

Different Patterns of Fluorescence in the Quantisation of Anti-Leishmania Infantum Antibodies by Indirect Immuno-Fluorescent Antibody Test (IFAT) in the Serological Diagnosis of Canine Leishmaniasis

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Abstract

Although Indirect Immuno-Fluorescent Antibody Test (IFAT), performed employing “*in house*” prepared antigen, is considered by several authors as the golden standard for the quantisation of anti-leishmania antibodies in dogs, there is a lack of papers reporting a description of the different patterns of fluorescence that can be observed. An incorrect identification of patterns of fluorescence may be an important source of bias in the interpretation of results. Previous papers report different criteria to define as “positive” a specific pattern of fluorescence, namely: membrane fluorescence, homogeneous fluorescence of the body, or homogeneous fluorescence of the body plus flagellum. In this paper, we report a detailed description of preparation of slides and of the patterns of fluorescence that can be obtained employing “*in house*” prepared antigen. At least six main patterns of fluorescence may be observed: 1): homogeneous cytoplasmatic green fluorescence; 2): membrane pattern, in which the fluorescence is mainly localized along the entire perimeter of the parasites; 3): coarse-speckled cytoplasmatic fluorescence; 4): flagellar pattern, in which the fluorescence is localized exclusively onto the flagellum; 5): punctiform pattern, in which the fluorescence is localized exclusively at the basis of the flagellum; 6): nuclear pattern, in which only the nucleus of the parasite shows a homogeneous green fluorescent. The significance of each pattern is discussed.

Keywords

Leishmaniasis, Leishmania, Infantum, IFAT, Dog, Diagnostics, Fluorescence, Pattern

1. Introduction

Indirect Immune-Fluorescent Antibody Test (IFAT) has been reported as the golden standard for the detection of anti-*leishmania* antibodies in dogs by several authors [1] [2]. The method is briefly described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [3]. Major criticism to IFAT is that it is an “operator dependent” method and, to such extent, results may be subject to different interpretations. An important source of bias in IFAT may be the interpretation of the different patterns of fluorescence that can be observed. Regarding this aspect, previous papers can be divided in four groups: 1): papers that do not report any consideration about this aspect [3] [4] [5]; 2): papers that consider “positive” only the samples in which the parasites show homogeneous green fluorescence [6]; 3): papers that consider “positive” the samples showing either cytoplasmatic or membrane fluorescence [7] [8] [9]; 4): papers that consider “positive” the samples showing homogeneous green fluorescence of the total promastigotes (body plus flagellum) [10]. Despite of this fact and to the best of our knowledge, there are no papers reporting a description of the different patterns of fluorescence that can be obtained by IFAT employing “*in house*” prepared antigen. The aim of this paper is to report a detailed protocol for the preparation of slides, fixation and a complete representation of the various patterns of fluorescence.

2. Materials and Methods

Agar, bacteriological peptone and beef extract were obtained from Liofilchem S.r.l. (Bacteriological products, Italy). Gentamycin and anti-dog IgG conjugated to fluorescein isothiocyanate were obtained from Sigma Chemical Company; St. Louis, MO, U.S.A.

Slides, (12 wells, 5 mm) were obtained from Thermo Fisher Scientific, Waltham, MA, U.S.A.

2.1. Cultures

The parasites were grown in 14 ml Falcon tubes (cat. N° 352059), 50 ml Falcon tubes (cat. N° 352070), Falcon multywell culture plates (6 well, flat bottom, cat. N° 353046). Cultures were performed at 22°C - 24°C.

Promastigotes of *L. infantum* were obtained from a dog that acquired the infection naturally and had not received treatment [11].

Parasites were grown in the standard biphasic culture media Evans' Modified Tobie's Medium (EMTM) [3] [12] with some modifications. Briefly, the solid phase was obtained by suspending in 100 ml of H₂O: beef extract, 0.3 g; bacteriological peptone, 0.5 g; NaCl, 0.8 g; agar, 2.0 g. The suspension was autoclaved at 121°C for 21 minutes and cooled in a water bath at 40°C. The culture media is completed by adding 18 ml of defibrinated horse blood obtained by jugular venipuncture (final concentration: 15%). The horse blood was used as an alternative of the rabbit blood, as it may be easily obtained with less stress to the animal. We found similar growth kinetics of parasites using either rabbit or horse blood

(not shown).

The liquid phase was constituted by RPMI 1640 containing L-Proline and supplemented with 15% of FBS. Gentamicin at a final concentration of 250 µg/ml was used as anti-microbial agent.

Tubes of 14 ml were utilized for ordinary maintenance of parasites, while either 50 ml falcon tubes or multiwell plates were used for mass cultures.

Tubes of 14 or 50 ml received respectively 1.5 ml and 5 ml of the solid phase and, respectively, up to 2 ml and up to 10 ml of RPMI/FBS. Multiwell plates received 2.5 ml of solid phase for each well and up to 8 ml of liquid media in each well.

Liquid phase was replaced each 2 days, by aspirating the whole supernatant and replacing it with fresh RPMI/FBS. By this way, the cultures may be easily maintained up to 60 days.

Parasites may be frozen and cryopreserved at -25°C , -80°C or by immersion in liquid nitrogen (-196°C). Briefly, sterile DMSO was added to the supernatant obtained from a two-day fresh culture at a final concentration of 9%. We were able to restore cultures of parasites after 60 days at -25°C or 12 months at -80°C . Liquid nitrogen was used for long-term storage. Briefly, frozen parasites (500 µl) were thawed at 25°C and diluted (1:1) with fresh FBS/RPMI. Then they were seeded in 14 ml tubes containing solid EMTM and checked daily for the presence of viable parasites.

2.2. Preparation of Slides

Parasites were collected after 3 - 4 days of culture [3] and immediately centrifuged at 4°C for 20 minutes at $300\times g$.

Pellet was suspended in cold PBS, washed two times and parasites suspended at the final concentration of $2 \times 10^6/\text{ml}$. The suspension (20 µl) was dispensed to each well. Slides were left to rapidly evaporate in a vertical laminar flow cabinet equipped with both HEPA and carbon filters. Then, cold acetone was dispensed onto the slides and left to evaporate in the vertical flow cabinet. After five minutes residual acetone was dried with clean 3 mm Whatman paper. Slides may be stored for two months at -30°C . Just prior the use, slides were washed with cold PBS.

2.3. IFAT

The assay procedure followed the protocol of the Office International des Epizooties [3]. Anti-Leishmania antibodies were detected using rabbit anti-dog IgG conjugated to fluorescein isothiocyanate. Briefly: 20 µl of appropriately diluted sera were dispensed to each well and incubated 30 minutes at 37°C . Slides were washed three times ($10'$) in cold PBS for 10 minutes. Then, 20 µl of diluted (1:500) anti-dog IgG conjugated to fluorescein isothiocyanate containing 0.0005% Evans blue was dispensed to each well, and slides were incubated again 30 minutes at 37°C . Counterstaining was essential for removing nonspecific background. After the incubation, slides were washed again three times with

cold PBS, and mounted with 50% glycerol.

Observation was performed at a magnification of 400 - 1000 \times .

3. Results

Figure 1 reports the patterns of fluorescence that could be observed in the serological diagnosis of *L. Infantum* by IFAT employing “in house” prepared antigen. We were able to identify at least six main patterns of fluorescence. The most frequent one was a diffuse homogeneous cytoplasmatic green fluorescence. A counterstained red nucleus was also visible. (**Figure 1(b)** & **Figure 1(c)**). Quite frequently could also be observed a membrane pattern in which the fluorescence was localized along the entire perimeter of the parasites (**Figure 1(d)** &

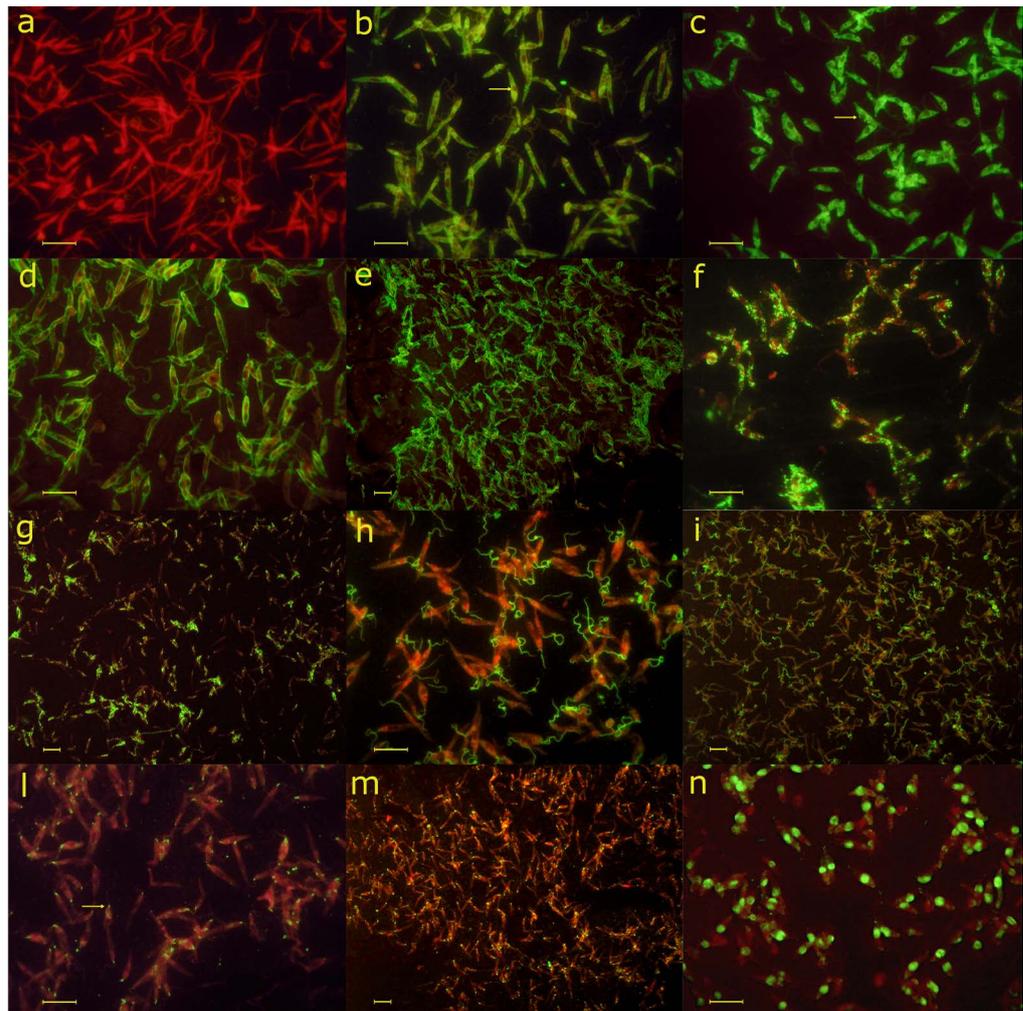


Figure 1. *Leishmania infantum* patterns of fluorescence. From left to right: (a): negative; (b): classic weak positive homogeneous cytoplasmatic pattern. A red nucleus is visible (arrow); (c): classic strong homogeneous positive pattern. A pale red nucleus is still visible (arrow); (d): membrane pattern: fluorescence is visible along the whole perimeter of *L. infantum*; (e): membrane pattern, lower magnification (400 \times); (f): coarse cytoplasmatic fluorescence; (g): coarse cytoplasmatic fluorescence, lower magnification (400 \times); (h): flagellar pattern; (i): flagellar pattern, lower magnification (400 \times); (l): punctiform fluorescence: the fluorescence is localized at the basis of flagellum (arrow), (m): punctiform fluorescence, lower magnification (400 \times); (n). Nuclear pattern. Bars: 20 μ m.

Figure 1(e)). A coarse-speckled cytoplasmatic fluorescence could also be observed, (**Figure 1(f)** & **Figure 1(g)**). In the flagellar pattern of fluorescence (**Figure 1(h)** & **Figure 1(i)**) the fluorescence was localized exclusively onto the flagellum. In a few samples, punctiform fluorescence was localized exclusively at the basis of the flagellum, (**Figure 1(l)** & **Figure 1(m)**). In the nuclear pattern, only the nucleus showed a homogeneous green fluorescence (**Figure 1(n)**).

4. Discussion

There are three main methods to reveal the presence of anti-leishmania antibodies in dog sera, namely Western Blot (WB), IFAT and ELISA. ELISA may be performed employing either a crude antigen extract or recombinant antigens. The use of specific antigens have some limitations [13], and a crude extract is widely employed for general diagnostic purpose [5] [7] [8]. ELISA is described as a method that does not require a specialized technician to understand the results obtained from the microplate reader, and non-specific reactions are controlled by the use of an appropriate cut-off [14]. It should be pointed out that ELISA could not identify an aspecific reaction generating a signal greater than the cut off.

On the contrary, a qualified technician is required to interpret the results obtained by WB and IFAT and to distinguish between specific and aspecific reactions. In particular, the analysis of the WB patterns is also suitable to distinguish between asymptomatic dogs and dogs with active infection and for the identification of new antigens involved in the different clinical phases of the disease [15] [16] [17]. In IFAT, the use of “*in house*” prepared antigen allows to distinguish between various patterns of fluorescence. Despite to this fact, and to the best of our knowledge there is no paper showing the different patterns of fluorescence that can be obtained by IFAT in the quantisation of anti-leishmania infantum antibodies in dogs.

The preparation of slides plays a pivotal role to preserve parasite structure. IFAT fixation reagents include methanol, acetone, paraformaldehyde, or formaldehyde. Fixation time varies between 5 - 15 minutes according to the characteristics of the substrate. Typically [3], “*in house*” *L. infantum* prepared slides are fixed 15 minutes in cold acetone (-25°C). Remarkably, several papers do not recommend acetone for immunofluorescence studies or report shorter time of fixation [18] [19] [20]. We have tested several fixing agents for the preparation of the antigen (not shown), namely acetone, methanol, acetone: methanol (1:1), formaldehyde, glutaraldehyde, and paraformaldehyde (at a final concentration ranging between 0.5% - 4%). Optimal results were obtained with cold acetone (-25°C), but differently with previously reported [3], with a shorter time of fixation (5 minutes). Longer time of fixation will result in a loss of parasite details and we were not able to clearly distinguish between the different patterns of fluorescence.

Once obtained a good preparation of the antigen, the first question was to establish what pattern of fluorescence had to be considered specific for the pres-

ence of anti-Leishmania antibodies. To our surprise, concerning this aspect, there is not a general agreement. Previous papers reported as “specific” the following patterns of fluorescence: a) homogeneous cytoplasmatic fluorescence [6]; b) homogeneous cytoplasmatic fluorescence or membrane fluorescence [7] [8] [9]; c) homogeneous fluorescence of the total promastigotes (body plus flagellum) [10]. A number of Authors do not report any consideration about this aspect [3] [4] [5].

We identified at least six patterns of fluorescence (**Figure 1**): a): diffuse homogeneous cytoplasmatic green fluorescence; b): membrane fluorescence, in which only a peripheral green fluorescence is visible; c): coarse-speckled cytoplasmatic fluorescence; d): flagellar fluorescence, in which only the flagellum is green fluorescent; e): punctiform fluorescence, in which fluorescence is localized exclusively at the basis of the flagellum, and may be related to the antibody binding to proteins localized nearby the basal portion of the flagellum (e.g.: basal body); f) nuclear fluorescence in which only the nucleus shows a homogeneous green fluorescence. Obviously it is possible to observe mixed patterns of fluorescence, such as homogeneous cytoplasmatic fluorescence or membrane fluorescence plus flagellum.

The significance of a specific pattern is related to the portion of the parasites that appears green-fluorescent. Namely, antibodies against nuclear proteins are not typically related to the immune response against leishmania spp., as nuclear proteins are highly conserved in all Eucaryotic cells. Thus, a nuclear pattern of fluorescence should be always considered as non-specific. Rather, samples showing a nuclear fluorescence are suggestive for the presence of anti-nuclear antibodies, to be confirmed by the use of a more specific substrate, such as the HEP-2 cells. With the same principle, a flagellar or punctiform fluorescence, is also suggestive of a poor specificity. This is in agreement with several authors that consider positive only the samples showing either homogeneous cytoplasmatic or membrane fluorescence [6] [7] [8] [9], but in disagreement with a single paper that consider as positive only the samples showing fluorescence of the total promastigotes (body plus flagellum) [10]. In addition, a coarse speckled cytoplasmatic fluorescence of the parasite should also be considered as a specific pattern of fluorescence, and the fact that this pattern has not been mentioned before may be related to different fixation methods employed.

In conclusion, both homogeneous cytoplasmatic, coarse speckled cytoplasmatic and membrane fluorescence should be considered as specific patterns. It should be pointed out that different opinions in the interpretation of fluorescence patterns might have a profound impact in the evaluation of performance of different tests in the serological diagnosis of canine leishmaniasis.

It is our opinion that also aspecific reactions should also be reported to the clinician, especially when a strong fluorescence is found, as it may explain the incongruity of results between ELISA and IFAT. Further studies showing the correlation between IFAT and WB patterns will unequivocally differentiate between specific and aspecific patterns of fluorescence, and establish if different

patterns of fluorescence in IFAT may be related to different clinical phases of the disease.

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Conflict of Interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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