive value. The specificity was 85% for flow cytometry, 65% for ELISA and 100% for other tests.

The flow cytometry results were then compared with the other four serological tests in other to evaluate the agreement between different sets of results (Table 3). There was substantial agreement ($\kappa > 0.6$; Agreement > 80%) between the DAT assay and rK39 rapid test when compared to flow cytometry for all groups, with lower values for the other tests. In fact, the IFAT had the lowest values of performance and only average agreement ($\kappa < 0.6$; Agreement < 80%) for the three VL positive serum groups.

We then aimed to evaluate the clinical value of flow cytometry for the differential diagnosis between VL and Chagas' disease in comparison with the other serological tests. As stated above, the analysis of PPFP values using the 1:4096 serum dilution with a PPFP = 2% as the cutoff point demonstrated that the percentage of cross reactivity for flow citometry was 33% (based on Fig. 3). As for the other tests, the one with the highest percentage of cross reactivity was ELISA (73%), while the other tests (DAT, rK39 and IFAT) did not show cross reactivity in our hands, at least with the serum available to us.

4. Discussion

Overall, the performance by the flow cytometry assay described here resulted in improved sensitivity for VL diagnosis when compared to the four serological tests used in the clinical procedure. This is especially relevant considering the results for the DG1 group, where flow cytometry had the best performance, similar to the rK39 rapid test, since this is the only group with all positive samples confirmed only after parasite visualization. This finding corroborates the early results by Garcia et al. (2009) and Lemos et al. (2007) indicating a better performance of flow cytometry for the diagnosis of VL, now with a substantially increase in the number of samples evaluated and including the comparison with other serological methods. Flow cytometry then

Table 1
Mean Intra-Assay and Inter-Assay Percentage Coefficients of Variation (CVs) and 95% Confidence Intervals (CIs) for high, medium and low controls across Multiple Assays of flow cytometer anti-IgG for *L. infantum*.

	Intra-assay			Inter-assay		
	No. of samples	Mean % PPFP (95% CI[a])	Mean % CV (95% CI)	No. of samples	Mean % PPFP (95% CI)	Mean % CV (95% CI)
High Control	5	75 (66–84)	4 (0.8–6.5)	27	71 (66–75)	13 (10–16)
Medium Controls	5	20 (7–27)	5 (3–7)	5	20 (13–26)	8 (3.5–12)
Low Controls	5	0.2 (0.1–0.2)	13(3 − 23)	5	0.2 (0.1–0.2)	10.5 (7–14)

[a] CI = Confidence Interval.

Table 2
Values of sensitivity, specificity, positive and negative predictive values, and accuracy of the serological tests used for the diagnosis of VL.

	DG1	DG2	DG3
Sensitivity% (95%CI)[a]			
Flow Cytometry	96 (87–99)	92 (83–96)	85 (76–91)
DAT	94 (84–98)	94.5 (87–98)	91.5 (83–96)
rK39 rapid test	92 (81.5–97)	96 (87–99)	88 (79–93)
ELISA	86 (74–93)	88 (78–93)	78 (68–86)
IFAT	63 (49–75)	73 (61–81.5)	66 (55–75)
Specificity% (95%CI)			
Flow Cytometry	85 (69–93)	85 (69–93)	85 (69–93)
DAT	100 (82–100)	100 (82–100)	100 (82–100)
rK39 rapid test	100 (82–100)	100 (82–100)	100 (82–100)
ELISA	67 (50–80)	67 (50–80)	67 (50–80)
IFAT	100 (82–100)	100 (82–100)	100 (82–100)
PPV %[b] *(95%CI)*			
Flow Cytometry	91 (80–96)	93 (85–97)	93 (85–97)
DAT	100 (93–100)	100 (95–100)	100 (95–100)
rK39 rapid test	100 (92–100)	100 (95–100)	100 (92–100)
ELISA	80 (68–88)	85 (76–92)	85 (76–92)
IFAT	100 (89–100)	100 (93–100)	100 (93–100)
NPV%[c] *(95%CI)*			
Flow Cytometry	93 (79–98)	82 (66.5–92)	70 (55–82)
DAT	92 (78–97)	89 (75–96)	82.5 (68–91)
rK39 rapid test	89 (75–96)	92 (78–97)	77 (62–87)
ELISA	76 (58–88)	71 (53–82)	55 (40–69)
IFAT	63.5 (50–75)	62 (49–74)	54 (42–66)
Accuracy % (95%CI)			
Flow Cytometry	92 (84–96)	90 (82–94)	85 (78–91)
DAT	96 (90–99)	96 (91–98)	94 (88–97)
rK39 rapid test	95 (88–98)	97 (92–99)	91 (85–95)
ELISA	79 (69–86)	81 (73–87)	75 (66–82)
IFAT	77 (67–85)	81 (73–87)	76 (67–83)

[a] CI = Confidence Interval.
[b] PPV = Predictive Value Positive.
[c] *NPV* = Predictive Value Negative.

emerges as an alternative test to be used for the serological diagnosis of VL. In addition, it proved to be a safe and less invasive alternative when compared to bone marrow aspiration, allowing early treatment and reduction in lethality, but adjustments are still necessary to reduce cross reactivity with trypanosomatids.

The appropriate choice of antigen is still one of the major challenges for serological tests and many of those, including ELISA, IFAT and DAT tests, use whole antigen extract from promastigote forms. It is, therefore, common to find false-positive reactions due to cross-reactions with other diseases (Caballero et al., 2007). Adjustments in antigen preparation can be made to minimize the issue of cross-reactivity and strategies were developed for live and fixed *Leishmania* promastigotes for the cytometric diagnosis of tegumentary or cutaneous leishmaniasis (Pissinate et al., 2008; Pereira et al., 2012; Oliveira et al., 2013). The use of live parasites may reduce the binding of antibodies to intracytoplasmic structures seen for the fixed parasites (which increases the likelihood of cross-reactions). However, the manipulation risks when using live parasites is a limiting factor, especially considering that the observed performance was similar in both approaches (Lemos et al., 2007; Garcia et al., 2009), leading us to use fixed parasites in the present study. Despite these adjustments in the antigen preparation, it is still necessary to evaluate how variable this methodology is, but the intra and inter assays carried out here found a coefficient of variation within acceptable parameters (Reed et al., 2002). However, it is still necessary to evaluate laboratory-to-laboratory variability, specially between different regions, to validate whether the performance of the method remains reproducible. In addition, in the cross reactivity evaluation, a percentage of positivity was observed in the group with Chagas' disease, but due to its great versatility, strategies using flow cytometry have already been proposed that can minimized false positive or inconclusive results (Teixeira-Carvalho et al., 2015).

Considering other serological tests based on grown promastigote forms for VL diagnosis, IFAT is the most used in South America, being one of the available tests in public health services in Brazil (Machado de Assis et al., 2016; Sakkas et al., 2016). Studies show a moderate IFAT

Table 3
Comparison between flow cytometry and the serological tests used for the diagnosis of VL for all populations analyzed.

	Group	Diagnostic Tests			
		DAT	rK39 rapid test	ELISA	IFAT
Agreement % (95%CI[a])	DG1	88 (79–93)	87 (78–92)	82 (73–89)	69 (58–78)
	DG2	91 (83.5–95)	89 (81–93)	85 (77–90)	76 (67.5–83)
	DG3	89 (82–93)	86 (79–91)	81(73–87)	74 (65–81)
Kappa Index (95%CI)	DG1	0.7(0.5–1.0)	0.7(0.5–1.0)	0.6(0.4–0.8)	0.4(0.2–0.6)
	DG2	0.8 (0.6–1.0)	0.7 (0.5–0.9)	0.6 (0.4–0.8)	0.5 (0.3–0.7)
	DG3	0.7 (0.6–0.9)	0.7 (0.5–0.9)	0.6 (0.4–0.7)	0.5(0.3–0.6)

[a] CI = Confidential interval.

performance with 88–92% sensitivity and 83–88% specificity (Pedras et al., 2008; Machado de Assis et al., 2016), but our study showed an even lower sensitivity, despite a higher specifiticity. As for the parasitological diagnosis, the IFAT requires a complex infrastructure and trained technicians, thus limiting access and delaying diagnosis and treatment (Machado de Assis et al., 2012). DAT is one of the simplest and most inexpensive tests ever developed for the diagnosis of VL. A meta-analysis, evaluating the performance of DAT in patients with VL, presented estimates of sensitivity and specificity of 94.8% (Romero and Boelaert, 2010), corroborating with the high performance observed in our study. Despite its high levels of sensitivity and specificity, DAT is associated with problems in antigen quality control, refrigeration and lack of standardization of the test reading (Srivastava et al., 2011; Sundar and Rai, 2002). Therefore, in the present study, a commercial DAT kit was used to minimize this issue and indeed it displayed an efficient performance. Nevertheless, the possibility of quantification from PPFP through flow cytometry minimizes limitations such as the subjectivity of the readings, which is observed in agglutination tests such as DAT. As for the ELISA assay, it has the lowest performance among the different tests evaluated here.

An alternative to crude lysate in serological tests is the use of recombinant antigens, such as rK39 used for the rapid test, which is used in several countries and the rK28 rapid test, which was developed as a novel antigen for VL diagnosis in east Africa (Pattabhi et al., 2010; Pedral-Sampaio et al., 2016). Machado de Assis et al. (2016) showed that the rK39 test presented the best sensitivity and cost-benefit in the diagnosis of VL. However, in some endemic countries such as Sudan, the sensitivity of this test is unsatisfactory, which may be related to low amounts of antibodies produced by individuals or to the below-ideal test format (Abass et al., 2013). Flow cytometry, possibly associated with the use of recombinant antigens, may emerge then as an important alternative for the specific diagnosis of VL.

Despite its higher sensitivity, the costs associated with the flow cytometer are still a limitation, since it requires a medium-sized laboratory apparatus, in addition to the maintenance and training of personnel (Shapiro, 2003). However, that is minimized due to the assurance of higher sensitivity. The presence of photomultiplier detectors provides technical support for quantitative antibody analysis and provide the management of assays with such high serum dilutions, when compared to those usually tested by standard methodologies (Vitelli-Avelar et al., 2007). In addition, flow cytometry has already been used in clinical laboratories of developing countries to diagnose HIV/AIDS, cancer and anemia (Mandy et al., 2002; Pati and Jain, 2013; Denes et al., 2015; Pang et al., 2018). Therefore, its use for the serological diagnosis of VL is technically possible and can be scaled up with the possibility of using microtiter plates, which allow the diagnosis of several patients simultaneously (Rocha et al., 2006). The performance observed by flow cytometry in the present study strongly reinforces this possibility, as well as opening up new avenues of research with this technique such as the understanding the humoral response in VL and VL-HIV/AIDS patients. The possibility still exists to improve the technique still further with, for example, the use of recombinant antigens associated with beads.

Acknowledgments

This work was supported by a Brazilian grant from MCTI/CNPq/MS-SCTIE - DECIT N° 40/2012 – Research in Neglected Diseases, and by scholarships from the Brazilian funding agency CAPES. We would like to thank Camila Queiroz from Instituto Aggeu Magalhães – FIOCRUZ/PE for the *L. infantum* strain and Walter Lins Barbosa Junior for reviewing this manuscript.

Conflict of interest

The authors report no conflict of interest.

References

Abass, E., Bollig, N., Reinhard, K., Camara, B., Mansour, D., Visekruna, A., Lohoff, M., Steinhoff, U., 2013. rKLO8, a novel Leishmania donovani - derived recombinant Immunodominant protein for sensitive detection of visceral Leishmaniasis in Sudan. PLoS Negl. Trop. Dis. 7. https://doi.org/10.1371/journal.pntd.0002322.

Abass, E., Kang, C., Martinkovic, F., Semião-Santos, S.J., Sundar, S., Walden, P., Piarroux, R., El Harith, A., Lohoff, M., Steinhoff, U., 2015. Heterogeneity of Leishmania donovani parasites complicates diagnosis of visceral leishmaniasis: comparison of different serological tests in three endemic regions. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0116408.

Andrade, R.A., Reis, A.B., Gontijo, C.M.F., Braga, L.B., Rocha, R.D.R., Araújo, M.S.S., Vianna, L.R., Martins-Filho, O.A., 2007. Clinical value of anti-Leishmania (Leishmania) chagasi IgG titers detected by flow cytometry to distinguish infected from vaccinated dogs. Vet. Immunol. Immunopathol. 116, 85–97. https://doi.org/10.1016/j.vetimm.2007.01.002.

Boelaert, M., Rijal, S., Regmi, S., Singh, R., Karki, B., Jacquet, D., Chappuis, F., Campino, L., Desjeux, P., Le Ray, D., Koirala, S., Van der Stuyft, P., 2004. A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. Am. J. Trop. Med. Hyg. 70, 72–77.

Boelaert, M., El-Safi, S., Hailu, A., Mukhtar, M., Rijal, S., Sundar, S., Wasunna, M., Aseffa, A., Mbui, J., Menten, J., Desjeux, P., Peeling, R.W., 2008. Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. Trans. R. Soc. Trop. Med. Hyg. 102, 32–40. https://doi.org/10.1016/j.trstmh.2007.09.003.

Caballero, Z.C., Sousa, O.E., Marques, W.P., Saez-Alquezar, A., Umezawa, E.S., 2007. Evaluation of serological tests to identify Trypanosoma cruzi infection in humans and determine cross-reactivity with Trypanosoma rangeli and Leishmania spp. Clin. Vaccine Immunol. 14, 1045–1049. https://doi.org/10.1128/CVI.00127-07.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R.W., Alvar, J., Boelaert, M., 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat. Rev. Microbiol. 5, 873–882. https://doi.org/10.1038/nrmicro1748.

Coura-Vital, W., de Araújo, V.E.M., Reis, I.A., Amancio, F.F., Reis, A.B., Carneiro, M., 2014. Prognostic factors and scoring system for death from visceral leishmaniasis: an historical cohort study in Brazil. PLoS Negl. Trop. Dis. 8, e3374. https://doi.org/10.1371/journal.pntd.0003374.

Cunningham, J., Hasker, E., Das, P., El Safi, S., Goto, H., Mondal, D., Mbuchi, M., Mukhtar, M., Rabello, A., Rijal, S., Sundar, S., Wasunna, M., Adams, E., Menten, J., Peeling, R., Khanal, B., Das, M., Oliveira, E., de Assis, T.M., Bhaskar, K.R., Huda, M.M., Hassan, M., Abdoun, A.O., Awad, A., Osman, M., Prajapati, D.K., Gidwani, K., Tiwary, P., Paniago, A.M.M., Sanchez, M.C.A., Celeste, B.J., Jacquet, D., Magiri, C., Muia, A., Kesusu, J., Ageed, A.F., Galal, N., Osman, O.S., Gupta, A.K., Bimal, A.S., Das, V.N.R., 2012. A global comparative evaluation of commercial immunochromatographic rapid diagnostic tests for visceral leishmaniasis. Clin. Infect. Dis. 55, 1312–1319. https://doi.org/10.1093/cid/cis716.

Da Silva, M.R.B., Stewart, J.M., Costa, C.H.N., 2005. Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. Am. J. Trop. Med. Hyg. 72, 811–814.

Denes, V., Lakk, M., Makarovskiy, A., Jakso, P., Szappanos, S., Graf, L., Mandel, L., Karadi, I., Geck, P., 2015. Metastasis blood test by flow cytometry: in vivo cancer spheroids and the role of hypoxia. Int. J. Cancer 136, 1528–1536. https://doi.org/10.1002/ijc.29155.

El Harith, A., Kolk, A.H.J., Leewenburg, J., Muigai, R., Huigen, E., Jelsma, T., Kager, P., 1988. Improvement of a direct agglutination test for field studies of visceral Leishmaniasis. J. Clin. Microbiol. 26, 1321–1325.

Eusebi, P., 2013. Diagnostic accuracy measures. Cerebrovasc. Dis. 267–272. https://doi.org/10.1159/000353863.

Garcia, L.M., Coelho-Dos-Reis, J.G.A., Peruhype-Magalhães, V., Teixeira-Carvalho, A., Rocha, R.D.R., Araújo, M.S.S., Gomes, I.T., Carvalho, S.F.G., Dietze, R., Lemos, E.M., Andrade, M.C., Martins-Filho, O.A., 2009. Anti-fixed Leishmania chagasi promastigotes IgG antibodies detected by flow cytometry (FC-AFPA-IgG) as a tool for serodiagnosis and for post-therapeutic cure assessment in American visceral leishmaniasis. J. Immunol. Methods 350, 36–45. https://doi.org/10.1016/j.jim.2009.07.004.

Ker, H.G., Coura-Vital, W., Aguiar-Soares, R.D.D.O., Roatt, B.M., das Dores Moreira, N., Carneiro, C.M., Machado, E.M.D.M., Teixeira-Carvalho, A., Martins-Filho, O.A., Giunchetti, R.C., Araújo, M.S.S., Coelho, E.A.F., da Silveira-Lemos, D., Reis, A.B., 2013. Evaluation of a prototype flow cytometry test for serodiagnosis of canine visceral leishmaniasis. Clin. Vaccine Immunol. 20, 1792–1798. https://doi.org/10.1128/CVI.00575-13.

Lemos, E.M., Gomes, I.T., Carvalho, S.F.G., Rocha, R.D.R., Pissinate, J.F., Martins-Filho, O.A., Dietze, R., 2007. Detection of anti-leishmania (Leishmania) chagasi immunoglobulin G by flow cytometry for cure assessment following chemotherapeutic treatment of American visceral leishmaniasis. Clin. Vaccine Immunol. 14, 569–576. https://doi.org/10.1128/CVI.00354-06.

Machado de Assis, T.S., Rabello, A., Werneck, G.L., 2012. Latent class analysis of diagnostic tests for visceral leishmaniasis in Brazil. Tropical Med. Int. Health 00, 1–6.

https://doi.org/10.1111/j.1365-3156.2012.03064.x.
Machado de Assis, T.S., Azeredo-da-Silva, A.L.F., Werneck, G.L., Rabello, A., 2016. Cost-effectiveness analysis of diagnostic tests for human visceral leishmaniasis in Brazil. Trans. R. Soc. Trop. Med. Hyg. 110, 464–471.
Maia, Z., Lírio, M., Mistro, S., Mendes, C.M.C., Mehta, S.R., Badaro, R., 2012. Comparative study of rK39 Leishmania antigen for serodiagnosis of visceral leishmaniasis: systematic review with meta-analysis. PLoS Negl. Trop. Dis. 6. https://doi.org/10.1371/journal.pntd.0001484.
Mandy, F., Nicholson, J., Autran, B., Janossy, G., 2002. T-cell subset counting and the fight against AIDS: reflections over a 20-year struggle. Clin. Cytom. 50, 39–45. https://doi.org/10.1002/cyto.10097.
Martins-Filho, O. a, Pereira, M.E., Carvalho, J.F., Cançado, J.R., Brener, Z., 1995. Flow cytometry, a new approach to detect anti-live trypomastigote antibodies and monitor the efficacy of specific treatment in human Chagas' disease. Clin. Diagn. Lab. Immunol. 2, 569–573.
Matos, C.S., Coelho-dos-Reis, J.G.A., Rassi, A., Luquetti, A.O., Dias, J.C.P., Eloi-Santos, S.M., Gomes, I.T., Vitelli-Avelar, D.M., Wendling, A.P.B., Rocha, R.D.R., Teixeira-Carvalho, A., Peruhype-Magalhães, V., Andrade, M.C., Martins-Filho, O.A., 2011. Applicability of an optimized non-conventional flow cytometry method to detect anti-Trypanosoma cruzi immunoglobulin G for the serological diagnosis and cure assessment following chemotherapeutic treatment of Chagas disease. J. Immunol. Methods 369, 22–32. https://doi.org/10.1016/j.jim.2011.03.007.
Oliveira, G.G.S., Magalhães, F.B., Teixeira, M.C.A., Pereira, A.M., Pinheiro, C.G.M., Santos, L.R., Nascimento, M.B., Bedor, C.N.G., Albuquerque, A.L., Dos-Santos, W.L.C., Gomes, Y.M., Moreira, E.D., Brito, M.E.F., Pontes De Carvalho, L.C., De Melo Neto, O.P., 2011. Characterization of novel Leishmania infantum recombinant proteins encoded by genes from five families with distinct capacities for serodiagnosis of canine and human visceral leishmaniasis. Am. J. Trop. Med. Hyg. 85, 1025–1034. https://doi.org/10.4269/ajtmh.2011.11-0102.
Oliveira, A.P., de Castro, M.C.A.B., de Almeida, A.F., Souza, M. de A., de Oliveira, B.C., Reis, L.C., Goto, H., de Brito, M.E.F., Celeste, B.J., Martins-Filho, O.A., Pereira, V.R.A., 2013. Comparison of flow cytometry and indirect immunofluorescence assay in the diagnosis and cure criterion after therapy of American tegumentary leishmaniasis by anti-live Leishmania (Viannia) braziliensis immunoglobulin G. J. Immunol. Methods 387, 245–253. https://doi.org/10.1016/j.jim.2012.11.002.
Organização Pan-Americana da Saúde, 2018. Leishmanioses: Informe Epidemiológico nas Américas. Organização Pan-Americana da Saúde, Washington. www.paho.org/leishmaniasis.
Pang, K., Xie, C., Yang, Z., Suo, Y., Zhu, X., Wei, D., Weng, X., Wei, X., Gu, Z., 2018. Monitoring circulating prostate cancer cells by in vivo flow cytometry assesses androgen deprivation therapy on metastasis. Cytom. Part A. https://doi.org/10.1002/cyto.a.23369.
Pati, H.P., Jain, S., 2013. Flow cytometry in hematological disorders. Indian J. Pediatr. 80, 772–778. https://doi.org/10.1007/s12098-013-1152-2.
Pattabhi, S., Whittle, J., Mohamath, R., El-Safi, S., Moulton, G.G., Guderian, J.A., Colombara, D., Abdoon, A.O., Mukhtar, M.M., Mondal, D., Esfandiari, J., Kumar, S., Chun, P., Reed, S.G., Bhatia, A., 2010. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. PLoS Negl. Trop. Dis. 4. https://doi.org/10.1371/journal.pntd.0000822.
Pedral-Sampaio, G., Alves, J.S., Schriefer, A., Magalhães, A., Meyer, R., Glesby, M.J., Carvalho, E.M., Carvalho, L.P., 2016. Detection of IgG anti-leishmania antigen by flow cytometry as a diagnostic test for cutaneous leishmaniasis. PLoS One 11, 1–9. https://doi.org/10.1371/journal.pone.0162793.
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. Trans. R. Soc. Trop. Med. Hyg. 102, 172–178. https://doi.org/10.1016/j.trstmh.2007.11.004.
Pereira, V.R.A., Reis, L.D.C., Souza, M.D.A., de Oliveira, A.P., de Brito, M.E.F., Lage, P.S., Andrade, M.C., Rocha, R.D.R., Martins-Filho, O.A., 2012. Evaluation of anti-lived and anti-fixed Leishmania (Viannia) braziliensis promastigote IgG antibodies detected by flow cytometry for diagnosis and post-therapeutic cure assessment in localized cutaneous leishmaniasis. Diagn. Microbiol. Infect. Dis. 74, 292–298. https://doi.org/10.1016/j.diagmicrobio.2012.06.025.
Pissinate, J.F., Gomes, I.T., Peruhype-Magalhães, V., Dietze, R., Martins-Filho, O.A., Lemos, E.M., 2008. Upgrading the flow-cytometric analysis of anti-Leishmania immunoglobulins for the diagnosis of American tegumentary leishmaniasis. J. Immunol. Methods 336, 193–202. https://doi.org/10.1016/j.jim.2008.04.018.
Reed, G.F., Lynn, F., Meade, B.D., 2002. Use of coefficient of variation in assess inVariability of quantitative assays. Clin. Diagn. Lab. Immunol. 9, 1235–1239. https://doi.org/10.1128/CDLI.9.6.1235.
Rocha, R., Gontijo, C.M.F., Elói-Santos, S.M., Carvalho, A.T., Corrôa-Oliveira, R., Marques, M.J., Genaro, O., Mayrink, W., Martins-Filho, O.A., 2002. Anticorpos antipromastigotas vivas de Leishmania (Viannia) braziliensis, detectados pela citometria de fluxo, para identificão da infecção ativa na leishmaniose tegumentar americana. Rev. Soc. Bras. Med. Trop. 35, 551–562. https://doi.org/10.1590/S0037-86822002000600002.
Rocha, R.D.R., Gontijo, C.M.F., Elói-Santos, S.M., Teixeira-Carvalho, A., Corrêa-Oliveira, R., Ferrari, T.C.A., Marques, M.J., Mayrink, W., Martins-Filho, O.A., 2006. Clinical value of anti-live Leishmania (Viannia) braziliensis immunoglobulin G subclasses, detected by flow cytometry, for diagnosing active localized cutaneous leishmaniasis. Trop. Med. Int. Heal. 11, 156–166. https://doi.org/10.1111/j.1365-3156.2005.01552.x.
Romero, G. a S., Boelaert, M., 2010. Control of visceral leishmaniasis in latin america-a systematic review. PLoS Negl. Trop. Dis. 4, e584. https://doi.org/10.1371/journal.pntd.0000584.
Sakkas, H., Gartzonika, C., Levidiotou, S., 2016. Laboratory diagnosis of human visceral Leishmaniasis. J. Vector Borne Dis. 53, 8–16.
Shapiro, H.M., 2003. Practical Flow Cytometry, fourth ed. New York.
Srivastava, P., Dayama, A., Mehrotra, S., Sundar, S., 2011. Diagnosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 105, 1–6. https://doi.org/10.1016/j.trstmh.2010.09.006.
Sundar, S., Rai, M., 2002. Laboratory Diagnosis of Visceral Leishmaniasis 9. pp. 951–958. https://doi.org/10.1128/CDLI.9.5.951.
Teixeira-Carvalho, A., Campos, F.M.F., Geiger, S.M., Rocha, R.D.R., Araújo, F.F., Vitelli Avelar, D.M., Andrade, M.C., Araújo, M.S.S., Lemos, E.M., Freitas Carneiro Proietti, A.B., Sabino, E.C., Caldas, R.G., Freitas, C.R.C., Campi-Azevedo, A.C., Elói-Santos, S.M., Martins-Filho, O.A., 2015. FC-TRIPLEX Chagas/Leish IgG1: a multiplexed flow cytometry method for differential serological diagnosis of chagas disease and leishmaniasis. PLoS One 10, e0122938. https://doi.org/10.1371/journal.pone.0122938.
Vitelli-Avelar, D.M., Sathler-Avelar, R., Wendling, A.P.B., Rocha, R.D.R., Teixeira-Carvalho, A., Martins, N.É., Dias, J.C.P., Rassi, A., Luquetti, A.O., Elói-Santos, S.M., Martins-Filho, O.A., 2007. Non-conventional flow cytometry approaches to detect anti-Trypanosoma cruzi immunoglobulin G in the clinical laboratory. J. Immunol. Methods 318, 102–112. https://doi.org/10.1016/j.jim.2006.10.009.