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Triple lines gold nanoparticle-based lateral flow assay for enhanced and simultaneous detection of *Leishmania* DNA and endogenous control

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ABSTRACT

A novel triple lines lateral-flow assay (LFA) with enhanced sensitivity for the detection of *Leishmania infantum* DNA in dog blood samples was designed and successfully applied. The enhanced LFA methodology takes advantage of the gold nanoparticle tags (AuNPs) conjugated to polyclonal secondary antibodies, which recognize anti-FITC antibodies. The polyclonal nature of the secondary antibodies allows for multiple binding to primary antibodies, leading to enhanced AuNP plasmonics signal. Furthermore, endogenous control consisting of the amplified dog 18S rRNA gene was introduced to avoid false negatives. Using this strategy, 0.038 spiked *Leishmania* parasites per DNA amplification reaction (1 parasite/100 μL of DNA sample) were detected. Detection limit of LFA was found to be lower than that of the conventional techniques. In summary, our novel LFA design is a universal and simple sensing alternative that can be extended to several other biosensing scenarios.

1 Introduction

Diagnostic devices that are low-cost and based on single-use paper platforms have gained much interest in recent years for their use as affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users (ASSURED) sensors [1]. Paper chromatography developed by Martin and Synge at the beginning of 1940s [2] is considered as the first paper-based sensor. Later on, semi-quantitative paper-based biosensors for the detection of glucose in urine [3], became the most common commercially

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available point-of-care (POC) lateral flow assay (LFA) device. Initially, the main application for LFAs was to serve as a pregnancy test [4]. However, recently, their application is extended to a wide variety of analytes including cancer biomarkers [5, 6], DNA [7, 8], toxins [9, 10], and metals [11, 12].

LFAs are characterized by their simple use, rapid result, low cost, good specificity and long shelf life. However, they suffer from analytical performance limitations mainly due to sensitivity and reproducibility issues. In this context, many efforts have been made in order to improve LFA sensitivity. Recently, special interests have been generated by potential tools developed in the field of nanotechnology. Improving immunosensing approaches based on gold nanoparticles (AuNPs) [13], such as immunogold silver staining [14, 15], dual AuNP conjugates [16], and AuNP loaded with enzymes [17–19] have been reported in the recent years. Apart from AuNPs, other NP labels such as fluorescent Eu(III) NPs [20] and quantum dots [21] have been also examined. Changes in paper architecture [22], the modification of detection pad with wax-printed pillars [23], and even paper integration into a Lab-in-a-Syringe [24] have also been proposed for improving the performance of LFAs. However, there is still a need for innovative enhancement strategies that would avoid the use of additional modification steps in the assay, which can maintain analytical performance while at the same time offer a simple and cost-effective solution.

In this context, we present here a very simple and universal enhancement approach based on the use of AuNPs conjugated to polyclonal secondary antibodies capable of recognizing multiple sites on primary antibodies that target the analyte of interest. This novel approach was applied to detect *Leishmania* DNA in dog blood samples. Leishmaniasis is a vector-borne and poverty-related disease, which can be potentially fatal for humans and dogs, and represents an important public health problem [25, 26]. There are two main forms of *Leishmania* infection, which are associated with varying clinical symptoms: cutaneous leishmaniasis, which can be cured but cause skin damage [27], and visceral leishmaniasis, which is fatal if left untreated [28]. According to the World Health Organization, an estimated 12 million people are infected around the world, and every year 1–2 million new cases arise [29]. More than 500,000 cases correspond to visceral leishmaniasis, and the mortality rate is estimated to be approximately 50,000 deaths per year [28, 29]. Canine Leishmaniasis (CanL) caused by *Leishmania infantum* is transmitted via the bite of insect vectors such as that by phlebotomine sand fly. The flagellated infective promastigote enters the host, and the intracellular amastigote form is subsequently developed and replicated in the mammal [30]. Different methods have been reported for the detection and diagnosis of CanL including parasitological [31–33], serological [34, 35], and molecular techniques [36–42]. LFA strips for specific CanL antigen (kR39) are also commercially available (InBiosInc®, CTK Biotec®, DiaMet IT®) for the detection of visceral leishmaniasis [43–46]. However, there are limited examples of detection in amplified (by PCR) CanL DNA using LFA (OligoC-test®) [47], which can offer enhanced assay sensitivity than that with other antigen based detection methods.

Given the importance of *Leishmania* DNA detection, we offer a novel LFA design with enhanced sensitivity that is able to detect very low quantities of DNA amplified from the analyte. DNA primers labeled with biotin and FITC are used for the amplification reaction to obtain DNA replicates that are double labeled with FITC/biotin. The enhanced methodology takes advantage of the use of AuNPs conjugated with polyclonal secondary antibodies that recognize anti-FITC primary antibodies. The polyclonal nature of the secondary antibodies allows for multiple bindings to different epitopes on primary antibodies, giving rise to enhancements of the AuNP signal. Furthermore, endogenous control in a third line of the LFA that corresponds to the amplification of the 18S rRNA gene was introduced to avoid false negatives.

2 Experimental

2.1 Chemicals and equipment

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9%), trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), and Streptavidin from *Streptomyces avidinii* were purchased from Sigma-Aldrich (Spain). Anti-goat IgG (polyclonal antibody produced in chicken; ab86245)

and anti-FITC IgG (polyclonal antibody produced in goat; ab19224) were purchased from Abcam (UK). All reagents used for the preparation of the buffers were supplied by Sigma Aldrich (Spain).

Boric acid (H_3BO_3, 99%) and sodium tetraborate decahydrate ($B_4NaO_7{\cdot}H_2O$, 99%) were used for the preparation of borate buffer (BB); Sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$, 99%) and sodium phosphate dibasic (Na_2HPO_4, 99%) were used for the preparation of phosphate buffer (PB); Trizma® HCl ($C_4H_{11}NO_3$, 99%) and sodium chloride (NaCl, 99.5%) were used for the preparation of tris buffer saline-tween (TBST); Tween®-20 ($C_{58}H_{114}O_{26}$), sucrose ($C_{12}H_{22}O_{11}$, 99.5%), bovine serum albumin (BSA, 96%), and sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$, 98.5%) were used for the preparation of blocking buffers. Phosphate buffer saline tablet was used for the preparation of PBS buffer.

All materials used for the production of LFIA strips were purchased from Millipore (Billerica, MA 08128, USA): sample and absorbent pads (CFSP001700), conjugate pad (GFCP00080000), detection pad (SHF1800425) and the backing card (HF000MC100). A guillotine Dahle 533 (Germany) was used to cut the strips. An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection and control lines. A strip reader (COZART—SpinReact, UK) was used for quantitative measurements. Milli-Q water, from a Milli-Q system (> 18.2 M/cm, from Millipore, Sweden), was used to prepare all solutions. The stirrer used was a TS-100 Thermo shaker (BioSan, Latvia). A thermostatic centrifuge (Sigma 2-16 PK, Fisher Bioblock Scientific, France) was used to purify the AuNP/antibody conjugates. A high resolution (XHR) scanning electron microscope (model FEI Magellan™ 400L, Nanolab Technologies, USA) was used to observe the AuNPs on the test line of the nitrocellulose strip.

All the size measurements and shape observation of AuNPs were conducted by using a Field Emission Gun Transmission Electronic Microscope (model TecnaiTM G2F20, FEI, USA). Spectrophotometer SpectraMax M2e (Molecular Devices, USA) was used to measure the UV–vis spectra of AuNPs suspensions.

2.2 Amplification of *Leishmania infantum* kinetoplast DNA

Different quantities of promastigote of *Leishmania* were spiked on DNA samples extracted from negative (previously analyzed by real time PCR [48]) dog blood. *Leishmania infantum* kinetoplast DNA amplification using reverse and forward primers labeled with biotin and FITC, respectively, was performed using Recombinase Polymerase Amplification (RPA) isothermal procedure (TwistDx's®) [49]. Samples from dogs without spiked parasite ("blank" samples) were also amplified to determine the specificity of the method.

For endogen control assays, an additional pair of primers that amplify the 18S rRNA gene (always present in both "positive" and "blank" samples), labeled in this case with FITC (forward) and digoxigenin (reverse) were also used.

2.3 Preparation of gold nanoparticles

AuNP 20 nm in size were prepared using the Turkevich's method [50]. Briefly, 50 mL aqueous solution of 0.1% $HAuCl_4$ was heated to boiling, and vigorously stirred in a 250 mL round-bottom flask; 1.25 mL sodium citrate (1%) was quickly added to this solution. The solution was boiled for an additional 10 min. The solution was then cooled to room temperature with continuous stirring. Colloids were stored in dark bottles at 4 ˚C. All glassware used in this preparation were previously cleaned with aqua regia overnight, and rinsed with double distilled H_2O. Reflux was used for all the procedure.

2.4 AuNPs modification with antibodies: Preparation of the double antibody solution

The conjugation of AuNPs with antibodies was performed according to the following procedure previously optimized by our group [13]. First, the pH of the suspension of AuNPs was adjusted to 9 with 0.1 M BB. Then, 100 μL of a 100 μg/mL anti-goat IgG aqueous solution was added to 1.5 mL of the AuNPs suspension. The resulting solution was incubated for 20 min at 650 rpm. This was followed by addition of 100 μL of 1 mg/mL BSA, and stirring was continued for other 20 min at 650 rpm. Finally, the solution was centrifuged at 14,000 rpm and 4 ˚C.

The supernatant was removed and the pellet of AuNP/anti-goat IgG was re-suspended in 300 μL of 3 μg/mL anti-FITC IgG solution in 2 mM BB (pH = 7.4, 10% sucrose). This method allows a suspension

containing both anti-FITC IgG and AuNPs/anti-goat IgG to be made, and can be used immediately for conjugate pad preparation. TEM micrographs and UV–vis spectra of AuNPs before and after their conjugation with antibodies are shown in Fig. S1 of the electronic supplementary material (ESM).

2.5 Preparation of the strips

Anti-goat IgG (1 mg/mL) and 10 mM streptavidin in PB (pH = 7.4) were spotted onto the detection pad at a dispensing rate of 0.05 μL/mm. An IsoFlow reagent dispensing system was used to form both the control and test lines. For the detection of the endogen control, an additional line with 1 mg/mL of anti-digoxigenin was also spotted. The detection pad was then dried at 37 ˚C for 1 h. The membrane was blocked using 2% BSA for 5 min. Finally, the membrane was washed for 15 min using PB (5 mM pH = 7.4) with 0.05% SDS, and dried at 37 ˚C for 2 h. The sample pad was prepared by dipping into 10 mM PBS, 5% BSA and 0.05% Tween®-20 and dried at 60 ˚C for 2 h. The conjugate pad was prepared by dipping it into the previously prepared double antibody solution (anti-FITC IgG and AuNP/anti-goat IgG), and drying under vacuum for 1 h. Different pads were sequentially laminated and pasted onto adhesive backing cards in the following order: detection, conjugation, sample and absorbent pads. Finally, the pads were cut into 7 mm wide strips and stored in dry conditions at 4 ˚C until further use.

2.6 Lateral-flow assay procedure

For evaluation of the signal enhancement, amplified *Leishmania* DNA sample solutions prepared from dog blood samples spiked with 20 parasites were diluted in 10 mM Tris buffered saline with Tween 20 (TBST, pH = 7.6 and 0.05% Tween®-20) at different dilution factors. Typical assay consisted in mixing 10 μL of the diluted sample (different dilution factors in TBST were assayed: 1:100, 1:250: 1:500 and 1:1,250) with 200 μL of TBST, immersing the strip into this solution for 10 min until the flow was stopped. Subsequently, 200 μL of TBST was dispensed to wash away the excess antibody solution.

For quantitative *Leishmania* parasite determination, amplified DNA (without any dilution) from dog blood samples spiked with varying quantities of parasites (0.04, 0.08, 0.4, 1, 2, 4 and 20 parasites) were examined.

Each sample was run in triplicates, and intensities of lines were read with the strip reader to obtain the corresponding calibration curve.

3 Results and discussion

3.1 Principle of enhanced detection

The principle of signal enhancement is based on indirect ELISA. In a typical assay, after the analyte is recognized by a capture antibody, the sandwich is completed with a label-free primary antibody. After that, an AuNP labeled polyclonal secondary antibody is used to detect the primary antibody. The polyclonal nature of the secondary antibody allows for multiple bindings between the primary and secondary antibodies as numerous epitopes on the primary antibody can be recognized. This leads an increase in the number of AuNP labels, and consequently, an enhancement in the analytical signal [51].

In our approach, the novel and simple strategy for signal amplification is based on immobilization of primary antibodies on the conjugation pad, which in turn are detected by secondary antibodies labeled with AuNPs. A complex is then formed consisting of the primary antibody bound to the analyte of interest and the secondary antibody tagged with AuNPs.

Two reagents are then deposited onto the nitrocellulose membrane: streptavidin and anti-goat IgG, forming the test line (TL1) and the assay control line (CL), respectively. As sample starts to flow through the strip, the primary antibody/secondary antibody complex is released, leading to the capture of the amplified DNA through tails labeled with FITC. While the sample migrates though the membrane, the test line becomes visible due to binding of streptavidin to the biotin present in the amplified DNA. The control line turns visible when the anti-species antibodies capture any excess antibody labeled with gold nanoparticles (Fig. 1).

Using the standard LFA design, a negative response (no signal in the detection line) can be due not only to the absence of *Leishmania* in the sample but also to errors in the DNA extraction amplification procedure.

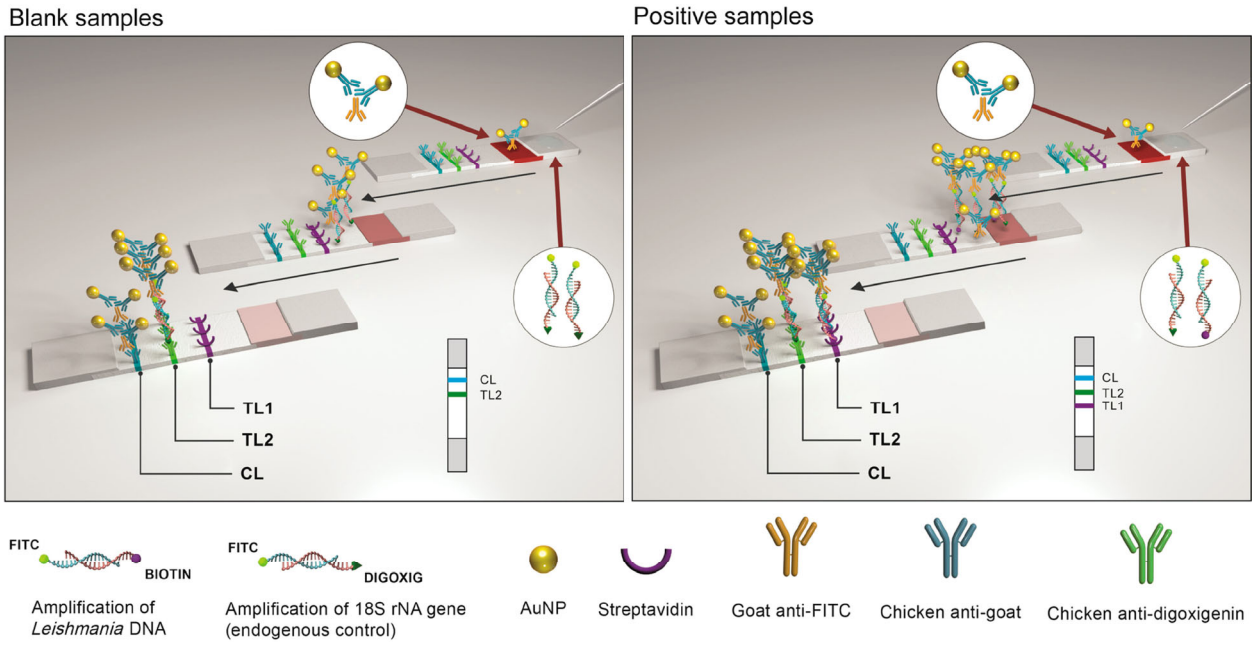

Figure 1 Schematic of the enhanced LFA based on the use of secondary antibodies for the detection of double labeled (FITC/biotin) *Leishmania* DNA (TL1) and 18S rNA gene (endogenous control) (TL2). CL stands for assay control line.

In order to resolve this, an endogenous control was also included in the LFA design. This consisted of an additional pair of primers that amplify the house keeping gene 18S rRNA, which will always be present in any DNA samples. These primers were labeled with FITC and digoxigenin as schematized in Fig. 1. As result, in the positive samples, both products (biotin/FITC and digoxigenin/FITC labeled) would be present while the blank samples would contain only digoxigenin-FITC.

In order to detect both products, the LFA strip was slightly modified with an additional line in the detection pad (TL2), where anti-digoxigenin antibodies were immobilized. This line is always visualized, even for blank samples, evidencing that the DNA amplification procedure was performed properly, as illustrated in Fig. 1.

3.2 Optimization of the enhanced lateral flow assay

Various parameters that affect the analytical signal such as blocking, antibody concentration, and make-up of running buffers were optimized. In order to minimize reagent consumption, this optimization was performed on assays without endogenous controls, and only TL1 and CL were visualized.

3.2.1 *Blocking agent*

Nitrocellulose membranes have been traditionally blocked with different agents (e.g. proteins, surfactants or polymers) not only to avoid non-specific bindings of nanoparticles over the membrane [52, 53], but also to control the flow rate as well as to stabilize the test and control line [54]. The best conditions for blocking the membrane for each specific approach must be thoroughly optimized. In our case, a solution containing 2% BSA was found to be a suitable blocking agent (Fig. 2(a)).

3.2.2 *Running buffer*

The formation of antigen-antibody complex can be affected by matrix parameters such as pH, temperature and ionic strength [55]. Tris buffer is well known for its capability to solubilize DNA and prevent its degradation. Three running buffers with various Tris concentrations and additives were tested for positive and blank samples, as shown in Fig. 2(a). Results showed that when the ionic strength of the running buffer increased, the test line became more intense, the sensitivity was improved, and no unspecific signal in the test line was observed. In addition, the presence of a surfactant (Tween-20®) allowed better flow of

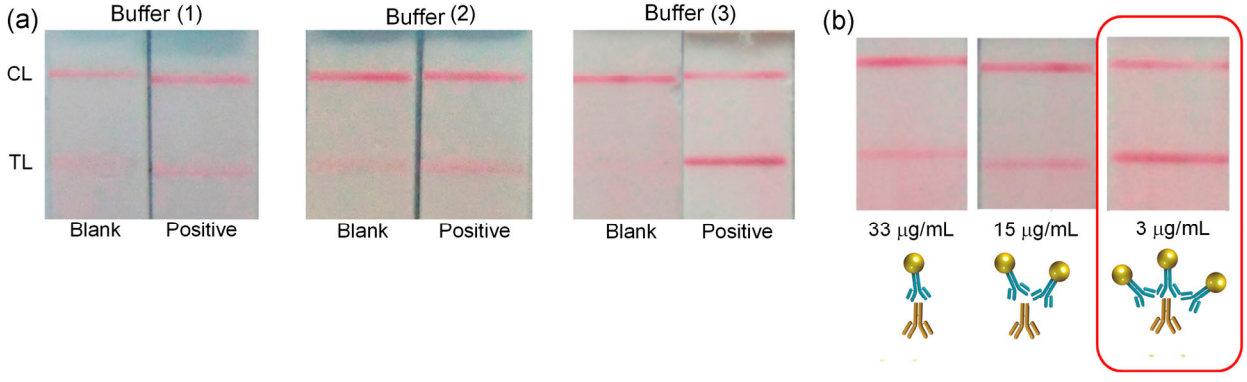

Figure 2 Optmization of the enhanced lateral-flow assay. (a) Effect of running buffer on an amplified *Leishmania* product diluted at 1:50. Buffers are: (1) 25 mM Tris, 150 mM NaCl, pH = 7.6, (2) 50 mM Tris, 138 mM NaCl, 0.27 mM KCl, 1% BSA and (3) 50 mM Tris, 150 mM NaCl, 0.05% Tween-20. (b) Effect of primary antibody concentration in double antibody-AuNP conjugate. Assays were performed for an amplified *Leishmania* DNA product diluted at 1:100. Other conditions are detailed in the experimental section.

nanoparticles through the membrane, avoiding non desirable adsorptions. Thus, the most suitable running buffer for this assay was found to be TrisHCl 50 mM, 150 mM NaCl and 0.05% Tween-20®. These observations are in agreement with previously reported studies showing that ionic strength of running buffer, together with the presence of surfactant, have an can significantly reduce background signal due to unspecific bindings over the membrane [56, 57].

3.2.3 Concentration of primary antibody

Different concentrations of the primary were evaluated in the presence of the secondary antibody/AuNPs. It was found that the most suitable concentration of goat anti-FITC in the double antibody solution was 3 μg/mL (Fig. 2(b)). This may be due to the fact that for higher concentrations of primary antibody, the primary Ab/secondary Ab ratio is close to 1:1, and consequently the concentration of AuNPs in the test line is lower, as schematized in Fig. 2(b).

3.3 Semi-quantitative assay: Evaluation of the signal enhancement

Preliminary tests for amplified DNA using the enhanced LFA strategy were performed under the optimized conditions. The results were compared with those obtained using a direct LFA without secondary antibodies, and are summarized in Fig. 3. In both cases, amplified DNA was serially diluted to evaluate the ability of the sensing system to detect lower quantities of the labeled products. As also shown in Fig. 3(a), TL2 corresponding to the endogenous control is clearly visualized. Furthermore, the intensity of color in the control line remains constant while the ones corresponding to the two test lines (TL1 and TL2) decreases when the dilution factor is increased, demonstrating that proper DNA amplification procedure was carried out.

Furthermore, a clear improvement in assay sensitivity was noticeable even with the naked eye.

This signal enhancement allows visual detection of amplified DNA diluted at 1:500, whereas with the direct assay only products diluted up to 1:250 can be visualized. The advantages of the enhanced strategy are even more evident with the measurements of optical density of TL1, which was performed with a scanner (Fig. 3(b)).

It is important to point out that with this novel strategy, not only is sensitivity improved, but the lines are also better defined, which is crucial for adequate and reproducible readings by the scanner.

Thanks to the optical density measurements, amplified DNA diluted up to a 1:1250 ratio can be detected, ensuring that false negatives are avoided by visualization of the endogenous TL2.

The enhanced methodology was also evaluated through examination by scanning electron microscopy (SEM) of TL1 on nitrocellulose strips after LFAs was performed on amplified *Leishmania* DNA, as shown in Fig. 4. For the direct assay, a small amount of dispersed AuNPs was found on the surface of the strip, which was observed as discrete spots (Fig. 4(a)). However, in the case of enhanced assay, a much higher density of

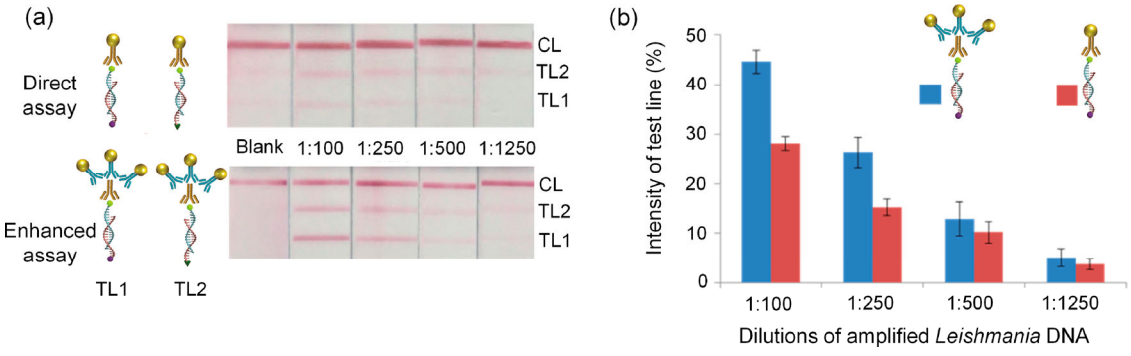

Figure 3 Evaluation of the signal enhancement. (a) Pictures of LFA strips after the assay performed on amplified *Leishmania* DNA at different dilution factors, including the endogenous control. The direct and the enhanced assays are also compared. TL1 corresponds to the test line while TL2 stands for the endogenous control. (b) The corresponding intensity values (TL1) obtained with the strip reader for both direct and enhanced assays.

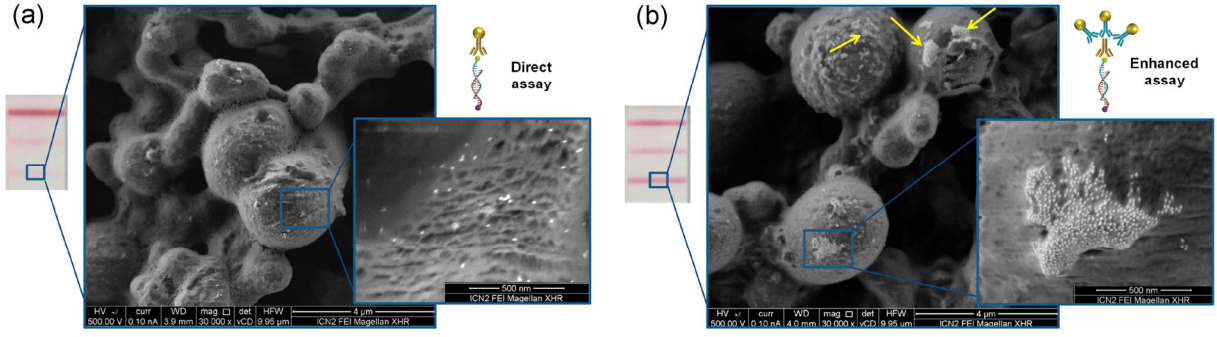

Figure 4 SEM characterization of signal enhancements. SEM images of TL1 of nitrocellulose strips after a direct (a) and an enhanced (b) LFA were performed for the amplified *Leishmania* DNA product 1:100 dilution. Pictures on the left belong to the corresponding strips. Arrows indicate areas with high density of AuNPs.

AuNPs was observed, as illustrated in Fig. 4(b). These observations are in agreement with the proposed enhancement principle, suggesting that the use of secondary antibodies highly increases density of AuNPs in the test line. This leads to important enhancements in the optical density, and consequently in the sensitivity of the assays.

3.4 Quantitative determination of *Leishmania infantum* parasites

The enhanced strategy in combination with the endogenous control was applied for the evaluation of amplified DNA prepared from samples containing different quantities of spiked parasite. As shown in Fig. 5(a), a gradual increase in the intensity of TL1 was observed for increasing amounts of parasite. As expected, intensity of the line corresponding to the endogenous TL2 remained almost constant independent of the quantity of parasites spiked in the sample. These evidences were also further corroborated by measurements performed with the scanner (Fig. 5(b), left).

The intensity values of TL1 vs. the logarithm of the number of parasites were plotted (Fig. 5(b), right), finding a linear relationship in the range 0.04 to 20 spiked parasites. A detection limit (LOD) of 0.038 parasites per DNA amplification reaction (1 parasite/100 μL of DNA) was estimated, which was equal to the blank signal plus three times its standard deviation. The reproducibility of responses (n = 3) for 2 spiked parasites was also studied, obtaining a relative standard deviation (RSD) of 4%.

The LOD obtained for two test lines was almost the same as the one obtained with only one test line for *Leishmania* parasite detection (see the study performed without endogenous control at the Figs. S2 and S3 in the ESM), demonstrating that the addition of a second test line is also a tool for *Leishmania* parasite detection without affecting the sensitivity of the enhanced LFA.

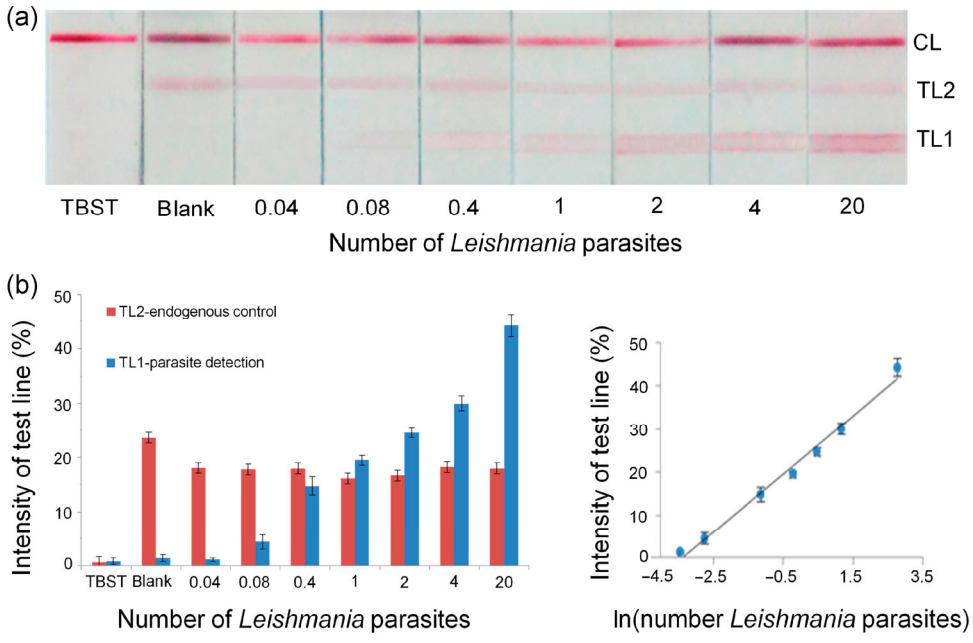

Figure 5 Quantitative *Leishmania* infantum parasite determination. (a) Pictures of LFA strips for an enhanced lateral flow assay containing the endogenous control. TL1 corresponds to the test line while TL2 stands for the endogenous control. (b) Corresponding bar intensity graphic (left) and logarithmic relationship between the number of *Leishmania* parasites and the % of intensity in the test line (right).

Currently, most of the LFA for *Leishmania* detection are used for qualitative tests, and only a few have been able to achieve semi-quantitative analytical response [39, 44, 58, 59]. Results of our AuNP based amplification approach are quite similar to those obtained in semi-quantitative test that uses nucleic acid sequence based amplification (NASBA) coupled to oligochromatography (OC) [60]. This method is even more sensitive than that offered by the OligoC-test® [47]. Furthermore, our technique provides a valuable proof of concept of this novel enhancement approach, which is a universal methodology that can be applied for any LFA design.

4 Conclusions

A novel LFA design based on the use of secondary antibodies in the conjugate pad was successfully accomplished and applied for the detection of amplified *Leishmania infantum* DNA extracted from dog blood samples. The use of labeled primers allows the generation of double labeled (FITC/biotin) products that can be detected in a LFA. The polyclonal nature of the secondary antibodies enables multiple connections with primary antibodies, giving rise to enhancements of the AuNP signal in the test line, and consequently increasing assay sensitivity up to 0.038 parasites per DNA amplification reaction (1 parasite/100 μL of DNA).

Furthermore, an endogen control was included in our assay to avoid false negatives. It was simply performed by the addition of a pair of primers that amplify the 18S rRNA gene (always present in the sample) labeled with FITC and digoxigenin and introducing an additional test line in the LFA strip, containing anti-digoxigenin antibodies. This approach was successfully implemented without losing the efficiency of signal enhancements. The proposed enhancement strategy is a versatile and universal methodology that can be applied for any LFA design. In addition, specific antibody against the analyte does not need to be directly labeled in this assay, which has clear advantages in terms of the cost of this technology.

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