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Clinical value of anti-*Leishmania* (*Leishmania*) *chagasi* IgG titers detected by flow cytometry to distinguish infected from vaccinated dogs

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Abstract

Leishmune® vaccination covers a broader number of endemic areas of canine visceral leishmaniasis (CVL) and therefore the development of new serological devices able to discriminate CVL from Leishmune® vaccinees becomes an urgent need considering the post-vaccine seroconversion detected throughout conventional methodologies. Herein, we have described the establishment of a flow cytometry based methodology to detect anti-fixed *L.* (*L.*) *chagasi* promastigotes antibodies (FC-AFPA-IgG, FC-AFPA-IgG1 and FC-AFPA-IgG2) in sera samples from *Leishmania* (*Leishmania*) *chagasi* infected dogs and Leishmune® vaccinees. The results of FC-AFPA were reported along the sera titration curve (1:128–1:524,288), as percentage-of-positive-fluorescent-parasite (PPFP). The use of PPFP = 20% as a cut-off edge to segregate negative and positive results at sera dilution 1:2048 revealed outstanding performance indexes that elect FC-AFPA-IgG and IgG2 (both detected by polyclonal FITC-labeled second step reagent) applicable to the serological diagnosis of CVL, with 100% of specificity for both IgG and IgG2 and 97 and 93% of sensitivity, respectively. Moreover, FC-AFPA-IgG, applied at sera dilution 1:2048, also appeared as a useful tool to discriminate *L. chagasi* infected dogs from Leishmune® vaccinees, with 76% of specificity. Outstanding likelihood indexes further support the performance of FC-AFPA-IgG for exclusion diagnosis of CVL in Leishmune® vaccinees. Analysis of FC-AFPA-IgG at sera dilution 1:8192 revealed the most outstanding indexes, demonstrating that besides the ability of PPFP ≤20% to exclude the diagnosis of CVL, a PPFP values higher 80%, mostly observed for infected dogs (INF) have a minimal change to come from a non-infected animal (NI) or Leishmune®

Abbreviations: AUC, area under the ROC curve; AD, asymptomatic dogs; CVL, canine visceral leishmaniasis; ELISA, enzime-linked immunosorbent assay; FC-AFPA, anti-fixed promastigote *L.* (*L.*) *chagasi* antibodies detected by flow cytometry; FITC, fluorescein isothiocyanate; FML, fucose-mannose ligand; IFAT, indirect fluorescence antibody title; Ig, immunoglobulin; INF, infected dogs; LR, likelihood ratios; NI, non-infected group; NPV, negative predictive value; OD, oligosymptomatic dogs; PCR, polymerase chain reaction; PPFP, percentage-of-positive-fluorescent-parasite; PPV, positive predictive value; ROC curve, receiver operating characteristic curve; VAC, vaccinated dogs; VL, visceral leishmaniasis

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vaccinees (VAC). Together, our findings showed the potential of both anti-*L. chagasi* FC-AFPA-IgG and IgG2 to distinguish the serological reactivity of *L. chagasi* infected dogs from Leishmune® vaccinees, which will further contribute for the differential diagnosis in the context of CVL immunoprophylaxis.

Keywords: *Leishmania* (*L.*) *chagasi*; Flow cytometry; Canine visceral leishmaniasis; Vaccine

1. Introduction

Canine visceral leishmaniasis (CVL) is a debilitating and often fatal disease, caused by *Leishmania* (*Leishmania*) *infantum syn. Leishmania* (*L.*) *chagasi* that affects wild and domestic animals as well as humans in several parts of the Old and New World (WHO, 2000). The disease is widespread in tropical and subtropical areas of Latin America, Europe, Africa and Asia. In Brazil, CVL becomes a serious public health problem with occurrence in rural and several urban areas of metropolitan cities (Arias et al., 1996; Tesh, 1995). From the epidemiological standing point, the canine visceral leishmaniasis is considered to be more important than the human disease, due to its higher prevalence and the fact that both asymptomatic and symptomatic dogs are equally infectious to the vectors (Molina et al., 1994). Moreover, the infected dogs, even those with asymptomatic disease display high frequency of *Leishmania* amastigotes in their skin (Reis et al., 2006a,b).

The major prophylactic practice to control the spread of the urban human visceral leishmaniasis (VL), recommended by the World Health Organization, involves a systematic treatment of human cases besides vector control by insecticide and elimination of seropositive dogs, the main domestic reservoir of VL (Tesh, 1995). However, it is important to mention that the serologic tests recommended by the Brazilian Healthy Ministry (Indirect Immunofluorescence Antibody Test, IFAT and conventional crude antigen enzyme-linked immunosorbent assay, ELISA) display a highly variable efficiency to detect canine *L. chagasi* infection, mainly due to the cross-reactivity of samples from dogs infected with other parasitic diseases and the low ability of those methods to detect seropositivity in asymptomatic dogs (Lira et al., 2006; Mancianti et al., 1995; Dye et al., 1993; Palatnik-De-Sousa et al., 2001). In clinical practice, the veterinarian is usually confronted with cases of positive serology not compatible with or suggestive of CVL according to lack of specific symptoms (Francino et al., 2006). Despite the specificity of the parasitological methods, including direct microscopic demonstration, culture isolation of *Leishmania* parasites from tissue aspirates, its use in clinical laboratory have some limitation, mainly due to the variable and relatively low sensitivity of such procedures (Schnur and Jacobson, 1987; Osman et al., 1997; Reale et al., 1999), besides the time-consuming and requirement of experienced personnel (Mettler et al., 2005). In the last decade, the implementation of molecular devices, based on the polymerase chain reaction (PCR) has contributed to the increment of sensitivity with confirmed specificity to detect *Leishmania* DNA in biological specimens (Pirmez et al., 1999). However, the development of new immunobiological tools to control CVL in endemic areas incited new challenges for the scientific community, considering that vaccines derived from whole crude *Leishmania* antigens as well as third generation DNA vaccines may lead to false positive results even when applying molecular based diagnosis. Therefore, the search toward the development of alternative serological devices still represents an important challenge for many investigators.

Besides these facts, the dog removal based on seropositivity, has been pointed out as drastic tool to reduce cases of VL mainly due to its impact in general society. Therefore, it has been considered that a protective vaccine for dogs against canine visceral leishmaniasis would represent the most effective control tool in eradication of the disease (Marzochi et al., 1985; Dye, 1996).

In this context, Palatnik-De-Sousa et al. (2001) have described the protective effect of the fucose–mannose ligand (FML)-vaccine on canine visceral leishmaniasis. This formulation had already shown to be safe, immunogenic and protective for dogs against *L. chagasi* infection. Recently, the FML-vaccine (Leishmune®, Fort Dodge, EUA) has been registered by the Brazilian Ministry of Agriculture and legally authorized for use by veterinary clinicians.

As expected for the use of saponin adjuvants, the Leishmune® canine vaccine induces a strong humoral immune response, soon after the complete vaccination (Cabrera et al., 1999). Therefore, an indistinct IgG mediated anti-*L. chagasi* immune response is detected in Leishmune® vaccinees, indistinct from that due to

natural infection with *L. chagasi*. As serological devices are still recommended as a tool for epidemiological surveys, the screening of canine population based on conventional serological approaches (IFAT or crude antigen ELISA), have become unconfident to identify naturally infected ones, reservoirs of *Leishmania*, that should be normally removed for sacrifice (Mendes et al., 2003). While some investigations have been able to demonstrate a dichotomous response to *Leishmania* antigens in the seropositive naturally infected dogs and Leishmune® vaccinees (Mendes et al., 2003), the development of new techniques still represent a broad field for investigations aiming to develop alternative methodologies of high specificity and sensitivity for an accurate differential diagnosis of CVL in a population that include vaccinated animals.

Since 1995 our groups have been working on flow cytometry based serological approaches applicable in clinical laboratories (Martins-Filho et al., 1995; Rocha et al., 2002; Bicalho et al., 2004; Reis et al., 2005). Flow cytometry is a well-established methodology in clinical laboratories with a large number of applications been available (Jaroszeski and Radcliff, 1999). While the term flow cytometry refers to the measurement of cells, the approach of making sensitive multiparameter optical measurements in a flowing sample stream is a very general analytical approach (Nolan and Mandy, 2006). The past few years we have worked to establishment of several applications of flow cytometry technology for molecular analysis in human and canine diseases and adapted the flow cytometry measurements using microparticles as solid supports (Martins-Filho et al., 1995; Rocha et al., 2002, 2006; Bicalho et al., 2004; Reis et al., 2005).

In this context, it is possible that IgG subclasses responses may provide a useful marker for disease underlying the post-vaccine seroconversion. Four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) have been described in canine sera samples (Mazza et al., 1993). Despite monoclonal antibodies have been previously used (Quinnell et al., 2003), they are not yet commercially available as fluorochrome-labeled second step reagent neither validated for flow cytometric use.

Herein, we evaluate the performance of the flow cytometry using pre-fixed promastigotes as tool for detection of anti-*L.* (*L.*) *chagasi* immunoglobulin G and subclasses to as the basis for the development of new diagnostic tools for canine visceral leismaniasis that allowed the discrimination between sera sample from Leishmune® vaccinees from *L. chagasi* infected dogs. Our findings support the use of FC-AFPA-IgG and IgG2 (both detected by polyclonal FITIC-labeled second step reagent) as a helpful tool to elucidate seropositivity during large-scale epidemiological screenings of canine visceral leishmaniasis. Moreover, these methodologies would support the use of Leishmune® to control canine kala-azar despite the seroconversion detected by conventional serological approaches following vaccination.

2. Materials and methods

2.1. Study population

Twenty-nine breed adult dogs of both genders, aging from 2 to 6 year-old naturally infected with *Leishmania* (*L.*) *chagasi*, presenting IFAT titer $\geq$1:40 and positive parasitological diagnosis to *Leishmania* in tissue smears (bone marrow, ear, skin, spleen, liver or popliteous lymph node), provided by Control Zoonosis Center at Belo Horizonte (Minas Gerais State, Brazil) were enrolled into the group of infected dogs (INF).

Twenty-one healthy German shepherd dogs, age ranging from 2 to 6 years, maintained at the kennel of Military Police of Minas Gerais, Belo Horizonte, Minas Gerais State, Brazil were submitted to a complete Leishmune® vaccination regimen, and included as a group of vaccinated dogs (VAC). All vaccinees displayed negative serologically test (IFAT and ELISA) prior vaccination procedure. Vaccinations were performed according to the manufacturer's instructions, i.e. all animals have received three doses of the vaccine with an interval of 21 days between each dose. Thirty days after the end of the complete vaccination regimen, sera samples were colleted to perform the flow cytometric assessment of the anti-*L. chagasi* Ig reactivity.

Fifteen age-matched non-infected mongrel dogs, displaying negative serological results (IFAT) and parasitological test to *Leishmania*, maintained in the kennel of Institute of Biological Science of Federal University of Minas Gerais were included as a control non-infected group (NI).

Blood samples (5 mL) with no anticoagulant were collected in disposable 10 cm^3 sterile syringes from jugular or cephalic veins. Sera samples were collected and stored in aliquots at 4 °C, until use for the serological examinations.

This study was approved by the Ethical Committee for the use of Experimental Animals of the Federal University of Minas Gerais—CETEA.

2.2. Serological tests

The indirect immunofluorescence antibody test (IFAT) to detect anti-*Leishmania* IgG antibodies was

carried out as described by Camargo (1966) using the IFAT-canine leishmaniasis kit. The enzyme-linked immunosorbent assay (ELISA) was performed using the EIE-canine leishmaniasis kit. Both kits are produced by Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, Brazil, and all procedures were performed according to the manufacturer's instructions.

2.3. Parasitological tests

Parasitological diagnoses in tissue smears (bone marrow, ear, skin, spleen, liver or popliteous lymph node) were performed after necropsy of the seropositive animals. The fragments of the tissues were used in slide smears by apposition of two microscopy slides. The smears were stained by Giemsa and examined under optical microscopy, for the identification of amastigote forms of *Leishmania.*

2.4. Detection of anti-fixed L. (L.) chagasi promastigotes antibodies by flow cytometry—FC-AFPA

2.4.1. Preparation of parasite suspension

Leishmania chagasi promastigote forms (MHOM/BR/1972/BH46) were grown in liver infusion tryptose medium (LIT) (Camargo, 1964) at 24 ± 1 °C temperature. Stationary-phase parasites (8 days of growth) were transferred to 50 mL polypropylene tubes (Falcon®, Becton Dickinson, San Diego), homogenized in low rotation vortex to dissolve clumps, and submitted to differential centrifugation (100 × *g*, 10 min, room temperature) to remove remaining clusters of parasites contaminants in the pellet. Prior to recover the single-cell parasite suspension, the supernatant was left to rest for 10 min at room temperature. The supernatant consisting of single-cell parasite suspension was transferred to another 50 mL polypropylene tube and spin down at high speed (1000 × *g*) for 10 min at 4 °C. The supernatant was discarded and the pellet washed twice (1000 × *g*, 10 min, 4 °C) in 0.15 M phosphate-buffered saline pH 7.2 (PBS), supplemented with 3% of heat-inactivated fetal bovine serum (FBS) – (GIBCO, Grand Island, NY) – PBS-3%FBS. The organisms were then resuspended immediately in equal volumes of PBS and FACS fix solution (per liter, 10 g of paraformaldehyde, 10.2 g of sodium cacodylate and 6.65 g of sodium chloride, pH 7.2, Sigma Chemical Corp., St. Louis, MO), and stored at 4 °C until use. The suspension of parasites was adjusted to approximately 1.0×10^6 promastigotes/mL and used for flow cytometric immunofluorescence assay.

2.4.2. Immunofluorescence by flow cytometry

The FC-AFPA procedure was carried out as proposed for the detection of anti-live promastigotes antibody by Rocha et al. (2002), modified to detect anti-pre-fixed promastigote *L.* (*L.*) *chagasi* immunoglobulins, as follows: in 96-well U-bottom plates (LINBRO, ICN Biomedicals, Inc. Aurora, OH), 50 μL aliquots of the pre-fixed parasite suspension 5.0×10^5 parasites/well) were incubated at 37 °C for 30 min in the presence of 50 μL of serial serum dilutions in PBS-3% FBS for IgG and IgG2 (1:256–1:524,288) and IgG1 (1:128–1:262,144) detection. All second step reagent used were polyclonal antibodies anti-dog immunoglobulins labeled with fluorescein isothiocyanate (FITC), purchased from Bethyl laboratories Inc. (Montgomery, TX, EUA), referred as cat# A40-105F, A40-120F, A40-121F for the anti-IgG, anti-IgG1 and anti-IgG2, respectively. Therefore, it is important to mention that the second step reagents used to detect the reactivity of IgG subclasses (goat-anti-dog IgG1 and sheep anti-dog IgG2) putatively detects canine IgG1 and IgG2 subclasses and that an optimization should be reconsidered when FITC-labeled monoclonal second step reagents become commercially available to detect canine IgG subclasses.

During the standardization procedures, we have worked thoroughly using different sera dilution starting at 1:32 up to 524,288. The analysis of IgG, IgG1 and IgG2 reactivity of poled sera samples from *L.* (*L.*) *chagasi* infected dogs allow us to identify 1:256, 1:128 and 1:256 as the first sera dilutions were no pro-zone effect was observed. Besides pro-zone effects leading to low PPFP reactivity at lower sera dilutions, agglutination of promastigotes were also another decisive factor taken into account to chose the selected sera dilutions.

Following the incubation with the diluted sera samples, parasites were washed twice with 150 μL of PBS-3% FBS (1000 × *g*, 10 min, 4 °C). The parasites were re-incubated in the dark, for 30 min at 37 °C in the presence of 50 μL of anti-canine IgG, IgG1 or IgG2 FITC-labeled polyclonal antibodies (Bethyl laboratories Inc., Montgomery, TX, EUA). During standardization procedure, a detailed titration of the secondary antibodies was performed to identify the best dilution factor for each second step reagent. Three different dilutions for each FITC-conjugated sheep (putatively anti-IgG and anti-IgG2) and goat (putatively anti-IgG1) polyclonal antibody anti-camine immunoglobulin were used, including 1:500, 1:1000 and 1:2000 for anti-IgG and anti-IgG2 and 1:250, 1:500 and 1:1000 for anti-IgG1.

After incubation with the second step fluorescent reagent, previously diluted PBS-3% FBS anti-IgG

(1:1000), IgG1 (1:500) and IgG2 (1:1000), two washing procedure were performed using 150 μL of PBS-3% FBS (1000 × *g*, 10 min, 4 °C). The FITC-labeled parasites were then fixed with a FACS fix solution for 30 min at 4 °C, and maintained for at least 30 min, at 4 °C in the dark, prior the flow cytometric data acquisition.

An internal control of the reaction, in which the parasites were incubated in the absence of dog serum, but in the presence of the FITC-labeled secondary reagents, was included in all set of experiments to monitor unspecific bindings. In all batches of FC-AFPA, positive and negative control samples were also run to certify the test performance.

2.4.3. Flow cytometric data acquisition and analysis

Flow cytometric measurements were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), interfaced to an Apple FACStation and the Cell-Quest™ software package used for data acquisition, storage and analysis.

A total of 5000 events were acquired for each sera sample dilution tested. *Leishmania* promastigotes were identified on the basis of their specific forward (size-FSC) and side (granularity-SSC) light scattering properties. Following FSC and SSC gain adjustments, parasites were found assuming a homogeneous and characteristic elliptical distribution on FSC × SSC dot plots. The standard profile was obtained by setting FSC gain at E00 and SSC gain at 300, both on LOG scale. Threshold was positioned at FSC channel ranging from 300 to 400. The fluorescence (FL1) was also set in LOG scale mode using gain of 547, as recommended value derived from the flow cytometer calibration with CaliBrite™ kit. Promastigotes were first selected by gating on the FSC × SSC dot plots and the relative FITC fluorescence intensity of each set of events quantified by single color histogram representation (FL1). On the basis of the histogram representing the internal control of unspecific binding of the second step reagent, a marker was set up in order to confine 98% of events into a region of lower fluorescence intensity. Once established this marker was further maintained in all data analysis to determine, for each test sample, the percentage of positive fluorescent parasites (PPFP).

2.5. Statistical analysis

The tests' performance was assessed by percentage, including:

Sensitivity = [true positives/(true positive samples + false negative samples)] × 100.
Specificity = [true negatives/(true negative samples + false positive samples) × 100.
Positive predictive value – PPV = (true positive samples/total of positive samples) × 100.
Negative predictive value – NPV = (true negative samples/total of negative samples) × 100.
Youden's J Index = Sensitivity + Specificity − 1; (Youden, 1950).

The *receiver operating characteristic curve* (ROC curve) was used to select the cut-off value to discriminate between positive and negative results of FC-AFPA-IgG (Greiner et al., 2000). The test's global accuracy was also evaluated by taking the area under the ROC curve (AUC) as proposed by Swets (1988).

The tests' performances were also investigated at distinct ranges of PPFP values by the determination of *likelihood ratios* (LR), as follow:

Positive LR = Sensitivity/(1 − Specificity).
Negative LR = (1 − Sensitivity)/Specificity.

The gold standard used for positive diagnosis of *L.* (*L.*) *chagasi* infection was the histological detection of the amastigote in the host tissue evaluated (bone marrow, ear, skin, spleen, liver or popliteous lymph node) besides positive serology detected throughout IFAT and ELISA, as formerly described.

3. Results

3.1. Performance indexes elect FC-AFPA-IgG and IgG2 applicable to the serological diagnosis of CVL

Sera samples from 29 *L. chagasi* naturally infected dogs (INF) and 15 non-infected healthy animals (NI) were tested by FC-AFPA-IgG, FC-AFPA-IgG1 and FC-AFPA-IgG2 in parallel experiments using FITC-labeled sheep (anti-IgG and anti-IgG2) and goat (anti-IgG1) polyclonal antibodies.

The mean values for the percentage of positive fluorescent parasites (PPFP) observed along the FC-AFPA-IgG, IgG1 and IgG2 titration curves are presented in Fig. 1. Data analysis demonstrated that the mean PPFP values obtained from FC-AFPA-IgG and IgG2 led to a clear segregation between INF and NI. Whereas NI was confined into a region of low PPFP values, INF displayed PPFP values restricted to a region of high reactivity with higher segregation amplitude at serum dilution 1:2048 (Fig. 1, top graphs). Despite the

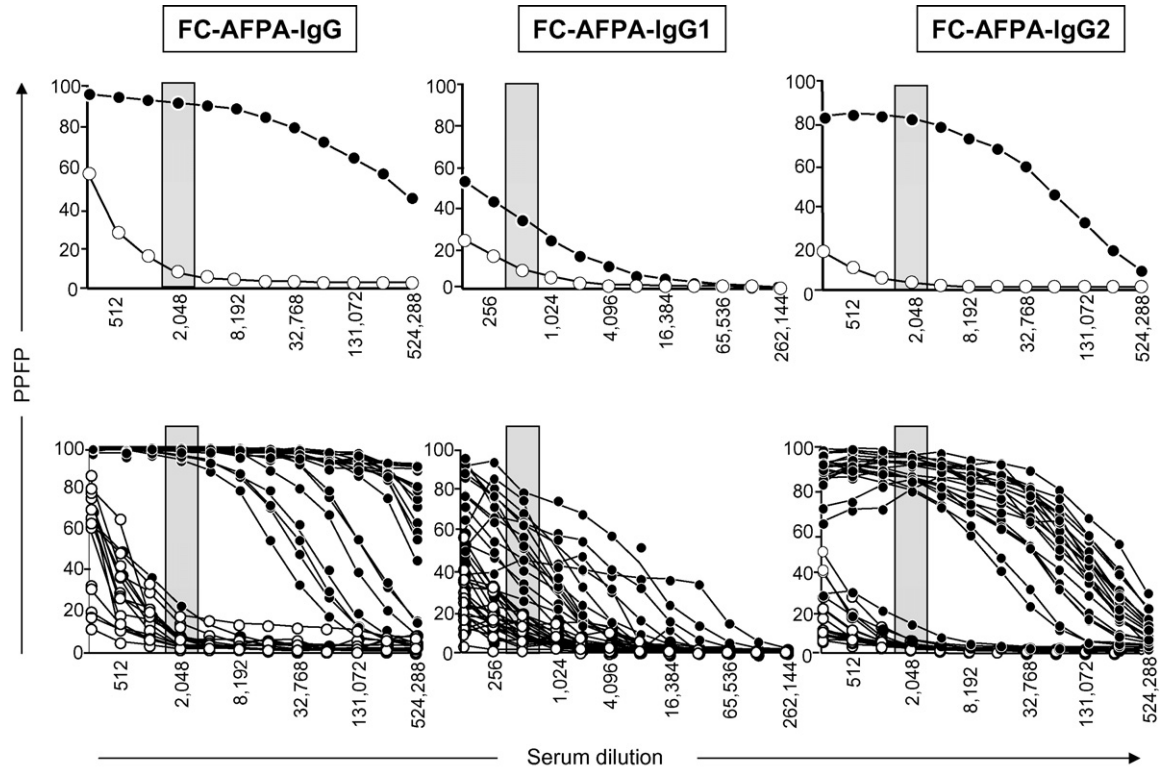

Fig. 1. FC-AFPA-IgG, FC-AFPA-IgG1 and FC-AFPA-IgG2 reactivity in sera samples from non-infected (NI (○)) and *L. chagasi* infected dogs (INF (●)). The reactivity of IgG and IgG subclasses were measured by FITC-labeled sheep (putatively anti-IgG and anti-IgG2) and goat (putatively anti-IgG1) anti-canine second step reagent. The results are expressed as mean PPFP (top graphs) and individual PPFP values (bottom graphs) from 1:256 to 1:524,288 sera dilutions for FC-AFPA-IgG and IgG2 and from 1:128 to 1:131,072 sera dilutions for FC-AFPA-IgG1. The rectangle represents the selected sera dilutions of higher segregation range between NI and INF (1:2048 for FC-AFPA-IgG and IgG2 and 1:512 for FC-AFPA-IgG1).

lower ability of FC-AFPA-IgG1 to segregated INF from NI, data analysis suggested the sera dilution 1:512 as the best choice to discriminate PPFP values form INF and NI (Fig. 1, middle top graph). Analysis of PPFP values at individual level further confirmed these findings (Fig. 1, bottom graphs).

Performance analysis of FC-AFPA IgG, IgG1 and IgG2 were initially taken using the *receiver operating curve* (ROC curve) as a basic statistical device to determine the better cut-off edge of PPFP values to segregate, at selected dilutions, the reactivity of sera samples from INF and NI (Fig. 2, top graphs). Data analysis suggested that PPFP = 20% was the most appropriated cut-off to discriminate INF from NI, throughout the proposed methodologies.

Furthermore, the analysis of the area under the ROC curve (AUC) demonstrated, as suggested by Swets (1988), high global accuracy for FC-AFPA-IgG (AUC = 0.98; 95% CI: 0.89–0.99) and FC-AFPA-IgG2 (AUC = 0.99; 95% CI: 0.91–1.0) with moderate score for FC-AFPA-IgG1 (AUC = 0.82; 95% CI: 0.67–0.92) (Fig. 2, top graphs).

Following the establishment of PPFP = 20%, as the cut-off line to classify the FC-AFPA data as negative (PPFP $\leq$ 20%) or positive results (PPFP $>$ 20%), our data further confirmed that FC-AFPA-IgG and IgG2 display better ability to generate positive results to INF (97 and 93%, respectively) than FC-AFPA-IgG1 (59%). However, all three proposed methodologies were able to identify with negative results all samples of non-infected control dogs (Fig. 2, bottom graphs).

In order to further validate these findings throughout additional statistical devices we have accessed a range of performance indexes for FC-AFPA-IgG and IgG2 applied at sera dilution 1:2048 (Table 1). Data analysis demonstrated, high sensitivity (97 and 93%, for FC-AFPA-IgG and IgG2, respectively) and specificity (100% for both methodologies), suggesting a clinical value for the use to these immunoglobulins, detected by FITC-labeld polyclonal second step reagent, applicable for the serological diagnosis of CVL. Analysis of predictive values further emphasize that while a positive result obtained by FC-AFPA-IgG and IgG2 contributes to confirm the diagnosis of CVL (PPV = 100% for both

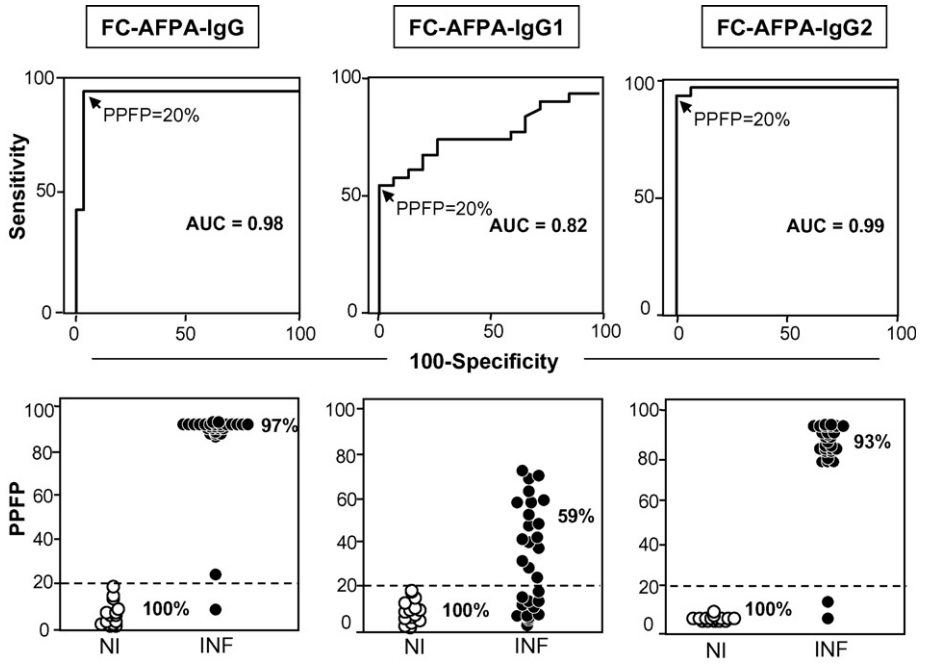

Fig. 2. ''Receiver operating curves''—ROC curves derived for FC-AFPA-IgG, FC-AFPA-IgG1 and FC-AFPA-IgG2 (top panels) indicating the area under the curve (AUC = global accuracy) and the location of the best PPFP value used as a cut-off edge (arrows) to analyze the anti-*Leishmania* immunoglobulin G. The IgG, IgG1 and IgG2 reactivity at individual level were measured by FITC-labeled sheep (putatively anti-IgG and anti-IgG2) and goat (putatively anti-IgG1) anti-canine second step reagent (bottom graphs). The results of FC-AFPA for individual samples from non-infected (NI (○)) and *L. chagasi* infected dogs (INF (●)) are expressed as PPFP at sera dilution 1:2048 for FC-AFPA-IgG and IgG2 and 1:512 for FC-AFPA-IgG1. The doted line represents the cut-off edge between negative and positive results. Sensitivity and specificity indexes are provided on graphs.

methodologies), a negative test also contributes to exclude the diagnosis of CVL (NPV = 94% and NPV = 88%, for FC-AFPA-IgG and IgG2, respectively) (Table 1). Additional analysis demonstrated that PPV and NPV of FC-AFPA-IgG and IgG2 have effective contribution in a wide range of pre-test probability including 5–95 and 5–80%, respectively (data not shown). Furthermore, analysis of accuracy (98 and 99% for FC-AFPA-IgG and IgG2, respectively) and Youden's J indexes (94 and 88% for FC-AFPA-IgG and IgG2, respectively) confirmed the excellent performance of both FC-AFPA-IgG and IgG2 (Table 1).

Table 1
Performance index of FC-AFPA-IgG and IgG2 applied in the serological diagnosis of CVL

Indexes[a]	FC-AFPA	
	IgG (1:2048)	IgG2 (1:2048)
Sensitivity	97 (83–99)	93 (78–98)
Specificity	100 (79–100)	100 (80–100)
PPV	100 (88–100)	100 (87–100)
NPV	94 (72–95)	88 (66–97)
Acuracy	98 (89–99)	99 (91–100)
J Youden	94 (81–99)	88 (74–95)
Lekelihood Ratio		
PPFP ≤20%	0.04[b]	0.03[b]
PPFP >20%	>10[b]	>10[b]

IgG and IgG2 reactivity was measured using FITC-labeled polyclonal sheep anti-canine imunoglobulins, that putatively detects total immunoglobulin G fraction and IgG2 subclasses, respectively.

[a] Performance indexes are expressed as percentage (confidence interval, CI 95%), except for LR values expressed in chances.

[b] LR values with high clinical significance.

The analysis of FC-AFPA-IgG and IgG2 performances according to the Likelihood ratio (LR) demonstrated that PPFP ≤20% practically exclude the possibility of CVL, whereas PPFP >20% basically confirm the diagnosis of CVL (Table 1).

3.2. FC-AFPA-IgG and IgG2 as a tool to discriminate L. chagasi infected dogs from Leishmune® vaccinees

A current challenge regarding the vaccine practice in canine visceral leishmaniasis is the urgent need for alternative serological devices able to discriminate dogs with active *L. chagasi* infection from those vaccinated with Leishmune®.

Aiming to contribute with this field of investigation, we have evaluated the clinical value of FC-AFPA-IgG and IgG2 to segregate the post-infection imunomediated

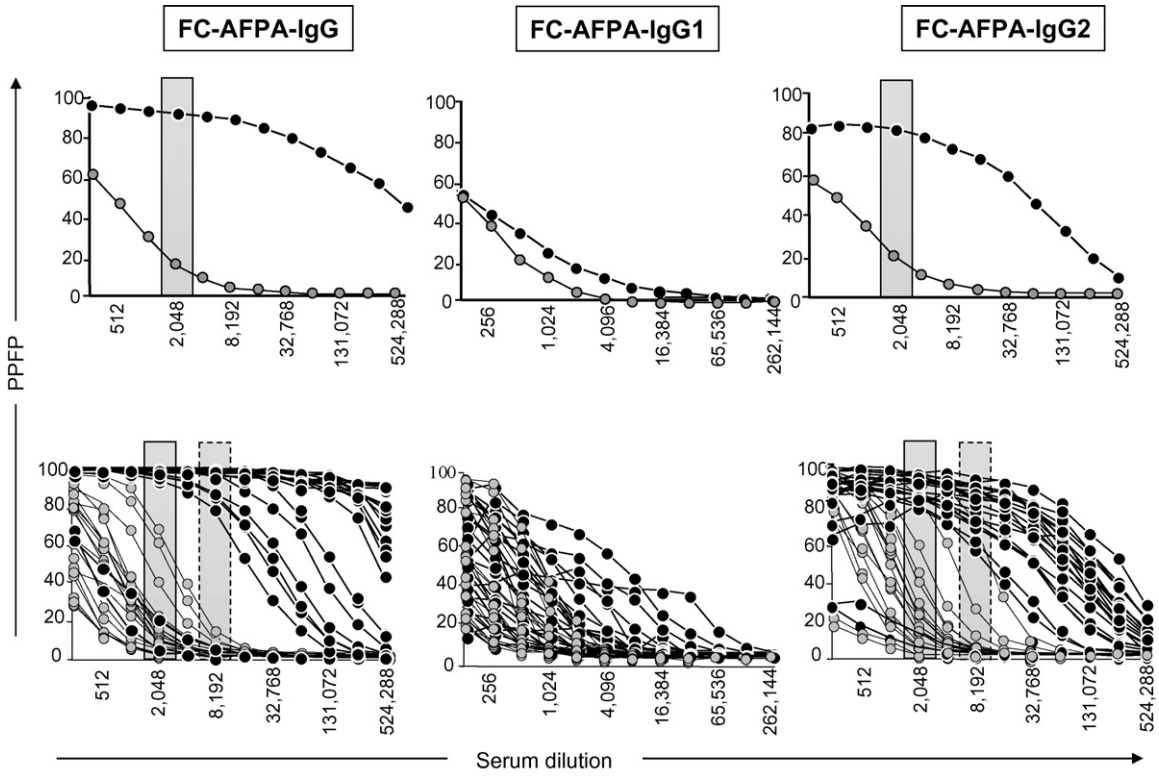

Fig. 3. FC-AFPA-IgG, FC-AFPA-IgG1 and FC-AFPA-IgG2 reactivity in sera samples from *L. chagasi* infected dogs (INF (●)) and Leishmune® vaccines (VAC (◍)). The reactivity of IgG and IgG subclasses were measured by FITC-labeled sheep (putatively anti-IgG and anti-IgG2) and goat (putatively anti-IgG1) anti-canine second step reagent. The results are expressed as mean PPFP (top graphs) and individual PPFP values (bottom graphs) from 1:256 to 1:524,288 sera dilutions for FC-AFPA-IgG and IgG2 and 1:128 to 1:131,072 sera dilutions for FC-AFPA-IgG1. The dotted rectangle represents the selected sera dilution 1:2048 of higher segregation range between NI and INF for FC-AFPA-IgG and IgG2.

immunoglobulin reactivity from that triggered by prophylactic immunization. For this purpose, sera samples from 29 *L. chagasi* infected dogs and 21 Leishmune® first time vaccinees at 30 days after vaccination were assayed by FC-AFPA and the results presented as PPFP values for a range of sera dilutions.

Titration curves displaying IgG and IgG2 reactivity, detected by FITC-labeled polyclonal second step reagent, for INF and VAC groups are presented in Fig. 3. Our data demonstrated differential mean (Fig. 3, top graphs) and individual (Fig. 3, bottom graphs) PPFP values for INF and VAC dogs, all along the FC-AFPA-IgG and IgG2 titration curves, confirming again the low performance of IgG1 to segregation purposes (Fig. 3). Data analysis was performed using the same criteria previously proposed for diagnostic application of FC-AFPA, i.e. considering PPFP = 20% as the cut-off line to segregate negative and positive results at sera dilution 1:2048. Additional analysis of the IgG and IgG2 titration curves allows us to further identify 1:8192 as an outstanding sera dilution to discriminate INF from VAC (rectangle Fig. 3, bottom graphs).

Analysis of PPFP values at individual level among NI, INF and VAC, at selected sera dilutions, further confirmed these findings and elected the dilution 1:8192 rather than 1:2048 with higher performance for the segregation purposes (Fig. 4). Taking PPFP = 20% as cut-off edge, to discriminate positive from negative results, our data demonstrated that FC-AFPA-IgG at serum dilution 1:2048 displayed co-positivity of 97% for INF (27/29) with co-negativity of 100 and 76% for NI (15/15) and VAC (15/21), respectively (Fig. 4, left top graph). Analysis of FC-AFPA-IgG2 at serum dilution 1:2048 displayed lower performance indexes co-positivity of 93% for INF (27/29) and co-negativity of 100 and 43% for NI (15/15) and VAC (9/21), respectively (Fig. 4, right top graph). Analysis of FC-AFPA-IgG2 at sera dilution 1:8192 in comparison to 1:2048, confirmed the co-negativity of 100% for NI (15/15), and highlighted the better performance for VAC, with increment of co-negativity from 43 to 95%. Outstanding was the ability of FC-AFPA-IgG at sera dilution 1:8192 to discriminate NI and VAC from INF with co-negativity of 100% (Fig. 4, left bottom graph).

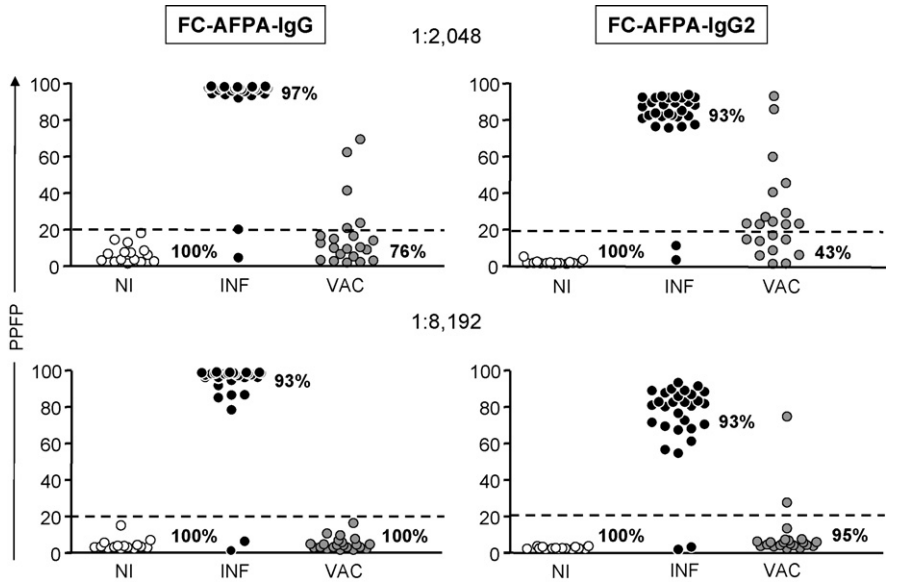

Fig. 4. Anti-*L. chagasi* FC-AFPA-IgG and IgG2 reactivity of individual sera samples from non-infected (NI (○)), *L. chagasi* infected (INF (●)) and Leishmune® vaccinees (VAC (◍)). The reactivity of IgG and IgG2 were measured by FITC-labeled sheep anti-canine second step reagent that putatively detects total IgG fraction IgG2 immunoglobulins, respectively. The results are expressed as PPFP for individual samples, at sera dilution 1:2048 (top graphs) and 1:8192 (bottom graphs). The doted line represents the cut-off edge between negative and positive results. Co-negativity and co-positivity indexes are provided on graphs.

It is important to mention that prior vaccination, all vaccines showed negative serology by ELISA, IFAT and by FC-AFPA. At day 30 after complete vaccination regimem (three 21-days apart consecutive doses) all Leishmune® vaccinees displayed seroconversion detectable by conventional methodologies, including ELISA and IFAT all Leishmune® vaccinees displayed seroconversion detectable by conventional methodologies, including ELISA and IFAT. Data analysis from conventional ELISA serology using a cut-off = 0.095, and the gray zone ranging from 0.076 to 0.114 demonstrated that all uninfected/unvaccinated dogs showed optical density from 0.062 to 0.070, whereas vaccinated dogs showed DO ranging from 0.144 to 0.221 and *L. chagasi* infected animals DO of 0.142–0.251. Data from IFAT demonstrated that while all uninfected/unvaccinated dogs demonstrated negative results at sera dilution 1/40, vaccinated animals showed positivity ranging from 1/40 to 1/320 whereas *L. chagasi* infected dogs displayed seropositivity up to sera dilution1/640.

Outstanding likelihood ratio indexes further support the performance of FC-AFPA-IgG for exclusion diagnosis of CVL in Leishmune® vaccinees.

Analysis of likelihood ration has been applied as an important statistical approach to validate the use of immunological features in clinical laboratories. Moreover, the possibility to calculate the LR indexes for a given set of results within the global data range allow a descriptive interpretation of different zones of PPFP able to confirms the applicability of both FC-AFPA-IgG and IgG2 to distinguish INF and VAC.

Although FC-AFPA-IgG2 applied at sera dilution 1:2048 did not contribute to discriminate NI and VAC from INF, clinical value of IgG2 can be observed at sera dilution 1:8192. Our data demonstrated that PPFP values lower or equal 20% practically excludes the diagnosis of CVL whereas PPFP >80% practically confirms the diagnosis of CVL (Table 2).

Higher performance of FC-AFPA-IgG was observed as compared to IgG2. Indeed, analysis of FC-AFPA-IgG at sera dilution 1:2048 demonstrated that PPFP values lower or equal 20% practically exclude the diagnosis of CVL for a give sample, whereas PPFP >80% practically confirms the diagnosis of CVL, excluding the possibility of similar results come from a Leishmune® vaccine. Additionally, analysis of FC-AFPA-IgG at sera dilution 1:8192 demonstrated the most outstanding results. Besides the ability of PPFP ≤20% to exclude the diagnosis of CVL, results confined into a broader range of PPFP values, starting over 20%, could also be applied as a complementary data to confirm the diagnosis of CVL. On the other hand a PPFP value higher 80%, mostly observed INF has a minimal change to come from a NI or VAC.

Table 2
Performance of FC-AFPA-IgG and IgG2, reported as likelihood ratio to distinguish *L. chagasi* infected dogs from Leishmune® vaccinees

Cut-off values	Sera dilution			
	1:2048		1:8192	
	FC-AFPA-IgG	FC-AFPA-IgG2	FC-AFPA-IgG	FC-AFPA-IgG2
PPFP ≤20%	0.05[a]	0.16	0.07[a]	0.08[a]
PPFP >20%	4.06	1.63	>10[a]	9.78
PPFP >80%	>10[a]	8.33	>10[a]	>10[a]

IgG and IgG2 reactivity was measured using FITC-labeled polyclonal sheep anti-canine imunoglobulins, that putatively detects total immunoglobulin G fraction and IgG2 subclasses, respectively.
[a] Likelyhood ratio values with high clinical significance.

4. Discussion

The accomplishment of CVL immunoprophylaxis with the availability of Leishmune® for veterinary uses in clinics surrounding the CVL endemic areas has prompted out many issues regarding the value of positive serological reactivity to distinguish CVL from Leishmune® vaccinees. In this context, the RIFI and conventional ELISA have been considered unable to discriminate *Leishmania*-infected from Leishmune® vaccinees causing serious problems for the public health domain. Despite the cumulative knowledge and the establishment of alternative serological approaches, including direct agglutination test—DAT (Cardoso et al., 2004; Silva et al., 2006) and rK39 based serology (Burns et al., 1993; Reis et al., 2006b), the differential diagnosis of CVL in endemic areas upon Leishmune® vaccination, still remains to be elucidated.

Previous reports have demonstrated that distinct mean IgG subclasses reactivity, specifically anti-FML IgG1/IgG2 ratio, was able to discriminate groups of *L. chagasi* infected animals from Leishmune® vaccinees (Mendes et al., 2003). Indeed, these authors were able to demonstrate a differential IgG1/IgG2 ratio by FML-ELISA, with higher IgG1 isotype associated with natural infection and IgG2 isotype correlated with highly protective vaccination. Despite the applicability of this serological approach to distinguish the mean serological reactivity between groups of animals, at clinical laboratories, the analysis of a given serological data should be considered at individual level, in order to release a conclusive report. Usually, the establishment of a cut-off edge for serological reactivity needs to be applied to segregate positive and negative samples.

Usually, the establishment of a cut-off edge for serological reactivity needs to be applied to segregate positive and negative samples. Therefore, the development of new serological approaches of high specificity and sensitivity for diagnosis of CVL that also could discriminate throughout differential IgG reactivity for *Leishmania*-infected and Leishmune® vaccinated dogs, at individual level, represents a relevant challenge in the field of CVL.

For this purposes, herein, we have presented a new flow cytometry based methodology using fixed promastigotes of *L. chagasi* as tool for detection of anti-immunoglobulin G and subclasses (IgG1 and IgG2) available for the first time with high performance to diagnose CVL in a canine population sample that also includes Leishmune® vaccinees. In is important to mention that all Leishmune® vaccinees displayed positive results at conventional serology (ELISA and IFAT) unable to discriminate them from INF.

Initially, the high specificity of FC-AFPA-IgG, IgG1 and IgG2 suggested their value to exclude the possibility of CVL. Moreover, the higher sensitivity of FC-AFPA-IgG and FC-AFPA-IgG2, but not FC-AFPA IgG1 demonstrated their excellence and clinical value to discriminate INF from NI. Outstanding performance indexes further confirmed the applicability of FC-AFPA IgG and IgG2, applied at the sera dilution 1:2048, taking PPFP = 20% as the cut-off edge to discriminate negative and positive results, as alternative tools in the diagnosis of CVL. Indeed, we have observed that a positive result of FC-AFPA-IgG and IgG2 contributes to the diagnosis of CVL and a negative FC-AFPA-IgG and IgG2 tests contributes to exclude the diagnosis of CVL in a broad zone of prevalence suggesting the applicability these methodologies in endemic areas despite the prevalence of the disease. Together, these results emphasize that our flow cytometry-based methodology (FC-AFPA-IgG and FC-AFPA-IgG2) have superior performance indexes, and therefore higher clinical values to identify CVL cases, than those conventional serological methods previously reported (ELISA and IFAT).

The low performance of FC-AFPA-IgG1 is in agreement with previous reports of extremely variable

levels of IgG1 in many *L. chagasi* infected dogs, including asymptomatic and symptomatic dogs (Deplazes et al., 1995; Solano-Gallego et al., 2001; Reis et al., 2006a). The two cases of seronegative FC-AFPA-IgG ($n = 1$) and FC-AFPA-IgG2 ($n = 2$) observed for INF, were further identified as a oligosymptomatic *L. chagasi* infected dog displaying low parasite density (data not shown) as described elsewhere (Reis et al., 2006a). We hypothesize that these oligosymptomatic dogs may represent cases of recent *L. chagasi* infection, based on previous reports that IgG specific antibody levels may take 1.5–3 months to appear after *L. infantum* experimental infection (Abranches et al., 1991) and that the sensitivity of antibody detection is generally lower in early canine infection (Mettler et al., 2005).

Additionally, we have evaluated the performance of AFPA-IgG and IgG2, applied at the sera dilution 1:2048, to distinguish INF from VAC at individual level. Our data demonstrated that FC-AFPA IgG has higher performance indexes in comparison to FC-AFPA-IgG2. Whereas most VAC presented negative results by FC-AFPA-IgG lower frequency of seronegative results was observed for IgG2. Differences inherent to imunofluorescence based methodologies, such as the dilution of second step fluorescent reagents may count for these differences since anti-IgG and anti-IgG2 secondary antibodies were used at distinct dilutions. It is important to mention that this apparently controversial result may indicate that the antibodies have different affinities for their ligands or that the polyclonal nature of the second step reagents used in our study may not be absolutely guarantee the specificity for the subclass recognition as previously described by Mazza et al. (1993). Moreover, as the PPFP reactivity reflect not only the amount of immunoglobulin attached to the promastigotes by also the number of fluorochrome molecules conjugated to each second step reagent, the fluorochrome/protein ration should be considered besides the stechiometry of the antigen/antibody binding, when performing comparative analysis between FC-AFPA IgG, IgG1 and IgG2. Sera dilution also represents a limiting factor in semi-quantitative serologic methodologies and therefore should be considered to take a precise data interpretation that leads to correct results. Indeed, we have observed that, at sera dilution 1:8192, both FC-AFPA-IgG and IgG2 displayed more outstanding performance as compared to 1:2:048. Additionally, descriptive analysis of likelihood ration allowed data interpretation at different zones of PPFP values that confirm the applicability of both FC-AFPA-IgG and IgG2 to distinguish INF and VAC, when applied at sera dilution 1:8192. As described by Jaeschke et al. (1994), LR values ranging from 5 to 10 generate moderate shifts pre-test to post-test probability, whereas LR lower than 0.1 generate large and often conclusive changes form pre to post-test probability. Herein, PPFP >80% generated from FC-AFPA-IgG and IgG2 at sera dilution 1:2048, are 10 and 8 times more likely to come from INF than VAC. Additionally, analysis of FC-AFPA-IgG at sera dilution 1:8192 demonstrated the most outstanding results. Besides the ability of PPFP ≤20% to exclude the diagnosis of CVL, a PPFP values higher 80%, mostly observed INF have a minimal change to come from a NI or VAC.

Together, our findings demonstrated that FC-AFPA-IgG and IgG2 have a better performance in comparison to conventional methodologies (IFAT and ELISA with crude antigen). It is important to mention that other ELISA-based methodologies, i.e. those using recombinant antigen preparations (rK39 and rK26) also represent an important field for investigations, considering its proven application in the diagnosis of CVL (Rosário et al., 2005). We have previously performed rK39-ELISA applied to the diagnosis on *L. chagasi* infection including animals bearing distinct clinical forms of the disease (Reis et al., 2006a,b). It has been already demonstrated that rK39-ELISA display restricted application to predict the active infection when testing dog samples displaying antibody titer between 1/40 and 1/320 (Genaro et al., 1997), major characteristics of asymptomatic dogs (Reis et al., 2006a,b). Those results pointed out that despite high specificity of rK39-ELISA, the low sensitivity, specially for asymptomatic dogs should be considered when confronting negative results from rK39-ELISA with a population that include cases of CVL, lacking specific symptoms, and animals vaccinated with Leishmune®. We are currently undertaking a parallel investigation to provide comparative evaluation of rK39-ELISA and FC-AFPA-IgG and IgG2 as concern its ability to discriminate *L. chagasi* infected and Leishmune® vaccinated animals. Considering that Leishmune® is a purified antigen based vaccine, it is highly expected that rK39-ELISA would also provide in most cases a powerful tool to discriminatory serological diagnosis on CVL.

In conclusion, we have presented the potential of both FC-AFPA-IgG and IgG2 regarding their applicability to distinguish the serological profile of *L. chagasi* infected dogs from Leishmune® vaccinees, which will further contribute for the confirmatory diagnosis in the context of CVL immunoprophylaxis. It is important to mention that the second step reagents

used to detect the reactivity of IgG and IgG subclasses (that putatively recognize canine IgG1 and IgG2) are polyclonal second step reagent and that an optimization should be re-considered when FITC-labeled monoclonal second step reagents become commercially available to detect canine IgG subclasses. Once monoclonal antibodies to canine IgG subclasses become commercially available as fluorochrome-conjugated second step reagent and validated for flow cytometric protocols further studies should be conduced to focus on IgG1 and IgG2 as well as the other relevant IgG subclasses, i.e. IgG3 and IgG4 (Mazza et al., 1993; Quinnell et al., 2003). The information generated by this study contributes for the trouble-free use of this vaccine in the public health, considering the lack of seroconversion after vaccination detectable by in FC-AFPA at sample dilution 1:8192.

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References

Abranches, P., Silva-Pereira, M.C.D., Conceição-Silva, F.M., Santos-Gomes, G.M., Janz, J.G., 1991. Canine Leishmaniasis: pathological and ecological factors influencing transmission of infection. J. Parasitol. 77, 557–561.

Arias, J.R., Monteiro, P.S., Zicker, F., 1996. The reemergence of visceral leishmaniasis in Brazil. Emerg. Infect. Dis. 2, 145–146.

Bicalho, K.A., Ribeiro, M.F., Martins-Filho, O.A., 2004. Molecular fluorescent approach to assessing intraerythrocytic hemoprotozoan Babesia canis infection in dogs. Vet. Parasitol. 125, 221–235.

Burns Jr., J.M., Shreffler, W.G., Benson, D.R., Ghalib, H.W., Badaró, R., Reed, S.G., 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci. U.S.A. 90, 775–779.

Cabrera, G.P., Da Silva, V.O., Da Costa, R.T., Reis, A.B., Mayrink, W., Genaro, O., Palatnik-de-Sousa, C.B., 1999. The fucose-mannose ligand-ELISA in the diagnosis and prognosis of canine visceral leishmaniasis in Brazil. Am. J. Trop. Med. Hyg. 61, 296–301.

Camargo, E.P., 1964. Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid media. Rev. Inst. Med. Trop. São Paulo 6, 93–100.

Camargo, E.P., 1966. Fluorescent antibody test for the serodiagnosis of American Trypanosomiasis: technical modification employing preserved culture forms of *Trypanosoma cruzi* in slide test. Rev. Inst. Méd. Trop. São Paulo 8, 227–235.

Cardoso, L., Schallig, H.D., Neto, F., Kroon, N., Rodrigues, M., 2004. Serological survey of *Leishmania* infection in dogs from the municipality of Peso da Regua (Alto Douro Portugal) using the direct agglutination test (DAT) and fast agglutination screening test (FAST). Acta Trop. 91, 95–100.

Deplazes, P., Smith, N.C., Arnold, P., Lutz, H., Eckert, J., 1995. Specific IgG1 and IgG2 antibody responses of dogs to *Leishmania infantum* and other parasites. Parasite Immunol. 17, 451–458.

Dye, C., Vidor, E., Dereure, J., 1993. Serological diagnosis of leishmaniasis: on detecting infection as well as disease. Epidemiol. Infect. 110, 647–656.

Dye, C., 1996. The logic of visceral leishmaniasis control. Am. J. Trop. Med. Hyg. 55, 125–130.

Francino, O., Altet, L., Sanchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sanchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. 137, 214–221.

Genaro, O., Costa, R.T., França-Silva, J.C., Reis, A.B., Silva, J.C., Vieira, E.P., Arias, J.R., Monteiro, P.S., Reed, S.G., Mayrink, W., Costa, C.A., Netto, E.M., Badaró, R., 1997. Evaluation of an immunochromatographic assay for the diagnosis of dogs experimentally and naturally infected with *Leishmania chagasi*, in Brasil. Acta Parasitol. Turcica 21, 93–193.

Greiner, M., Pfeiffer, D., Smith, R.D., 2000. Principles and pratical application of the receiver-operating characteristic analysis for diagnostic tests. Prev. Vet. Med. 45, 23–41.

Jaeschke, R., Guyatt, G.H., Sackett, D.L., 1994. Users' guides to the medical literature. III. How to use an article about a diagnostic test. B. What are the results and will they help me in caring for my patients? The Evidence-Based Medicine Working Group. JAMA 271, 703–707.

Jaroszeski, M.J., Radcliff, G., 1999. Fundamentals of flow cytometry. Mol. Biotechnol. 11, 37–53.

Lira, R.A., Cavalcanti, M.P., Nakazawa, M., Ferreira, A.G., Silva, E.D., Abath, F.G., Alves, L.C., Souza, W.V., Gomes, Y.M., 2006. Canine visceral leishmaniosis: a comparative analysis of the EIE-leishmaniose-visceral-canina-Bio-Manguinhos and the IFI-leishmaniose-visceral-canina-Bio-Manguinhos kits. Vet. Parasitol. 137, 11–16.

Martins-Filho, O.A., Pereira, M.E.S., Carvalho, J.F., Cançado, J.R., Brener, Z., 1995. Flow cytometry, a new approach to detect anti-live trypomastigote antibodies and monitor the efficacy treatment in human Chagas disease. Clin. Diagn. Lab. Immunol. 2, 569–573.

Mancianti, F., Falcone, M.L., Giannelli, C., Poli, A., 1995. Comparison between an enzyme-linked immunosorbent assay using a detergent-soluble *Leishmania infantum* antigen and indirect immunofluorescence for the diagnosis of canine leishmaniosis. Vet. Parasitol. 59, 13–21.

Marzochi, M.C., Coutinho, S.G., De Souza, W.J., De Toledo, L.M., Grimaldi Junior, G., Momen, H., Pacheco, R., Da, S., Sabroza, P.C., De Souza, M.A., Rangel Junior, F.B., 1985. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977–1983). Mem. Inst. Oswaldo Cruz. 80, 349–357.

Mazza, G., Duffus, W.P., Elson, C.J., Stokes, C.R., Wilson, A.D., Whiting, A.H., 1993. The separation and identification by monoclonal antibodies of dog IgG fractions. J. Immunol. Methods 161, 193–203.

Mendes, C.O., Paraguai de Souza, E., Borja-Cabrera, G.P., Mello Batista, M.L., Santos, M.A., Parra, L.E., Menz, I., Palatnik de Souza, C.B., 2003. IgG1/IgG2 antibody dichotomy in sera of vaccinated or naturally infected dogs with visceral leishmaniosis. Vaccine 21, 2589–2597.

Mettler, M., Grimm, F., Capelli, G., Camp, H., Deplazes, P., 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J. Clin. Microbiol. 43, 5515–5519.

Molina, R., Amela, C., Nieto, J., San-Andres, M., Gonzalez, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Trans. R. Soc. Trop. Med. Hyg. 88, 491–493.

Nolan, J.P., Mandy, F., 2006. Multiplexed and microparticle-based analyses: quantitative tools for the large-scale analysis of biological systems. Cytometry A. 69, 318–325.

Osman, O.F., Kager, P.A., Zijlstra, E.E., El-Hassan, A.M., Oskam, L., 1997. Use of PCR on lymph-node sample as test of cure of visceral leishmaniasis. Ann. Trop. Med. Parasitol. 91, 845–850.

Palatnik-De-Sousa, C.B., Dos Santos, W.R., Franca-Silva, J.C., Da Costa, R.T., Reis, A.B., Palatnik, M., Mayrink, W., Genaro, O., 2001. Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. Am. J. Trop. Med. Hyg. 65, 510–517.

Pirmez, C., da Silva Trajano, V., Paes-Oliveira Neto, M., da-Cruz, A.M., Goncalves-da-Costa, S.C., Catanho, M., Degrave, W., Fernandes, O., 1999. Use of PCR in diagnosis of human american tegumentary leishmaniasis in Rio de Janeiro. Brazil. J. Clin. Microbiol. 37, 1819–1823.

Quinnell, R.J., Courtenay, O., Garcez, L.M., Kaye, P.M., Shaw, M.A., Dye, C., Day, M.J., 2003. IgG subclass responses in a longitudinal study of canine visceral leishmaniasis. Vet. Immunol. Immunopathol. 91, 161–168.

Reale, S., Maxia, L., Vitale, F., Glorioso, N.S., Caracappa, S., Vesco, G., 1999. Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. J. Clin. Microbiol. 37, 2931–2935.

Rosário, E.Y., Genaro, O., França-Silva, J.C., Da Costa, R.T., Mayrink, W., Reis, A.B., Carneiro, M., 2005. Evaluation of enzyme-linked immunosorbent assay using crude Leishmania and recombinant antigens as a diagnostic marker for canine visceral leishmaniasis. Mem. Inst. Oswaldo Cruz. 100, 197–203.

Reis, A.B., Carneiro, C.M., Carvalho, M.G., Teixeira-Carvalho, A., Giunchetti, R.C., Mayrink, W., Genaro, O., Correa-Oliveira, R., Martins-Filho, O.A., 2005. Establishment of a microplate assay for flow cytometric assessment and it is use for the evaluation of age-related phenotypic changes in canine whole blood leukocytes. Vet. Immunol. Immunopathol. 103, 173–185.

Reis, A.B., Martins-Filho, O.A., Teixeira-Carvalho, A., Carvalho, M.G., Mayrink, W., Franca-Silva, J.C., Giunchetti, R.C., Genaro, O., Correa-Oliveira, R., 2006a. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. Res. Vet. Sci. 81, 68–75.

Reis, A.B., Teixeira-Carvalho, A., Vale, A.M., Marques, M.J., Giunchetti, R.C., Mayrink, W., Guerra, L.L., Andrade, R.A., Correa-Oliveira, R., Martins-Filho, O.A., 2006b. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet. Immunol. Immunopathol. 112, 102–116.

Rocha, R.D., Gontijo, C.M., Eloi-Santos, S.M., Carvalho, A.T., Correa-Oliveira, R., Marques, M.J., 2002. Anti-live *Leishmania* (*Viannia*) *braziliensis* promastigote antibodies, detected by flow cytometry, to identify active infection in american cutaneous leishmaniasis. Rev Soc. Bras. Med. Trop. 35, 551–562.

Rocha, R.D., Gontijo, C.M., Eloi-Santos, S.M., Teixeira-Carvalho, A., Correa-Oliveira, R., Ferrari, T.C., 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. Health 11, 156–166.

Schnur, L., Jacobson, R.L., 1987. Parasitological techniques. In: The Leishmaniasis in Biology and Medicine/Clinical Aspects and Control, Academic press, New York.

Silva, E.S., Meide, W.F., Schoone, G.J., Gontijo, C.M., Schallig, H.D., Brazil, R.P., 2006. Diagnosis of canine leishmaniasis in the endemic area of Belo Horizonte, Minas Gerais, Brazil by parasite, antibody and DNA detection assays. Vet. Res. Commun. 30, 637–643.

Solano-Gallego, L., Moprel, P., Arboix, M., Alberola, J., Ferrer, L., 2001. Prevalence of *Leishmania infantum* infection in dogs living an Area of canine leishmaniasis endemicity using PCR on several tissue and serology. J. Clin. Microbiol. 39, 560–563.

Swets, J.A., 1988. Measuring the accuracy of diagnostic systems. Science 240, 1285–1293.

Tesh, R.B., 1995. Control of zoonotic visceral leishmaniasis: is it time to change strategies? Am. J. Trop. Med. Hyg. 52, 287–292.

World Health Organization, 2000. Leishmania/HIV co-infection South-Western Europe 1990–1998: retrospective analysis of 965 cases. WHO/LEISH/2000, Geneva. Eletronic access: http://whqlibdoc.who.int/hq/2000/WHO_LEISH_2000.42.pdf.

Youden, W.J., 1950. Index for rating diagnostic tests. Cancer 3, 32.