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Abstract: Leishmaniasis is a disease, caused by *Leishmania* parasites, which infect humans and animals, posing a major social and economic burden worldwide. The need for accurate and sensitive disease diagnosis led to the widespread adoption of PCR amplification. Detection of the amplification products (i.e. gel electrophoresis) require time-consuming protocols performed by trained personnel, with high cost. Aim of the present study was the simplification of PCR product detection, using a nucleic acid lateral flow, combined with functionalized gold nanoparticles. Amplification reactions targeting kinetoplastid DNA of *Leishmania spp* were performed on canine blood samples and a positive signal was formed as a red test zone. The visual detection was completed in 20 min. Extensive optimization enabled the detection of 100 fmol of target DNA. Clinical samples of infected dog blood were analyzed with high specificity. Overall, the proposed lateral flow biosensor can be considered an appealing alternative platform for *Leishmania*-specific amplification products detection with low cost and attractive simplicity.

Keywords: Lateral flow strip biosensor; gold nanoparticles; *Leishmania;* kinetoplast gene.

1. Introduction

Leishmaniasis is a parasitic, vector-borne disease caused by protozoa of the genus *Leishmania (L.)*. The parasites are transmitted through the bites of female phlebotomine sandflies and infect humans and various animal species, like dogs (Akhoundi, et al., 2016). It is endemic in more than 98 countries of the tropics, subtropics and the Mediterranean basin, and affects 14 million people, with approximately 350 million having a potential risk. The disease constitutes a major social and economic burden in India, Sudan, Brazil, and Bangladesh, as reported by the World Health Organization (WHO) [(Alvar, et al., 2012); (Hussain, et al., 2014); (Castellanos-Gonzalez, et al., 2015); (Okwor & Uzonna, 2016)]. There are three main forms of the disease caused by more than 20 different species: cutaneous (CL), mucocutaneous (ML), and the most severe, visceral leishmaniasis (VL), also called kala-azar (Hindi for Black fever) (Gutiérrez, et al., 2015).

Canine leishmaniasis (CanL) is caused by *L. infantum*. The parasite infects domestic dogs (*Canis familiaris*) which are its main reservoir hosts. It is an important disease due to its zoonotic potential and results in significant mortality and morbidity of the host. CanL is endemic in more than 70 countries and about 2.5 million dogs suffer from the disease (Solano-Gallego, et al., 2011). Different specific diagnostic methods have been described for the detection of *L. infantum* infection in dogs and they are detailed in (Solano-Gallego, et al., 2011). Immunochromatography-based assays are easy to use and provide rapid qualitative results on the spot [(Mettler, et al., 2005); (Pattabhi, et al., 2010)]. Lateral flow strips for the antigen (rK39) are commercially available (InBiosInc®, CTK Biotec® DiaMet IT®) for detection of VL (Welch, et al.,

2008), but their performance is still not optimal (Solano-Gallego, et al., 2011). Alternative methodologies include the use of interdigitated electrodes for antibodies detection (Perinoto, et al., 2010), application of paper biosensors (Costa, et al., 2014) or nanoparticles for detection of *Leishmania* antigens and unamplified DNA [(Andreadou M, 2014); (Andreadou M., 2016)].

Polymerase chain reaction (PCR) has evolved into one of the most specific and sensitive methods for *Leishmania* detection, especially for asymptomatic and early-stage infections [(Rodgers, et al., 1990); (Smyth, et al., 1992); (Schulz, et al., 2003); (Mary, et al., 2004); (Francino, et al., 2006); (de Paiva Cavalcanti, et al., 2009); (da Silva Solcà, et al., 2012); (Lombardo, et al., 2012); (Andreadou M., 2012); (Tellevik, et al., 2014); (de Ruiter, et al., 2014); (Koltas, et al., 2016)]. Isothermal methods have been proposed for *Leishmania* DNA amplification but PCR remains the main choice for diagnostic tests [(van der Meide, et al., 2005); (Takagi, et al., 2009); (de Ruiter, et al., 2014)]. In most cases the PCR products detection is based on gel electrophoresis followed by ultraviolet transillumination in the presence of the carcinogenic ethidium bromide (Deborggraeve, 2008). In order to simplify the existing methodologies, detection of the amplification products with lateral flow biosensors has been introduced.

Lateral flow biosensors (LFBs) are diagnostic devices based on single use paper carrier material, where dry reagents are activated by applying any fluid sample. LFBs are important for diagnostic purposes since they are affordable, sensitive, specific, user-friendly, rapid, robust and equipment-free (Parolo & Merkoçi, 2013). They are an ideal platform for single use at point of care, where a positive/negative signal is

sufficient and results usually come within 20 min (Posthuma-Trumpie, et al., 2009). A few lateral flow assays have been proposed for detection of PCR amplified *Leishmania* specific DNA, including the OligoC-TesT® PCR-oligochromatographic test (Coris BioConcept, Gembloux, Belgium) (Deborggraeve, 2008); an OligoC-TesT variation for *Leishmania* typing (Laurent, et al., 2009); a generic lateral flow oligochromatograhic dipstick paper (Milenia® GenLine HybriDetect, Milenia Biotec GmbH, Germany) (Castellanos-Gonzalez, et al., 2015) and a triple-line LFB (Rivas L., 2015).

Aim of the present study was the development and evaluation of an alternative lateral flow biosensor-based assay for detection of kinetoplast DNA amplification products in canine blood samples. The proposed lateral flow biosensor enables visual detection of double stranded DNA within minutes. Biotinylated PCR products are hybridized with a poly(dA)-tailed oligonucleotide probe and applied on the biosensor. Gold nanoparticles functionalized with oligo(dT) segments interact with the hybridized complex of target DNA – poly(dA) probe and then gets captured by immobilized streptavidin in the test zone of the LFB, indicating a positive signal.

2. Materials and Methods

2.1. Oligonucleotides

The oligonucleotides used in this study were synthesized by Eurofins genomics AT (Vienna, Austria). A 5'-thiolated $(dT)_{30}$ oligonucleotide (5'-SH-TTTTTTTTTTTTTTTTTTTT

TTTTTTTTTT-3') was used for conjugation to gold nanoparticles. A $(dA)_{20}$ oligonucleotide (5' -AAAAAAAAAAAAAAAAAAAA-3') was used for construction of the control zone of the biosensor, while the biotinylated poly-(dA) reference oligonucleotide (B-dA_{20}: 5'-B-AAAAAAAAAAAAAAAAAAAA-3') was labeled at the 5'-end with biotin and used as a positive control for biosensor signal generation. The 22-mer 5'-CTTTTCTGGTCCTCCGGGTAGG-3' was labeled at the 5' end with biotin and used as the upstream primer (UpLei_B) for *Leishmania* kinetoplast DNA amplification, while the 24-mer 5'-CCACCCGGCCCTATTTTACACCAA-3' was used as the downstream primer (DpLei). The 24-mer 5'-TTTTCGCAGAACGCCCCTACCCGC-3' was used as the *Leishmania*-specific probe (Probe Lei). The oligonucleotide primers were adapted from (Ravel S., 1995) and (le Fichoux Y., 1999). The probe was designed based on the published sequence for the kinetoplast minicircle DNA (GenBank accession number: AF169140); the same used for primers design since it is highly conserved among *Leishmania* species (Ravel S., 1995).

2.2. Tailing of oligonucleotide probes with dTTP or dATP

The 5' thiol modified $(dT)_{30}$ oligonucleotide was tailed enzymatically with dTTP, whereas the $(dA)_{20}$ oligonucleotide, and the *Leishmania*-specific oligonucleotide probe were tailed with dATP. The reactions were performed in a total volume of 20 µL, containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate (pH 7.9), 0.25 mM $CoCl_2$, 3.5 mM dTTP or dATP (2 mM dATP for probe Lei), 10 units of terminal deoxynucleotidyl transferase (TdT; New England Biolabs, Ipswich, MA, USA), and 700 pmol of oligonucleotide (400 pmol for probe Lei). The reactions were carried out at 37 °C for 60 min and terminated by heating to 70 °C for

10 min. After completion of the tailing reaction the poly(dA)-tailed Lei probe was mixed with 1.5 µL of 40 mM N-methylmaleimide (Sigma-Aldrich, Steinhem, Germany) solution in dimethyl-sulfoxide (DMSO, 2.7 mM final concentration). The tailed probes were stored at -20 °C.

2.3. Preparation of oligonucleotide conjugated gold nanoparticles

Gold nanoparticles (40 nm, 7.2×10^{10} particles/mL; Sigma-Aldrich, Steinhem, Germany) were modified with oligonucleotide addition, by thiol group conjugation based on previously published protocols [(Glynou, et al., 2003); (Toubanaki, et al., 2015)]. The reactions were carried out by adding 75 pmol of tailed SH-$(dT)_{30}$ oligonucleotide and 0.8 µL of absolute pyridine (Sigma-Aldrich, Steinhem, Germany) to 1 mL of the gold nanoparticle solution (~ 0.12 pmol). The mixture was incubated at 4 °C for 24 h. Subsequently, the solution was subjected to aging by addition of NaCl up to 90 mM. The solution was incubated for another 24 h at 4 °C, and then centrifuged at 1,300×g for 30 min. The supernatant was discarded, and the pellet was re-dispersed in 100 µL of an aqueous solution containing 30% sucrose, 0.25% Tween-20, 0.25% sodium dodecyl sulfate (SDS) and 45 mM NaCl, by vortexing and brief sonication.

2.4. Preparation of the nucleic acid lateral flow biosensors

The dry reagent lateral flow biosensors (4×60 mm) were prepared as described before [(Toubanaki, 2008); (Toubanaki, et al., 2015)]. Briefly, LFBs consisted of an immersion pad (CFSPOO1700, 17 mm; Millipore, Billerica, MA, USA), a glass-fiber conjugate pad (GFCP000800, 8 mm; Millipore, Billerica, MA, USA), the nitrocellulose

diagnostic membrane (HF240MC100, 25 mm in length; Millipore, Billerica, MA, USA), and an absorbent pad (same as immersion pad). The parts were assembled on a plastic adhesive backing as follows: The diagnostic membrane was placed on the center of the backing by the manufacturer. The conjugate pad was then placed below the membrane. The immersion pad was placed below the conjugate pad and the absorbent pad was positioned above the membrane. Each part overlapped 2 mm to ensure that the solution could migrate through the biosensor. The TLC applicator, Linomat 5 and WinCats software (Camag, Muttenz, Switzerland), was employed to construct the test zone and the control zone by spraying streptavidin and poly(dA), respectively, on the membrane. A solution containing 4 g/L streptavidin (from *Streptomyces avidinii*; Sigma-Aldrich, Steinhem, Germany), 150 mL/L methanol, and 20 g/L sucrose was loaded at a density of 1.6 µg/4 mm membrane and a solution containing 4 µM poly(dA) oligonucleotide, 500 mL/L methanol, and 20 g/L sucrose was loaded at a density of 2.4 pmol/4 mm membrane. The membrane was dried at 80 °C for 1 hr. LFBs with a 4-mm width were cut by using SmartCut A425 cutter (Rexel) and stored dry at ambient temperature.

2.5. Culture of *L. infantum* promastigotes

L. infantum (MON-1 zymodeme) strain IMT244 (MCAN/PT/1998/IMT244) used in this study was isolated from a dog suffering from CanL and parasite virulence was maintained by monthly passage of 1×10^7 stationary phase promastigotes in BALB/c mice (Agallou, et al., 2011). Briefly, BALB/c mice were injected intravenously via the lateral tail vein, with 1×10^7 second-passage promastigotes suspended in 100 µL sterile phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium

phosphate and 1.7 mM potassium phosphate, pH 7.4). At indicated time points, the amastigotes were isolated from the spleens of infected animals and allowed to transform into promastigotes at 26 °C in complete RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 % (v/v) heat inactivated fetal bovine serum (FBS; Gibco, Paisley, UK). Promastigotes were then cultured in the above growth medium at 26 °C.

2.6. Ethics statement

The collection of biological samples from domestic dogs (*Canis familiaris*) was conducted by licensed veterinarians properly informed, as described in the Supplemental Data file.

2.7. Samples

Blood samples from domestic adult dogs (*C. familiaris*) that varied in age, weight, and breed were used. Samples were collected as described in (Agallou, et al., 2016). Biological samples from healthy dogs living in a non-endemic area (Bristol, UK) were kindly provided by Dr. Tasker (School of Veterinary Sciences, University of Bristol, UK). Classification of the animals as healthy, asymptomatic, or symptomatic is described in (Agallou, et al., 2016).

2.8. DNA extraction

Genomic DNA was extracted either from stationary phase *L. infantum* promastigotes (10^5 promastigotes per sample), or leukocytes isolated from peripheral blood samples of healthy, asymptomatic, or symptomatic dogs, using ammonium chloride

lysis buffer, pH 7.2 (ACK: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) for the removal of erythrocytes. Blood samples in heparin tubes were mixed with fresh cold ACK Buffer (volume ratio 1:3) and incubated at room temperature for 10 min under continuous agitation. Cell suspension was centrifuged (10 min, 250xg, 4 °C) and the cell pellet was re-suspended in ACK buffer until the complete lysis of erythrocytes. DNA extraction was performed on the pellet of leukocytes by using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer instructions. DNA was eluted in 100 µL of the provided elution buffer and stored at -20 °C, until use.

The QIAamp DNA mini kit was selected after comparison between three commercially available kits; details on the comparison are provided in Supplemental Data file. Extracted DNA concentration and purity were determined by measuring the absorbance at 260 nm (A_{260}) using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The purified aliquots had concentrations in the range of 5-200 ng/mL of DNA. The A_{260}/A_{280} ratios of the purified aliquots were 1.8-2.0, indicating that the isolated DNA was efficiently purified for further use. Samples were stored at −20 °C, until use. Good laboratory practice was used to avoid DNA-cross contamination, and negative controls were included during all DNA extraction procedures.

2.9. PCR amplification of *Leishmania* kinetoplast DNA from canine samples

The extracted genomic DNA was subjected to PCR for kinetoplast DNA amplification. The reaction mixture (25 µL) contained KapaTaq Buffer C, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dNTPs: dATP, dTTP, dCTP, dGTP; HT Biotechnology, Cambridge, UK), 1 µM of each of the upstream and downstream primers, 2 – 4 µL of DNA sample

(containing 2 – 15 ng/µL DNA), and 5 U of KapaTaq DNA polymerase (Kapa Biosystems, Wilmington, MA, USA). The cycling conditions were 94 °C for 4 min, followed by 40 cycles at 94 °C, 30 s; 59 °C, 30 s; and 72 °C, 30 s. At the end of the cycling, the mixture was incubated at 72 °C for 10 min and cooled down to 4 °C. PCRs were performed in GeneAmp PCR System 9700 cycler (Applied Biosystems, New York, USA). Negative controls (containing water instead of DNA) were included in each series of PCRs to confirm the absence of contamination. The KapaTaq DNA polymerase was adopted after the comparison between three DNA polymerases. The KapaTaq and KapaTaq Hot Start DNA polymerase (Kapa Biosystems, Wilmington, MA, USA), along with the GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) were used to amplify genomic DNA isolated from 10^5 *L. infantum* promastigotes. All PCR products were visualized by 2% agarose gel electrophoresis and quantified based on the ϕX174 DNA *HaeIII* digest DNA molecular weight marker (HT Biotechnology, Cambridge, UK). The Sub-Cell GT agarose gel electrophoresis system was from Bio-Rad (New York, USA). The digital camera and UV transilluminator, AlphaEase FC Imaging System, was from Alpha Innotech (California, USA) and the gel images were analyzed with AlphaImager and ImageJ softwares (Abramoff, et al., 2004).

2.10. Lateral flow based detection assay of *Leishmania* amplification product

For detection of *Leishmania* amplification products, a 2-µL aliquot of PCR solution was mixed with 1 µL of 0.9 M NaCl, 0.5 pmol of dATP-tailed *Leishmania*-specific probe and ddH_2O in a total volume of 5 µL. The mixture was heated at 95 °C for 3 min and then hybridization was allowed to proceed for 10 min at 37 °C. The above

mixture was applied to the conjugation pad next to the poly(dT) conjugated gold nanoparticles. The immersion pad of the biosensor was then dipped into 250 µL of the developing solution. The developing solution contained 40 mL/L glycerol, and 10 g/L SDS in PBS. The visual detection was completed within 20 min. Longer times did not affect the assay results. After completion of the assay, the LFBs were scanned with a desktop scanner (HP Scanjet G4050, HP, California, USA) and the band densities were quantified using ImageJ software (Abramoff, et al., 2004).

3. Results

3.1. Assay principle

The gold nanoparticle-based lateral flow biosensor (LFB) is illustrated, in figure 1. DNA isolated from canine samples is subjected to PCR amplification. The resulting products are end-labeled with biotin, since a 5´ biotinylated primer is used. The amplification products are denatured and hybridize with a specific poly(dA)-tailed oligonucleotide probe. Subsequently, the hybridization solution is applied on the conjugate pad, where poly(dT)-conjugated gold nanoparticles (Au NPs) have been added. The biosensor is immersed in the developing solution which moves along the LFB by capillary action. The continuous liquid flow moves the poly(dT) functionalized nanoparticles towards the sample – poly(dA)-probe hybrid. The poly(dT) oligonucleotides which are immobilized on the surface of the gold nanoparticles, hybridize with the poly(dA) segment of the sample complex. The hybrids are finally captured from immobilized streptavidin at the test zone, since the amplification products are biotinylated. A characteristic red line is formed at the test zone as a

result of the gold nanoparticles accumulation. The excess nanoparticles are captured by immobilized oligo(dA) strands at the control zone of the biosensor generating a second red line that confirms its proper function. In the absence of target DNA, no red band is observed at the test zone.

3.2. Evaluation of genomic DNA extracted with different commercial extraction kits

Three commercial DNA extraction kits were compared and the extraction efficiency was assessed by measuring the resulting genomic DNA amount and purity, as well as the amount of PCR amplified products for four different sample types. Sample type 1 consisted of pure *L. infantum* promastigotes obtained from culture, to measure the kits differences in parasitic DNA isolation. Sample type 2 contained the *L. infantum* promastigotes mixed with leukocytes isolated from a healthy dog in order to have a sample with defined parasites number. Sample types 3 and 4 were leukocytes obtained from infected symptomatic and asymptomatic dogs, respectively, to assess the kits performance in real sample conditions. The use of those four different types of samples was considered essential since their analysis would allow the most realistic evaluation of the extraction kits. The comparison results are depicted in supplemental figure S1 and analyzed in the supplemental data file. It was shown that DNA extracted with the QIAamp kit resulted in higher amounts of PCR products. This finding indicated that the sample with the highest purity resulted in higher amounts of PCR products even though the starting DNA amount was lower. For that reason, the QIAamp kit was chosen as the optimum kit for that specific application.

3.3. Comparison of different DNA polymerases for effective *Leishmania* PCR amplification

A comparison study of three DNA polymerases was performed in an attempt to further optimize the *Leishmania* PCR amplification. The GoTaq Flexi and the KapaTaq DNA polymerases were tested along with the Hot Start analog of the KapaTaq DNA polymerase. A specific amount of extracted DNA from *Leishmania*-infected dog leukocytes (10^5 leukocytes) was subjected to standard PCR amplification with the three polymerases. Subsequently, to assess the optimum DNA polymerase for use in combination with the lateral flow biosensor detection assay, hybridization mixtures containing 1 pmol of each PCR product were applied on the biosensors. The results are shown in supplemental figure S2 and analyzed in the supplemental data file. The results indicated that the KapaTaq DNA polymerase amplified more efficiently the target DNA and the difference between the two amounts used (0.5 and 1 u) was not dramatic. Therefore less quantity could be used in cases where the assay cost needs to be reduced.

3.4. Optimization of the lateral flow biosensor assay with *Leishmania* amplification products

Optimization studies were performed with PCR products from amplification of 10^5 leukocytes from a *Leishmania*-infected dog.

3.4.1. Effect of the hybridization temperature

The effect of target PCR product and specific oligonucleotide probe hybridization temperature was studied in the range of 25 – 55 °C. The results are presented in figure S3A. The density of the test zone was optimum at 37 °C and slightly decreased

in higher temperatures, probably due to hybridization inhibition. Therefore, all subsequent hybridizations were performed at 37 °C.

3.4.2. Effect of the specific oligonucleotide probe to target ratio

The influence of the oligonucleotide probe to target ratio in the hybridization mixture was studied in the range of 0.1 – 10 pmol probe/ 1 pmol of target (figure S3B). The density of the test zone was optimum with a ratio of 0.5 pmol probe and decreased at higher amounts. The observed effect was due to the fact that at high levels, the amount of poly(dA) oligonucleotide probe may exceed the binding capacity of the poly(dT)-functionalized gold nanoparticles. As a consequence, poly(dA) oligonucleotide that is hybridized to the target sequence competes with the free (unhybridized) poly(dA) oligo for binding to limited poly(dT) strands on the nanoparticles. The higher the amount of poly(dA) oligo used, the larger the fraction of nanoparticles that bind to the free probe. These nanoparticles then migrate along the entire length of the biosensor without being captured from immobilized streptavidin and poly(dA). As a result, the red bands of both the test zone and the control zone become fainter (Glynou, et al., 2003).

3.5. Analytical characteristics of the lateral flow biosensor

3.5.1. *Leishmania* lateral flow biosensor limit of detection

In order to study the biosensor limit of detection, a *Leishmania* positive PCR product was used (145 bp). Sample solutions containing different concentrations of target PCR products were tested under the optimized experimental conditions. Specifically, various amounts of PCR products ranging from 0 to 2000 fmol were applied per

biosensor. The results are presented in figure 2. As low as 100 fmol of target DNA (signal = 1604.8) were detectable by the naked eye on the biosensor. To define a positive result, we used the signal/noise (S/N) ratio (S/N = 3) as reference. Signal is the optical intensity of the test line with detected sample and noise is the intensity of the test line with 1×KapaTaq Buffer C (signal = 517).

3.5.2. *Leishmania* lateral flow biosensor reproducibility

To determine the reproducibility of the LFB, constant amounts of PCR products hybridization mixtures were loaded on 6 LFBs that had been kept dry at room temperature. The biosensors were produced in different batches. The assay results are presented in supplemental figure S4. Densitometric analysis of the test zones of the biosensors resulted in a Coefficient of variation (CV) of 8.4 %, while analysis of the control zones resulted in CV = 9.7%.

3.5.3. Leishmania lateral flow biosensor specificity

The specificity of the LFB for *Leishmania* amplification products detection was determined by analyzing the cross-reactivity of the assay with DNA extracted from leukocytes from healthy dogs. In all cases, amplification products were detected only on infected symptomatic and asymptomatic dogs (figure 3).

3.6. *Leishmania* amplification products from canine peripheral blood samples detection

The proposed biosensor was used to detect *Leishmania* amplification products in canine peripheral blood samples with the optimized reaction conditions. Nine indicative samples i.e. two healthy (N1, N2) and seven infected (S3-S9) dog samples,

were analyzed along with 10^5 isolated leukocytes from a symptomatic dog as positive control (CS), as shown in figure 3. The healthy dog samples were obtained from healthy dogs living in a non-endemic area (Bristol, UK), in order to assure that no infection had occurred. In each run a negative control (NC), containing 1× Kapa Taq Buffer C instead of a PCR product, and a positive control, containing 500 fmol of B-dA_{20} reference oligonucleotide (PC), were included. The mixtures used in the present study contained PCR products in the range of 300 – 993 fmol/ 2 µL. None of the healthy dog samples showed any signal in the test zone, higher that the detection limit. All infected dog samples were positive with varying signal intensities, corresponding to different amounts of *Leishmania* in the sample.

4. Discussion

Rapid and accurate detection of *Leishmania* amplification products is of great importance for the effective diagnosis of human and canine leishmaniasis. A gold nanoparticle-based paper lateral flow biosensor was developed for simple and specific visual detection of *Leishmania* kinetoplast DNA PCR products in canine blood samples. The target amplification products could be easily detected by observing the formation of an intense red colored test zone.

The widely used methodologies for *Leishmania* detection consists of three steps: DNA extraction, amplification of *Leishmania*-specific DNA target and amplification products detection. Three commercial DNA extraction kits were compared and the results indicated that the optimum extraction conditions to obtain higher PCR

products amount, were dependent on the sample purity rather than the starting DNA amount.

Several regions of *Leishmania* genome, have been used for specific amplification, including kinetoplast DNA (kDNA), small subunit rRNA (SSU rRNA), internal transcribed spacer (ITS), mini exon RNA, β-tubulin, glycoprotein 63 (gp63), heat-shock protein (HSP70) and arginine permease genes [(Srividya, et al., 2012); (Tellevik, et al., 2014); (Koltas, et al., 2016)]. Especially the kDNA is widely used to identify *Leishmania*, since this genomic region corresponds to genus-specific conserved sequences. The target sequence is multicopy (there are about 10^4 minicircle copies per parasite) and allows detection of about one parasite per 1 mL of blood [(Ravel S., 1995); (Koltas, et al., 2016)]. Since kDNA is considered to be the most sensitive for diagnosis of leishmaniasis, it was chosen as the target and three different polymerases were tested, in order to increase PCR efficiency.

Immunochromatographic lateral flow biosensors for *Leishmania* detection have been developed and commercialized [(Mettler, et al., 2005); (Pattabhi, et al., 2010)]. However, the low sensitivity of the serological methods (rK39 tests) [(Mettler, et al., 2005); (Castellanos-Gonzalez, et al., 2015)] dictated the need of nucleic acid LFBs for *Leishmania* DNA detection (Deborggraeve, 2008). The first lateral flow test introduced for *Leishmania* PCR product detection was the OligoC-TesT (Deborggraeve, 2008). A custom-made oligochromatographic test, based on OligoC-TesT, was, also, developed for *L. donovani* and *L. infantum* detection and typing (Laurent, et al., 2009). In a recent study, the amplification product of recombinase polymerase amplification (RPA; TwistDx Ltd., Cambridge, UK) was detected using a

generic lateral flow oligochromatograhic dipstick paper (Milenia strip) (Castellanos-Gonzalez, et al., 2015). A triple-line LFB for simultaneous detection of *Leishmania* DNA and the endogenous control (18S rRNA), utilized gold nanoparticles connected with polyclonal secondary antibodies which were used as enhancers of the obtained signal. In that case, amplification was, also, achieved with RPA reactions (Rivas L., 2015).

None of the above mentioned biosensors have been widely adapted, leaving a gap between effective diagnostic tools development and application in the point of need, dictating the need for introduction of alternative LFB platforms. In that aspect, a nucleic lateral flow biosensor based on oligonucleotide-functionalized gold nanoparticles was combined with kDNA PCR for effective *Leishmania* detection. The proposed biosensor has already been evaluated in pathogens detection [(Glynou, et al., 2003); (Kalogianni, 2007); (Toubanaki, et al., 2015)], genetically modified organisms (GMO) detection (Kalogianni, 2006), food authentication (Trantakis, et al., 2012) and single nucleotide polymorphisms (SNPs) genotyping [(Toubanaki, 2008); (Toubanaki, et al., 2009)].

In order to determine the biosensors' limit of detection, sample solutions containing various amounts of PCR products (0 – 2000 fmol) were applied to the biosensors. Under optimal conditions, 100 fmol of target DNA, corresponding to ~ 3 parasites/ μL of sample, were detectable by the naked eye within 20 min, by using a standard PCR machine and the low cost LFB. The lower detection limit of the OligoC-TesT was 10 fg of *Leishmania* DNA per assay, which is approximately $1/20^{th}$ of the DNA content of 1 parasite. When the assay was evaluated with DNA extracted from blood

samples spiked with *L. donovani* promastigotes it could detect 1 parasite/ 180 µL of blood (Deborggraeve, 2008), whereas application of the OligoC-TesT assay to canine bone marrow DNA samples could detect 74 parasites/mL of sample (Carson, et al., 2010). NASBA - OligoC-TesT was able to detect 1 and 10 parasites per mL using cultured parasites or spiked blood, respectively (Mugasa, et al., 2010). The customized OligoC-TesT for *L. donovani* and *L. infantum* typing detected 14 promastigotes/ µL of spiked blood, for both assays (Laurent, et al., 2009). (Castellanos-Gonzalez, et al., 2015) reported that DNA from 0.1 parasites spiked in dog blood (equivalent to 40 parasites/mL) was successfully detected, while 0.038 parasites per amplification reaction were visible with the triple-line LFB (Rivas L., 2015).

Even though a direct comparison of the detection limits would not be accurate since different sample types (amplified products of cultured *L. infantum* vs spiked blood samples) or amplification methods were used, it should be noted that the proposed method is capable to detect fmol of target DNA, which is within the requirements for animal diagnostic purposes (Andreadou M., 2016).

The LFB assay reproducibility was expressed with CV values of 8.4% and 9.7%, for test and control zones, respectively. The total assay time including DNA extraction, PCR and detection with the proposed LFB was less than 3 h. All tested positive dog blood samples had a positive signal whereas none of the negative control samples resulted in signal higher than the detection limit. Even though the proposed assay was evaluated with a small number of veterinary samples, the performance of the biosensor is promising, and further evaluations using a wider panel of clinical

samples are our future goal. It should be noted that blood was chosen for the evaluation of the proposed assay, because it contains large amounts of debris DNA and in cases of leishmaniasis, it harbors the pathogen in low concentrations compared to lymph nodes or bone marrow aspirates (Andreadou M, 2014), providing an indication of the clinical applicability of the developed methodology.

Compared to the well-established methods for *Leishmania* amplification products detection (i.e. gel electrophoresis and real-time PCR), the presented methodology offers a simple, fast, selective, sensitive and cost-effective approach to detect *Leishmania* kDNA PCR products. As described for other applications of the proposed biosensor (Toubanaki, et al., 2015), detection of the PCR products provides sequence confirmation by target-probe hybridization while electrophoresis is based on the size of the amplified fragments; the pre-hybridized sample can be applied directly on the biosensor, a small amount of developing solution is needed, reducing the wastes amount, and only a small amount of gold nanoparticles is required for visualization, eliminating the need for the highly toxic/ mutagenic ethidium bromide. The LFB is based on nucleic acids for gold nanoparticles functionalization and control zone fabrication, eliminating the need for expensive antibodies. Therefore it has higher stability and no need for special storage. The cost of the assay in terms of reagents and the biosensor is less than 3 €. The biosensor allows visual detection of *Leishmania*-specific amplification products within minutes without the need of any instruments.

5. Conclusions

Lateral flow biosensor-based assays are being increasingly adapted as promising platforms for nucleic acid amplification products detection allowing accurate, simple, selective, sensitive, rapid, portable, easy-to-operate, long self life and low cost testing of infectious pathogens. The combination of simple PCR assays with nucleic acid LFBs are, currently, being exploited for improving the diagnosis and management of leishmaniasis. The present describes the development of a sensitive and specific molecular detection method for CanL, based on a kinetoplast DNA specific PCR method and an alternative lateral flow biosensor for amplification products detection. The biosensor was evaluated with actual clinical samples of infected dog blood and successfully confirmed the presence of *Leishmania*, while no product was detected for negative samples. Ongoing studies include further evaluation of the LFB assay using a wider panel of clinical samples, the adaptation of isothermal methods for *Leishmania*-specific nucleic acids amplification, which combined with the proposed biosensor will allow easiest detection on the point-of care, and improvements in the sensitivity of the LFB by using gold nanoparticles dual labels and other LFB signal enhancement systems, based on previously developed methodologies (Toubanaki, et al., 2016).

The effective diagnosis of canine leishmaniasis is essential for surveillance and control programs. Since accurate diagnosis is the first step to achieve the disease elimination, by effective treatment and follow up, it is anticipated that the proposed assay would be useful to small scale research labs and veterinary specialist facilities.

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Author Contributions

D.K.T. conceived and designed the experiments; D.K.T and E.A. performed the experiments; D.K.T. and E.K. analyzed the data; D.K.T. wrote the paper; E.A. and E.K. proof-read the manuscript; D.K.T. and E.K. supervised the work. All authors have approved the present article.

Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results

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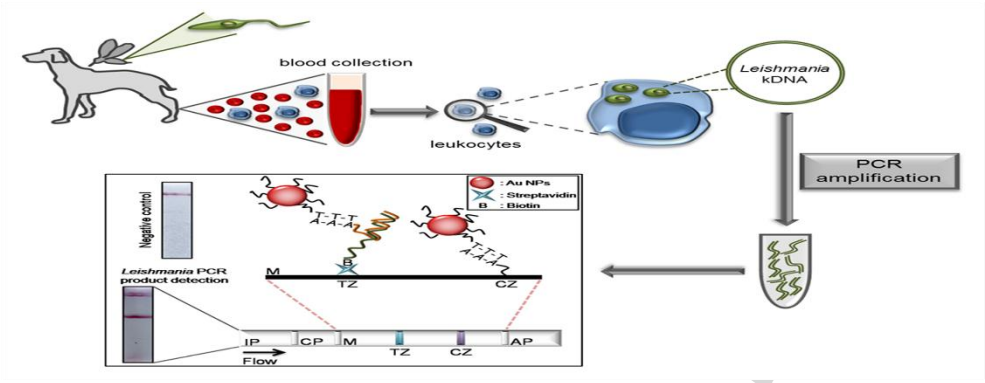
FIGURE LEGENDS

Figure 1. Principle of the gold nanoparticle-based lateral flow biosensor for *Leishmania* amplification products detection. Front view of the lateral flow biosensor: IP: immersion pad; CP: conjugation pad; M: diagnostic membrane; AP: absorbent pad. Streptavidin has been immobilized on the test zone (TZ) and the poly(dA) oligonucleotide has been immobilized on the control zone (CZ) on the biosensors' diagnostic membrane. The sample hybrid consists of the target analyte (PCR product), labelled with biotin (B), and a hybridized poly(dA)-tailed oligonucleotide probe. The sample is applied on the conjugation pad next to poly(dT) functionalized gold nanoparticles (Au NPs). The biosensor is dipped on the developing solutions, the sample and the nanoparticles are captured on the test zone, giving a characteristic red line as a positive signal. The excess nanoparticles bind to the control zone. The assay components are not in scale.

Figure 2. Study of *Leishmania* lateral flow biosensor limit of detection. A. Typical images of lateral flow biosensors after applying different amounts of *Leishmania* amplification PCR products. **B.** Graph of the intensities of the LFBs test zones versus the *Leishmania* amplification PCR products.

Figure 3. *Leishmania* detection in canine peripheral blood samples. Visual detection of *Leishmania* amplification products with lateral flow biosensors. NC: Negative control; PC: Positive control (reference oligonucleotide; N1-2: Negative samples; CS: 10^5 isolated leukocytes from a symptomatic dog sample, S3-9: *Leishmania* positive samples.



Graphical abstract

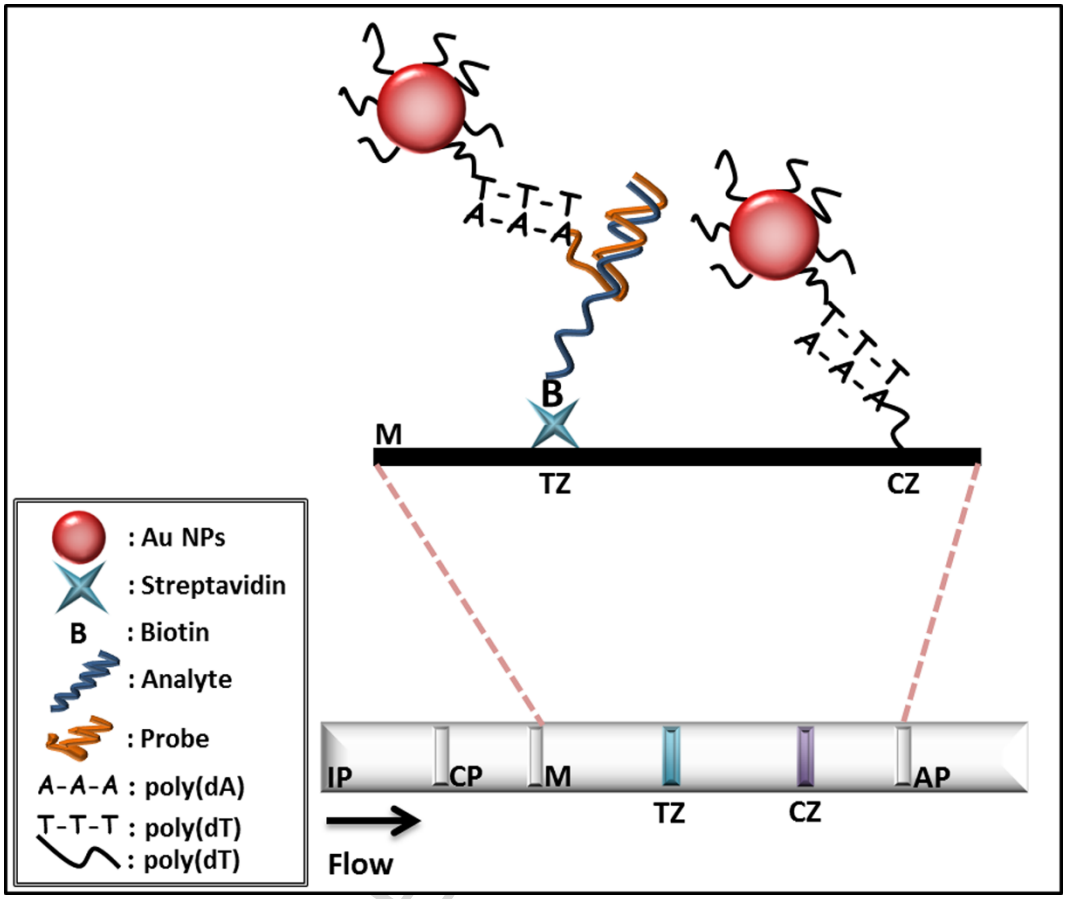


Fig. 1

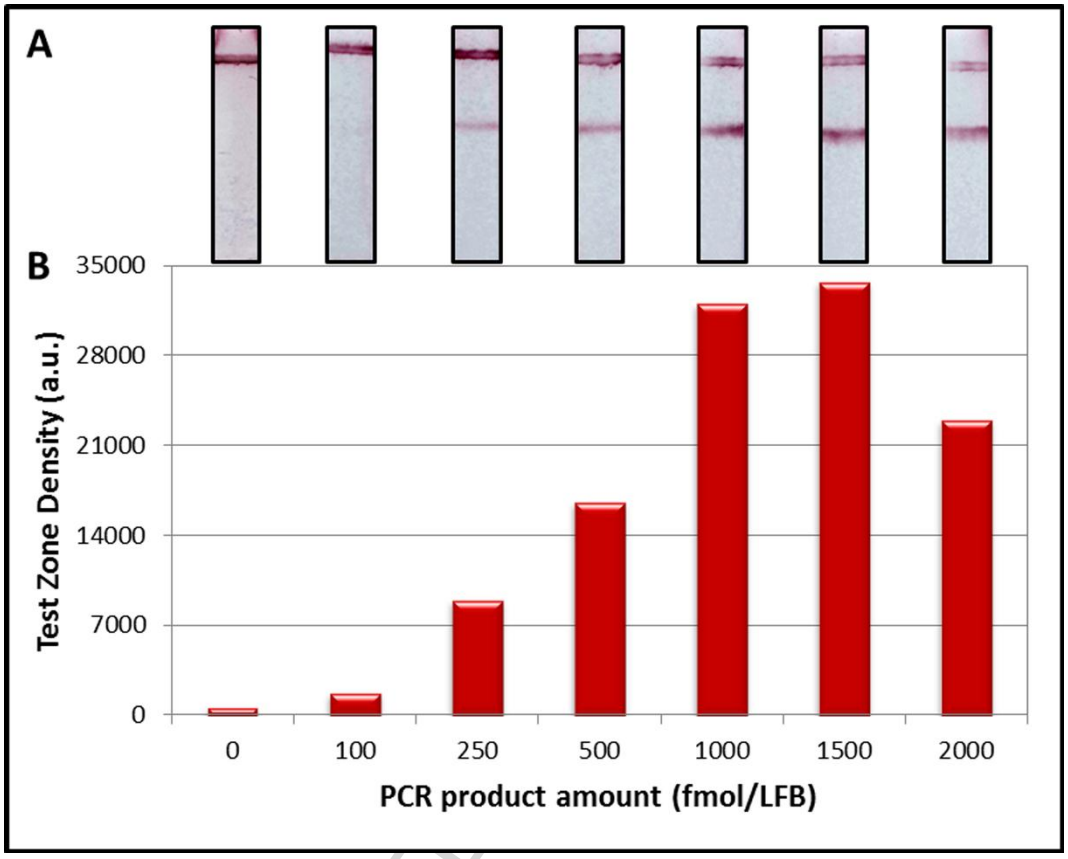


Fig. 2

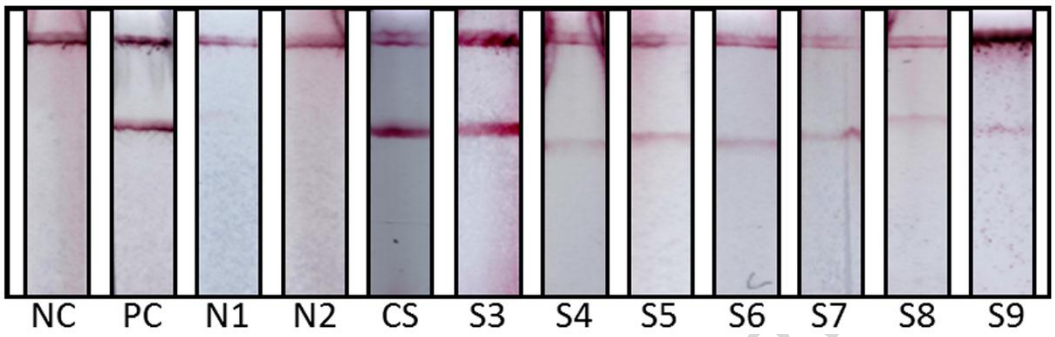


Fig. 3

HIGHLIGHTS

- A gold nanoparticle-based LFB was developed for *Leishmania* DNA detection.
- The biosensor allows visual detection of amplification products without special instrumentation.
- The detection limit was 100 fmol of target DNA, corresponding to 3 parasites/µL of sample.
- Results were visible within 20 min and amplification products were specifically detected on infected dogs.
- The proposed assay is considered a step forward to the wider use of sensitive PCR detection of infectious pathogens, as an effective tool for clinical application in human and veterinary medicine.