A versatile and sensitive lateral flow immunoassay for the rapid diagnosis of visceral leishmaniasis

A VERSATILE AND SENSITIVE LATERAL FLOW IMMUNOASSAY FOR THE RAPID DIAGNOSIS OF VISCERAL LEISHMANIASIS

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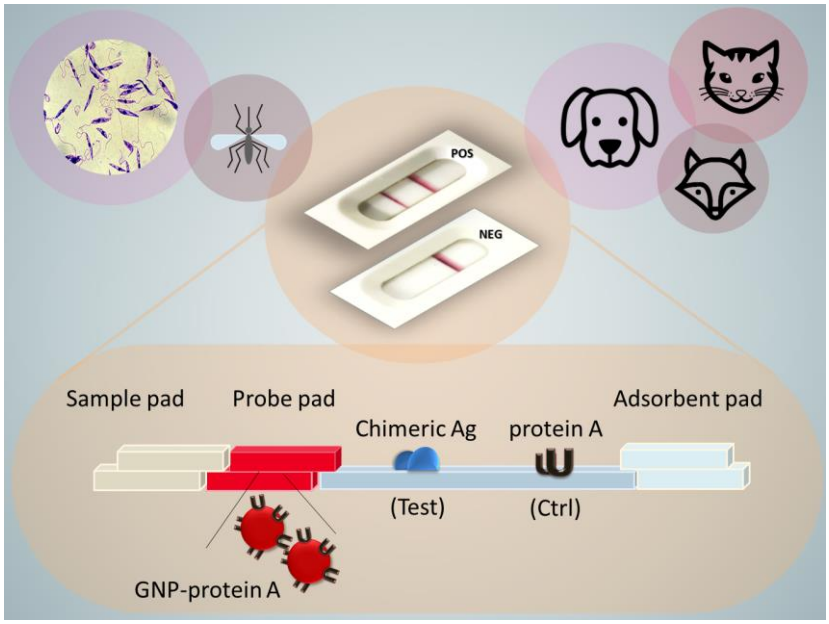

A rapid and portable diagnostic tool for Visceral leishmaniasis (VL) based on the lateral flow immunoassay (LFIA) technology: anti-leishmanial antibodies are revealed by their binding to a highly specific chimeric antigen; while the broad-specific signal reporter (protein A labelled with gold nanoparticles) enables the facile adaptation of the LFIA to VL diagnosis in different animals.

Abstract

Visceral leishmaniasis (VL) is a zoonotic infectious disease with severe impact on humans and animals. Infection is transmitted by phlebotomine sand-flies and several domestic and wild mammals act as reservoirs for the infection, therefore the prompt detection of infected hosts is crucial for the prevention and control of the spread of the disease and of transmission to humans. A rapid and portable diagnostic tool for VL diagnosis is described based on the lateral flow immunoassay (LFIA) technology. The device exploits a highly specific chimeric recombinant antigen as the recognition element for capturing anti-leishmanial antibodies and protein A labelled with gold nanoparticle as the signal reporter. The LFIA shows excellent diagnostic sensitivity (98.4%), specificity (98.9%) and agreement with serological reference methods for diagnosing canine VL. The long-term stability of the LFIA device was confirmed for six-month storage at room temperature and 4°C and the qualitative response was not affected by limited thermal stress. The use of the broad-specific protein A enables the versatile application of the LFIA to VL diagnosis in dogs, which represent the main reservoir for human infection, and in other mammals assuring the opportunity of efficiently controlling the spreading of the infection.

Keywords

Immunochromatographic test, validation, point-of-care test, shelf-life, fox, cat

Introduction

Visceral leishmaniasis (VL) is a zoonotic disease, caused by the protozoan parasite *Leishmania infantum* that is transmitted to vertebrate hosts through the bites of infected female phlebotomine sand flies, endemic in many countries throughout Latin America and Asia [1]. It ranked second in mortality and fourth in morbidity among tropical diseases and is considered as one of the world's most neglected diseases by the WHO (World Health Organization) [2]. In European countries, the incidence risk of VL is still relatively low, although the disease is spreading to regions previously referred as non-endemic, probably because of climate change and population movements [3-4]. However VL impact on human health is severe (HVL) and is characterized by fever, weight loss, splenomegaly, hepatomegaly and anaemia [1]. It is estimated that more than 700,000 new cases and about 20,000-30,000 deaths occur annually worldwide due to HVL [5]. Although Leishmania amastigotes parasites more than 70 vertebrate hosts; domestic dogs are considered the main reservoir for human infection [1-3]. Infected dogs have very variable clinical manifestations that range from apparently healthy to severely diseased. Many infected dogs may never exhibit clinical signs, thus making difficult to early detect canine visceral leishmaniasis (CVL) [6]. Nevertheless, both symptomatic and asymptomatic dogs are able to transmit the parasite to other dogs and humans [3]. Therefore, early detection and prompt treatment of infected animals help to reduce spreading of transmission and represent a relevant part of the prevention and control of the burden of disease in humans.

CVL can be diagnosed by combining clinical and epidemiological parameters with parasitological, serological, or molecular methods [1]. According to the World Organisation for Animal Health [7], serology is the preferred diagnostic method for CVL. In particular, detection of anti-leishmanial antibodies is commonly realized by three techniques: the immunofluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and the lateral-flow immunoassay (LFIA). The IFAT is considered as the reference method for anti-leishmanial serology in dogs and is used as the reference test for the validation of new diagnostic methods, though data on its diagnostic sensitivity and specificity are controversial [3]. Furthermore, IFAT suffers from operator-dependent variability. The ELISA is also very sensitive and specific, with the advantage of easier standardization [8]. Both IFAT and ELISA provide quantitative results, defined as the antibody titre (the last 2-fold serial dilution of sample providing a positive result). However, the rapid and cost-effective detection of infected dogs is a key point in the control of infection and infection transmission. LFIA, also known as immunochromatographic assay (ICA) and immunochromatographic strip test (ICST), is the most popular diagnostic tool for rapid onsite assays. Advantage of LFIA is represented by its perfect match with ASSURED criteria required for point-of-care testing (Affordable, Sensitive, Specific, User-friendly, Rapid/Robust, Equipment-free and Deliverable to end users) [9], which explains its rapid spreading and huge commercial success. Nevertheless, LFIA only provide qualitative results (i.e.: healthy /sick subject) that need to be completed by quantitative information to ensure the correct management of the disease. Several commercial LFIA devices are available [10-13] that varies for simplicity of use, rapidity and sensitivity [6]. The specificity of these tests is generally high, while the diagnostic sensitivity is usually low (30–70%) and largely dependent on leishmaniasis stage [6,8]. A limited sensitivity strongly reduces the effectiveness of control of infection transmission, also frustrating attempts of preventing the spread of the disease in humans.

In addition to inadequate sensitivity, existing LFIA kits for diagnosing leishmaniasis are designed for detecting specifically CVL and HVL. Although dogs are considered the most important domestic reservoirs of *L. infantum*, several species of wild mammals have been recognized as hosts and potential reservoirs of Leishmania parasites. [14]. In addition, domestic mammals (sheep, goats, cattle and donkey) has been suggested as the reservoir hosts responsible of a HVL outbreak occurred in 2008-2009 in China. [15] Therefore, versatile diagnostic tools that can be adapted for VL diagnosis in other mammals beside dogs are also demanded.

In this work, we describe the design of a rapid diagnostic tool for detecting anti-leishmanial antibodies that shows high diagnostic sensitivity and versatility to be adapted for use with other mammalians besides dogs and humans.

The rapid test for diagnosing VL is a lateral flow immunoassay based on the one-site immunometric assay format. The specific recognition element is represented by a recombinant chimeric antigen (rCAg), comprising three Leishmania antigens, which has been shown to be highly specific for VL [16-17]. The signal

reporter is constituted by staphylococcal protein A (pA) labelled with gold nanoparticles (GNPs) that are used as coloured probes for the visual interpretation of the qualitative result. Anti-leishmanial antibodies present in the sample bind to the chimeric antigen coated in the so-called Test zone, and the rate of formation of the complex is measured by reaction with the labelled protein A. The protein A, which also forms the Control line, captures any excess of immunoglobulins, regardless of their specificity towards the leishmanial antigen. Again, the captured immunoglobulins are revealed by the protein A labelled with GNPs. Therefore, two coloured lines form if anti-leishmanial antibodies are present in the sample, thanks to the accumulation of GNP-pA at both the Test and Control zones. Vice versa, only the Control line is visible if the sample does not contain any anti-leishmanial antibodies (Fig. 1).

The use of protein A as a generic recognition element for conferring versatility to the assay, thanks to the pA ability of binding immunoglobulins from various animal species, is reported for ELISA methods [18-20]. Some LFIAs also employed protein A/G as the labelled probe, in combination with immunoglobulins as the capturing reagent at the Control line [21]. However, since VL is commonly associated with hypergammaglobulinemia [22], the high levels of gamma-globulins can saturate the binding capacity of the pA-GNP probe, thus preventing its ability to react with immunoglobulins forming the Control line. The effect is an unacceptably rate of invalid result (Control line not visible, Fig 2). To overcome this limitation, we used pA also as the capturing reagent to form the Control line. In such a way, the LFIA for diagnosing VL maintains the capacity of adaptation for detecting anti-leishmanial antibodies belonging to different mammalian species while also assuring validity of the test even for subject showing abnormal levels of immunoglobulins.

Materials and methods

Immunoreagents, chemicals and materials

Gold (III) chloride trihydrate (ACS reagent), protein A (pA), bovine serum albumin (BSA), rabbit immunoglobulins, swine immunoglobulins and polyvinyl alcohol (PVA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Triton X-100 and other chemicals were purchased from VWR International (Milan, Italy). Anti-dog IgG were purchased from Sigma Aldrich.

Nitrocellulose membranes (HF180 plus card), cellulose absorbent pad and glass fibre conjugate pad were obtained from Merck Millipore (Billerica, MA, USA). Standard 14 glass fibre pads from Whatman (XX) were used as sample pads.

K9-K39-K26 recombinant chimeric antigen (rCAg) was prepared as described in [17].

Statistical calculations were carried out with SigmaPlot 11.0 software.

Preparation of GNPs and GNP-protein A conjugates (GNP-pA)

GNPs with a SPR band at 525 nm and mean diameter of ca. 30 nm were prepared by tetrachloroauric acid reduction with sodium citrate [23]. Briefly, 1 mL of 1% w/v sodium citrate was added to 0.01% of boiling tetrachloroauric acid under vigorous stirring. The colour of the solution changed gradually from light yellow to red thus confirming the successful formation of gold nanoparticles. Signal reporters used in the LFIA were prepared by adsorbing protein A onto GNPs. In details, 8 µg of pA and 1 ml of borate buffer (pH 7.4) were mixed with 10 ml of GNPs and incubated for 30 min at 37°C. Then, 1 ml of BSA (1% in borate buffer) was added and reacted for 10 min at 37°C to saturate free GNP surface. GNP-pA conjugates were recovered by centrifugation (14000 rpm, 15 min) and washed twice with borate buffer supplemented with 0.1% BSA. Finally, GNP-pA were re-suspended in GNP storage buffer (borate buffer with 1% BSA, 0.25% Tween 20, 2% sucrose, and 0.02% sodium azide) and stored at 4°C until use.

Fabrication of the LFIA device

The recombinant chimeric antigen [17] was applied to the nitrocellulose (NC) membrane to form the Test line (0.5 mg/ml). Protein A (0.2 mg/ml) was used as the capturing reagent at the Control line. Reagents were dotted at 1 µL cm^{-1} by means of a XYZ3050 platform (Biodot, Irvine, CA, USA), equipped with BioJet Quanti™ 3000 Line Dispenser for non-contact dispensing, keeping a distance of 4 mm between the lines. The signal reporters (GNP-pA conjugates) were absorbed onto the glass fibre conjugate pad previously saturated with GNP storage buffer. The pad was dipped into GNP-pA solution (optical density 1) and dried for 3 hours at room temperature. NC membranes were dried at 37°C for 60 minutes under vacuum, layered with sample, conjugate and absorbent pads (Fig. 1), cut into strips (4.2 mm width) by means of a CM4000 guillotine (Biodot, Irvine, CA, USA) and inserted into plastic cassettes (Kinbio, China) to fabricate the ready-to-use LFIA device. Cassettes were stored in the dark in plastic bags containing silica at room temperature until use.

The Lateral Flow ImmunoAssay for canine leishmaniasis diagnosis

Assays to detect anti-leishmanial antibodies were carried out at room temperature, by applying 70 µl of diluted serum to the sample well. For the analysis, samples were thawed at room temperature, carefully mixed and diluted by 1:20 using the running buffer (phosphate buffer 20 mM, pH 7.4, 50mM NaCl, 1% BSA, 0.5% PVA, 0.1% Triton X-100).

Qualitative results were judged by the naked eye after 15 minutes (Fig. 2). Samples were analysed in duplicate and results were observed by three operators. Images of LFIA devices were also acquired by a portable scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and the area of the coloured lines was quantified by means of the QuantiScan 3.0 software (Biosoft, Cambridge, UK).

Serum samples

A total of 167 canine sera were used in the study; 37 serum samples were collected from an endemic region (West Liguria, Italy), while 130 samples belonged on non-endemic regions (Piemonte and Valle d'Aosta, Italy). Most canine sera were characterized by analysing them through more than one reference method: IFAT titration was carried out on 157 samples, PCR and western blot (WB) were carried out on 120 samples and 141 canine sera were analysed by a previously validated ELISA (Enzyme-Linked ImmunoSorbent Assay) that was based on the same recombinant chimeric antigen employed for fabricating the LFIA [20]. Samples belonging to the non-endemic area were characterized by IFAT, PCR and WB. In order to classify canine sera, IFAT cut-off was set at 1/80 [3,6,8,20], while PCR and WB were carried out according to the protocols described in Ferroglio et al [24].

In details, 70 samples showed IFAT titre < 1/40 and were negative also according with PCR and WB methods; 40 samples showed IFAT titre > 1/80 and were positive also according with PCR and WB methods; 10 samples were analysed through WB and PCR and resulted as positive. These samples were also analysed by the ELISA that classified 5 samples as positive and 5 as negative.

Samples belonging to the non-endemic area were classified according with either the IFAT titre or the ELISA score; in particular, 7 with inconclusive classification (IFAT = 1/80) were analysed by the ELISA method. Further 102 samples were randomly chosen among those already characterized by other reference methods and submitted to ELISA qualification, as well.

To evaluate the potential application of the assay to different animal species, 2 red fox sera (1 IFAT positive and 1 negative) and 9 cat sera were also analysed. Cat sera were characterized by PCR and WB; however results were ambiguous and were considered inconclusive. Fox and cat samples were analysed by the versatile LFIA and by the reference ELISA (which also employed protein A as the probe, although conjugated to horseradish peroxidase).

Validation of the LFIA device for detecting leishmaniasis in canine serum

The impact of serum matrix on the assay was studied by variably diluting a pool of positive and a pool of negative canine sera with phosphate buffer supplemented with various additives. In order to limit matrix interference, the following chemicals were considered: proteins (BSA, casein), surfactants (Tween 20, Triton X-100), polymers (polyvinyl alcohol), and salts (NaCl). Each additive was added to phosphate buffer at three different levels and used to dilute pooled sera 1:10 before LFIA analysis. In addition, the same compounds were also used for impregnating the sample pad, as an alternative to sample dilution.

Accuracy of the assay was calculated as the rate of results agreeing with those provided by the reference methods (IFAT and ELISA) on canine sera. The Cohen's K parameter was calculated to evaluate concordance of the new LFIA with serological reference methods.

The imprecision of the LFIA was considered to be due to the sum of 3 components: the within- and between-day variations due to the assay, and the biological variability. Accordingly, overall imprecision was

estimated by an experimental design approach firstly proposed by Lattanzio et al. [25-26], with minor modifications due to availability of biological samples [27]. The study was conducted by analysing 11 sets of canine serum, of which 7 were positive and 4 negative. The samples were analysed on two days. On each day, samples were analysed in triplicate. Negative samples and positive samples were used to calculate the rate of false positivity (n=24) and false negativity (n=42), respectively.

Robustness, in terms of the reliability of the assay response over time, was also studied: for that purpose, 10 serum samples (5 positive and 5 negative) were analysed in duplicate and the result was observed after 10, 20 and 60 minutes from sample application. The rate of false positive (n=10) and false negative (n=10) was calculated at each observation time.

Stability of the LFIA device

With the aim of evaluating the shelf-life of the LFIA device, real-time stability and accelerated ageing experiments were carried out as follows [27-28]. For the accelerated ageing experiment, LFIA cassettes were kept at 37°C for 7 days and tested on day 0, 1, 3 and 7. For the real-time stability experiment, LFIA cassettes were stored at room temperature and at 4°C for 6 months, and tested on week 0, 1, 2, 4, 8, 12, and 24. For each experiment, a pool of positive samples and a pool of negative samples were analysed in duplicate. For all experiments, LFIA devices were stored in the dark and with desiccant added.

Results and discussion

Optimization of the LFIA device

The LFIA device was designed as a versatile tool for diagnosing Leishmaniasis in various animal species. Hence, protein A was used as a broad selective recognition element and labelled with gold nanoparticles to fabricate the signal reporter (Fig.1). The same protein A was used also as the capturing reagent forming the Control line. Attempts were made using immunoglobulins from other animal species known to bind pA (rabbit and swine) as the C-line capturing reagent, according to the strategy proposed by Intaramat et al. [21]. However, the rate of invalid test (i.e.: test in which the Control line is not visible) was unacceptably high, due to the unavailability of the GNP-pA probe for binding to the immunoglobulins immobilized at the C-line. In fact, subjects infected by VL also show hypergammaglobulinemia [22] that saturated the binding capacity of the labelled pA. The use of an anti-canine antibody partially solved the problem, however at the expenses of assay versatility. Therefore, we opted to use the same pA as the C-line reagent. In such a way, the LFIA is putatively able to reveal immunoglobulins of all animal species that are bound by pA.

The specificity of the LFIA is connected to the recognition element deposed at the Test zone, which is a recombinant chimeric antigen (rCAg) from the amastigote form of Lehismania parasite [17]. In details, the rCAg comprises three antigenic domains (K9, K39, and K26) from *L. Infantum* [11] and has proved to allow the highly sensitive and specific detection of anti-leishmanial antibodies by ELISA [20]. Most importantly,

234 the rCAg is representative of the form of the Leishmanial amastigote antigens that are expressed in
235 vertebrates, enabling to discriminate infected subjects from those who just underwent into contact with
236 the phlebotomine vector. This is particularly relevant for correctly identifying infected subjects in endemic
237 areas, where the probability of accidental contact with the vector is high, although not necessarily
238 connected to the actual development of the infection [29].
239 The setting up and tuning of LFIA parameters in order to produce a rapid, sensitive and easy-to-handle LFIA
240 device followed a checkerboard strategy, in which concentrations of the signal reporter (GNP-pA), the
241 recognition element for the Test line (rCAg) and the capturing reagent (pA) for the Control line were
242 variously combined. Pooled positive and negative canine sera were used during the optimization work to
243 mitigate the influence of biological variability. Preliminary, the experimental conditions for optimal
244 conjugation of pA with gold nanoparticles were defined. In details, pH and amounts of the pA were defined
245 based on a compromise providing stable GNP-pA conjugates [30] and high detectability in the LFIA device
246 [31].
247 The protocol for executing the assay includes serum dilution with a running buffer. This additional step
248 limits simplicity of use of the LFIA device for non-trained personnel and in low-resource settings. However,
249 it was required for two main reasons. On one hand, serum is a viscous liquid that hardly flows across the
250 LFIA membrane. As a consequence, the application of undiluted samples resulted in the lengthening of
251 analysis time and increasing of rate of irreproducible results. Most importantly, the rate of false negative
252 samples was unacceptably high due to the hook effect, associated to the hypergammablobulinemia of
253 subjects infected by VL [22]. The minimal sample dilution required for obtaining a clearly visible signal at
254 the Test lines for most positive samples and in a reasonable time (15 minutes) was established as 1:20.
255 Lower dilution factors (e.g.: 1:10) allowed for acceptable diagnostic sensitivity to be reached, however at
256 the expenses of rapidity (accurate results were observed after 60 minutes from sample applications).
257 Commercial LFIA kits also involve some dilution of the serum, typically realized by applying a limited
258 volume of the sample immediately followed by the addition of a larger volume of a diluent [
259 The composition of the running buffer was defined with the aim of guarantee rapidity, high detectability
260 and reduced sample-to-sample result variation. At the purpose, several modifiers were added to the
261 phosphate buffer, such as BSA, PVA, NaCl and Triton X-100. PVA was especially helpful for the rapid and
262 complete re-dissolution of the dried GNP-pA; while NaCl efficiently abated non-specific binding of GNP-pA
263 to the rCAg at the Test line, thus contributed to dramatically reducing false positive results.
264
265 *Analytical parameters of the LFIA for the qualitative detection of anti-Leishmanial antibodies in canine*
266 *serum*
267 The precision of the LFIA device was investigated following the approach firstly proposed by Lattanzio et al
268 [25] and widely applied for assessing performances of qualitative LFIAs [26-27]. The strategy is based on

designing a set of experiments to include three factors that contribute potentially to the overall precision of the assay, namely: the within-day, the between-day, and the biological variability.. Therefore, seven positive and four negative serum samples were tested in replicate on the same day and on two distinct days. Positive samples included serum with high and low IFAT titres. No false positive (n=42) nor false negative (n=24) results were registered during the assessment, as a confirmation that the LFIA is precise enough for enabling reproducible detection of anti-leishmanial antibodies in canine serum. The mean coefficients of variation were calculated for positive canine sera by digitalizing images of the cassettes and converting them into quantitative data [23]. The area under the Test line (AreaT) was measured and used as the quantitative parameter for verifying repeatability and reproducibility. Mean coefficient of variations were calculated as 14.6% (n=12) and 15.5% (n=6) for the within- and between-day experiments, respectively (Fig. 3a). The mean values of AreaT for the two days were compared by a one way analysis of variance (ANOVA). The observed difference among the days was not statistically significant (P=0.145), although the power of the performed test was below the desired value (power of performed test with alpha=0.050: 0.199).

A larger variability was observed among the various canine samples; however, this was expected since samples were expressly chosen for having variable IFAT titres. Indeed, even if the LFIA is not able to provide quantitative results, obviously, the variable content of anti-leishmanial antibodies of samples reflected into differently coloured Test lines, where the higher the content of antibodies, the more coloured the Test line and vice versa.

A frequent issue of LFIAs for serological application is represented by the modification of the visual result over time. In particular, negative results (i.e.: assay in which only the Control line is visibly coloured) have the tendency to become positive (the Test line becomes coloured, as well). In order to define the minimum time required for observing a reliable response by the LFIA, and to verify the robustness of the response over time, the LFIA was used to detect anti-leishmanial antibodies in ten canine sera and the observation of the results was repeated after 10, 20 and 60 minutes from sample application. Four negative and six positive samples were analysed. Among positive samples, three were characterized by high IFAT titres (equal or above 1/640) and three by low IFAT titres (below 1/640). Each sample was tested in duplicate and results were observed by the naked eye. The colour at the Test line indicating positivity was detectable after ten minutes for all positive samples, increased in the following 10 minutes, and then stabilized (Fig. 3b). Precautionary, we set 15 minutes as the time for achieving a reliable response for low positive samples. Most interestingly, no colour at the Test line was visible for negative samples even after 60 minutes from sample application and this observation was confirmed for all negative samples analysed during LFIA validation. Therefore, the LFIA demonstrated to provide responses very stable over time.

Validation of the LFIA as a rapid tool for serological diagnosis of canine Leishmaniasis

The capability of the LFIA to correctly identify subjects infected by CVL was studied by analysing sera from a total of 167 dogs, belonging to both endemic (37 subjects) and non-endemic (130 subjects) areas.

Considering that a gold standard reference method for diagnosing VL is still missing and that each of the analytical method usually employed show some limitations [1,3,8,32-35], we opted to classify canine serum based on IFAT and ELISA responses. Actually, IFAT method is commonly regarded as the reference for validation of new diagnostic tools [1-3]. On the other hand, the ELISA used in the study was based on the same capturing antigen exploited for fabricating the LFIA device. Thus we considered that any discordance between the two methods should be attributed to the functioning of the LFIA itself and not to the specificity of the capturing reagent. For the same reason, we considered just the qualitative interpretation of the LFIA result (i.e.: colour present at the Test line) and we did not attempted to correlate quantitatively the LFIA output with IFAT titre.

Most samples were characterized by the serological reference method and their classification as positive/negative was based on the general assumption that IFAT titres above 1/160 and below 1/40 are considered unequivocally positive and negative, respectively. IFAT titres comprise between 1/40 and 1/80 are considered as controversial [6,33] and, commonly, the 1/80 level is considered as the decision cut-off [3,6,8]. The sensitive and specific ELISA was used to confirm classification of samples with an ambiguous titre and to assign samples without IFAT titre. Further 102 sera were also analysed by the ELISA. Most results obtained by the ELISA method were in agreement with those provided by the IFAT method. However, 11 samples gave conflicting results among the two reference methods. These samples belonged to a non-endemic area and were classified as positive based on the IFAT method, while negative according to the ELISA. The difference can be explained considering the different antigen used by the two reference techniques. In particular, the ELISA employed the same chimeric antigen as the LFIA and therefore is more specifically directed to detect antibodies against the amastigote form of Leishmanial parasite, while the IFAT method employs an antigen from the promastigote form of the parasite. In conclusion, samples were classified as truly negative if having the IFAT titre below the cut-off titre (1/80) and a negative ELISA score [20]. Positivity was assigned to samples having the IFAT titre above the cut-off titre (>1/80) and a positive ELISA score. Sera with IFAT titre at the cut-off level (1/80) and without IFAT titre were classified based on the ELISA score only. Accordingly, 93 truly negative samples, 63 truly positive samples, and 11 ambiguous samples (positive according to IFAT, negative according to ELISA) were analysed by the LFIA during the study.

Samples were blindly analysed through the LFIA in duplicate and were judged positive based on the presence of two visible lines. The visual result was assessed by three different operators, who observed the LFIA devices by the naked eye after 15 min from the application of the sample. Agreeing results were obtained within replicate measurements and between observations of the three operators for all canine sera.

From these results, we obtained the figures of merits for the validation of the qualitative LFIA (Table 1). In particular, we calculated: the diagnostic sensitivity (Se) of the test, defined as the rate of truly positive results and the diagnostic specificity (Sp) of the test, defined as the rate of truly negative results [36]. The LFIA furnished one false negative result for a canine serum belonging to the endemic region. This sample had an IFAT titre equal to 1/80, which is considered as controversial and, especially for animals living in endemic area, can be related to an initial phase of the infection. A false positive result was observed for a sample belonging to the non-endemic area and, furthermore, classified as negative by both reference methods. Nevertheless, the LFIA demonstrated very high diagnostic sensitivity (98.4%, 95% confidence interval 91.47-99.96%) and specificity (98.9%, 95% confidence interval 94.15-99.97%), thus confirmed its applicability for accurately diagnosing CVL. Achieved sensitivity is higher than those of other rapid test kits, especially considering that the LFIA was able to correctly discriminate also samples with very low IFAT titres (1/40 and 1/80) while previously reported assays failed in classifying such kind of samples. Low IFAT titres can be associated to the early stage of the infection; so the LFIA candidate as an effective tool for the prevention and control of CVL infection transmission by enabling early diagnosis.

Canine sera with conflicting attribution based on the reference methods were not considered for calculation of diagnostic sensibility and specificity of the LFIA. Conversely, they were included in the comparison of LFIA with reference methods (Table 2) to calculate the accuracy. For this purpose, samples were classified differently, in accordance with the response of each individual reference method considered. The LFIA judgement provided 8 positive and 3 negative results on these ambiguous samples, thus the agreement was higher with the IFAT reference compared to the ELISA, despite the fact that the ELISA method used the same antigen specific for the amastigote VL parasite as the LFIA. In details, the accuracy of the test, defined as the fraction of tests correctly classified, was ca 93% and 97% assuming the IFAT or the ELISA method as the reference, respectively.

The concordance with the two reference methods was estimated by the Cohen's k [36]. Excellent concordance was calculated with both reference methods. Moreover, by comparing the LFIA to the IFAT method, which is generally considered as the gold standard reference for Leishmaniasis diagnosis, the k value exceeded 0.9.

Shelf-life study

Long-term and thermal stability are crucial factors for LFIA devices because they are designed for working on field. However, most materials and bio-reagents included in the device are intrinsically sensitive to environmental conditions.

The long-term stability of the LFIA device stored at 4 °C and at room temperature was investigated within six months. LFIA cassettes were individually packed, with light protection and in the presence of a desiccant. In details, a positive and a negative control were correctly attributed, based on the visual

observation of the colour at the Test lines. Also, the quantification of coloured areas confirmed the visual observation (Fig. 3c). Although we observed a slight decrease of Test line colour starting from day 7 compared to that measured at day 0 for both temperatures, we concluded that the LFIA is acceptably stable for six months and does not require a specific storage temperature.

In addition, accelerated ageing of the LFIA was carried out by keeping the LFIA device at 37°C for one week. The experiment allowed us to conclude that the LFIA device is insensitive to limited increase of the temperature (Fig. 3d) that can occur due to particular ambient conditions (i.e.: use during summer season, storage in non-conditioned environment for short periods) and, therefore, is robust enough for the on-field usage.

Application of the LFIA for the diagnosis of VL in other animal species

Although dogs are considered the main reservoir for VL, other mammalian reservoirs have been reported and incriminated for transmission of the infection to humans [14-15]. Companion animals like cats can be infected by *L. infantum* and transmit infection to sand flies [37] and several species of wild animals have been found infected in Europe [38]. In the past, wild species were considered as secondary reservoirs or occasional hosts; however the recent focus in Madrid with more than 600 human cases highlights the risk of VL spreading from wildlife to humans [38]. One major limitation for better understanding the dynamic of interaction between VL hosts and reservoirs is represented by the availability of diagnostic methods applicable for animals other than dogs. Indeed, most serological methods use probes that are specific for revealing canine immunoglobulins and necessitate modifying protocols for enabling detection of anti-leishmanial antibodies from other mammalians. An example of a broad-specific serological assay for VL diagnosis in dogs and humans has been previously developed by the group in the ELISA format [20], based on a similar strategy. Protein A labelled with an enzyme was exploited as a versatile probe capable of revealing both human and canine anti-leishmanial antibodies. To demonstrate the versatility of the LFIA and its adaptability to detect anti-leishmanial antibodies produced by diverse animal species, two sera from red fox and nine from cat were analysed by the protocol optimized for CVL diagnosis. Red fox sera were characterized by IFAT titration and were known as one being positive and one negative. PCR and WB analyses provided controversial classification of feline sera, which were thus analysed by the reference ELISA. The ELISA provided a positive response for the red fox sample classified as positive by IFAT method and for three feline samples. The observed disagreement between molecular and serological techniques is frequent when cats are tested by both methods [37], due to the immune response of cats that differ from dogs as the low number of clinical cases demonstrates.

LFIA analysis was conducted in two replicates; no invalid tests were observed, which means that the GNP-pA probe is suitable for adapting the LFIA for diagnosing VL in cat and red fox sera. Furthermore, LFIA judgement on red fox sera matched those obtained by both reference methods (ELISA and IFAT).

Regarding feline sera, three samples were classified as positive and six as negative (Fig. 4), thus providing an excellent concordance with the reference ELISA. The observed discordance with respect to PCR was attributed to the variability of results among molecular and serological diagnostic methods, often underlined in the few available literature [37]. More interestingly, the strategy used to develop the LFIA based on the broad-specific GNP-pA probe enabled the detection of immunoglobulins from different mammalians (dogs, cats and red fox) and the recombinant chimeric antigen was able to capture anti-leishmanial antibodies from other carnivores.

Conclusions

Performance of rapid tests available on the Brazilian market for the rapid diagnosis of CVL has been reviewed by Woyame-Pinto et al. [6]. Although validation studies were heterogeneous in sample size and in regards to the reference methods used to classify samples (IFAT, ELISA, PCR) some conclusions can be drawn. Not considering the Rapidtest assay, which validation precedes the others by about ten years, the diagnostic specificity of existing point-of-care-test for CVL varied between 90.6% provided by the SNAP Leishmania test [10] to 100% shown by the Kalazar Detect assay [11]. Noticeably, sensitivity ranged between 32.6% (Kalazar Detect) to 98% (Dual-path platform, dpp®) [12], with a strong variability associated to the phase of the disease. In particular, asymptomatic dogs were hardly recognized as sick by most of the rapid tests reviewed (sensitivity: 32.6-94.7%) while symptomatic animals were more easily identified as infected (sensitivity: 77-98%). The highest sensitivity value was provided by the dpp® test (98%). Another LFIA kit for CVL diagnosis, the Speed leish K [13], has been validated in a study by Ferroglio et al. [35]. The sensitivity and specificity were 96.3% and 100%, respectively, when calculated for canine sera with high IFAT titres (>1/160), which likely correspond to animals showing clinical signs of infection or in advanced stage of disease [35]. Otranto et al. validated a LFIA for CVL based on a recombinant K39 antigen that provided 97.06% and 100% sensitivity and specificity, respectively [36].

The LFIA for detecting canine anti-leishmanial antibodies developed in this study has higher sensitivity (98.4%) compared to other LFIAs, and the validation also included subjects with low IFAT titre. Therefore, it candidates as a reliable tool for the accurate early detection of CVL. Specificity is comparable to the mean of LFIA kits available on the market for Leishmaniasis diagnosis. The LFIA device is also robust, as the visual output demonstrated to be stable over time and not influenced by the occasional increase of the temperature. It show long-term stability (up to six months), without requiring refrigeration. In conclusion, it is suitable for on field applications by non- trained personnel and in low-resources settings.

Furthermore, the design of the assay allows for its facile adaptation to diagnosing VL in other companion animals and wild carnivores that have been confirmed to have a role in the spreading of VL transmission. This versatility represents a further benefit for keeping the broadening of infection transmission under control in a timely and efficient way.

The LFIA provides a qualitative yes/no response that may be used as a first screening test. In case of a positive result, a quantitative serology (ELISA or IFAT) should be performed to better discriminate the stage of infection and direct treatments.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest: The author Chiara Nogarol works at In3diagnostic company (L.go P. Braccini 2, Grugliasco (TO), Italy) which may commercialize the device. However, this does not alter the authors' adherence to the principles of good scientific practice and to relevant policies on sharing data and materials. The authors declare no other competing interest.

Research involving Animal Participants.

Informed consent: Blood samples were obtained during routine activities at the Veterinary Teaching Hospital of the Department of Veterinary Science or performed by veterinary practitioners. The study encompassed dogs from a private kennel/small animal veterinary clinics and informed consent was obtained from the owner. The consent was provided in oral form. No additional permission was required. All procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments, as well as subject to informed owner consent.

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571 **Tables**

572

573 **Table 1.** Classification of canine sera by the LFIA

574

	N of positive result LFIA / reference method	Se (%)	False negative rate (%)	N of negative result LFIA / reference method	Sp (%)	False positive rate (%)
Endemic	23 / 24	95.8	4.2	13 / 13	100	0
Non-endemic	39 / 39	100	0	79 / 80	98.8	1.2
	62 / 63	**98.4**	**1.6**	**92 / 93**	**98.9**	**1.1**

575

576

577

578

579 **Table 2.** LFIA compared to the reference IFAT and ELISA methods

580

	vs IFAT	vs ELISA
N samples	140	140
(pos / neg)	(61 / 79)	(62 / 78)
Accuracy (%)	97.1	92.9
K	0.94	0.86

581

582 **Figure captions**

583

584 **Figure 1.** Scheme of the LFIA device for the rapid diagnosis of VL. The strip is composed of the analytical
585 membrane onto which the recombinant chimeric antigen (rCAg) and protein A (pA) are coated to form the
586 Test and Control line, respectively. The signal reporter is made of pA labelled with gold nanoparticles that
587 are red coloured due to their surface resonance band at 525 nm. GNP-pA is included in the device in dried
588 form by pre-impregnation of the probe pad. The device also comprises a sample pad, that adsorbs the
589 sample and distributes it homogeneously to the membrane, and an adsorbent pad to decrease background
590 colour by incrementing the volume of the flowing sample. A single visible line (Control) is expected for a
591 canine serum that does not contain any anti-leishmanial antibodies (negative sample) due to interaction of
592 generic immunoglobulins with the labelled pA and with the pA immobilized onto the membrane. The
593 presence of specific anti-leishmanial antibodies is revealed by their specific binding to the rCAg, which is
594 made visible as a second red line (Test line).

595

596 **Figure 2.** Typical results provided by the LFIA for detecting anti-leishmanial antibodies for a positive and a
597 negative canine serum. Negativity is represented by the presence of single red line (the Control line), while
598 positivity is observed as the presence of two red lines (Test and Control lines) after sample running. The
599 strip is included into a plastic cassette providing a sample well and a reading window. When only the Test
600 line is visibly coloured the test is invalid.

601

602 **Figure 3.** In-house validation of the LFIA for detecting anti-leishmanial antibodies: **(a)** within- and between-
603 day variability of the LFIA response, bar represent standard deviations of the three replicates obtained on
604 each day for the between-day experiment; **(b)** stability of the LFIA response over time for six positive
605 samples; **(c)** shelf-life of the LFIA device as measured at 4 °C (circle) and room temperature (square) **(d)**
606 thermal stability of the LFIA device at 37 °C. Bars in (b), (c), and (d) represent standard deviations of
607 replicates experiments (n=2)

608

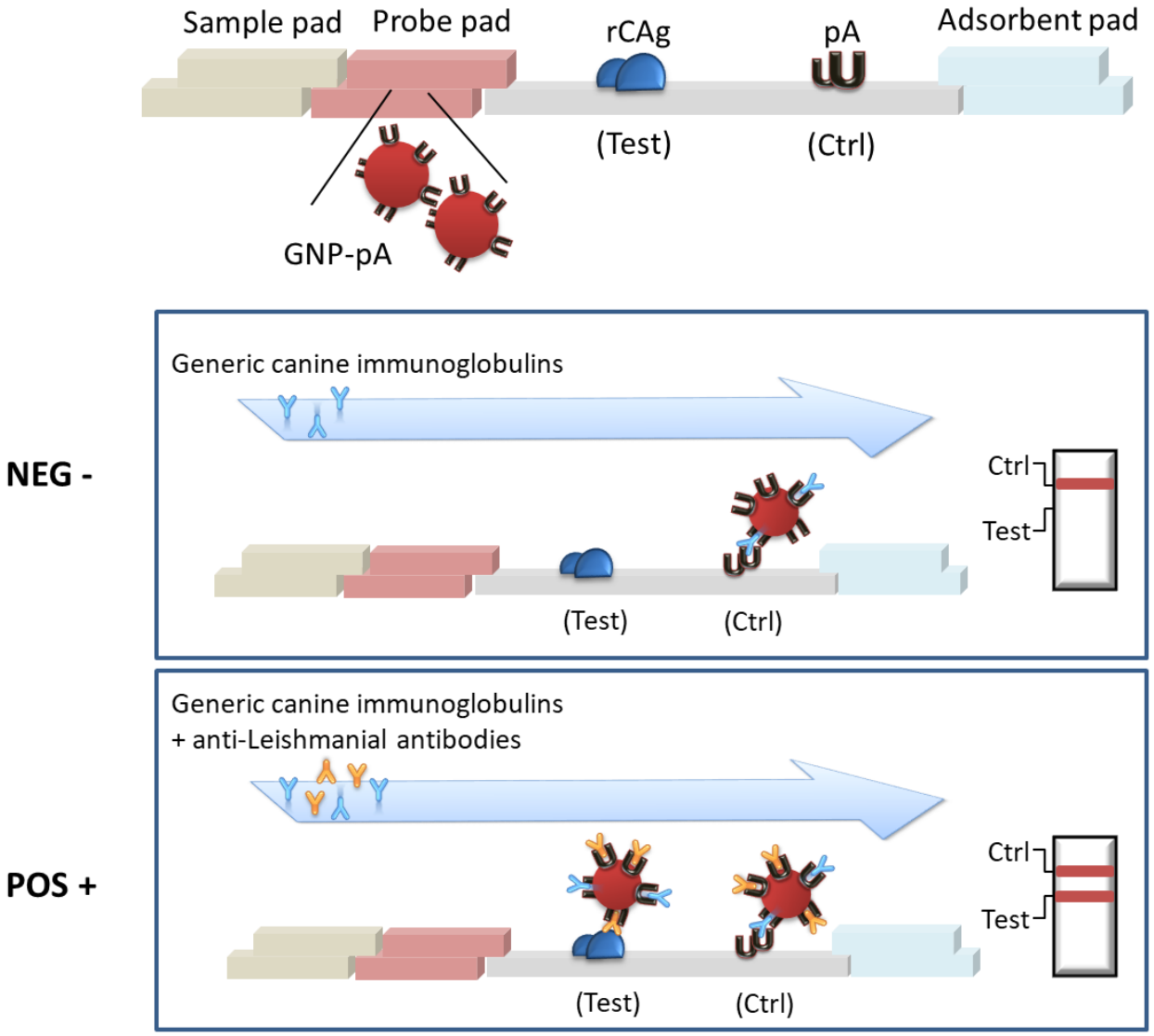
609 **Figure 4.** LFIA results obtained by analysing sera from two foxes (lines #1-2) and nine cats (lines #3-11). A
610 clearly visible Test line indicated positivity for three samples (lines #1, #3 and #9). One sample (line #10)
611 was weakly positive.

612
613

614

615 **Figure 1**

616



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620 **Figure 2.**

621

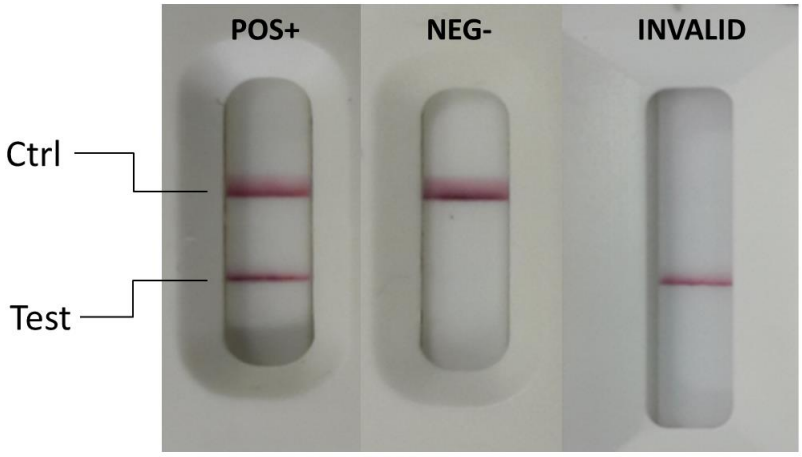

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Figure 3.

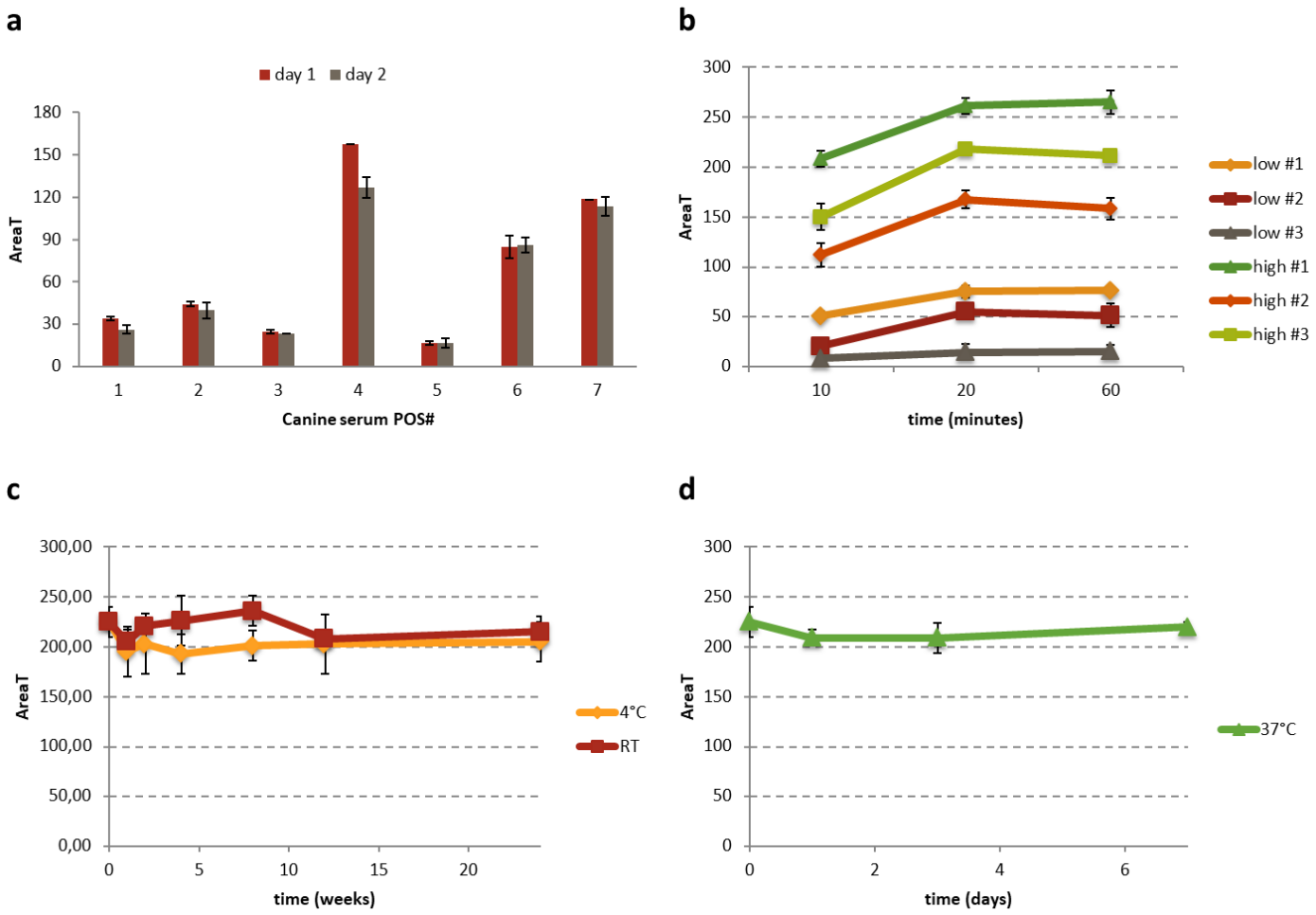
a
day 1
day 2
AreaT
Canine serum POS#
b
AreaT
time (minutes)
low #1
low #2
low #3
high #1
high #2
high #3
c
AreaT
time (weeks)
4°C
RT
d
AreaT
time (days)
37°C

Figure 4.


F PlG
F916
C80
C28
C 88
C 00
C01
C 52
C 108
C 111
Ctrl
Test
Line
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11