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ORIGINAL PAPER

Development of a Fluorescent Enzyme-Linked DNA Aptamer-Magnetic Bead Sandwich Assay and Portable Fluorometer for Sensitive and Rapid *Leishmania* Detection in Sandflies

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Abstract A fluorescent peroxidase-linked DNA aptamer-magnetic bead sandwich assay is described which detects as little as 100 ng of soluble protein extracted from *Leishmania major* promastigotes with a high molarity chaotropic salt. Lessons learned during development of the assay are described and elucidate the pros and cons of using fluorescent dyes or nanoparticles and quantum dots versus a more consistent peroxidase-linked Amplex Ultra Red (AUR; similar to resazurin) fluorescence version of the assay. While all versions of the assays were highly sensitive, the AUR-based version exhibited lower variability between tests. We hypothesize that the AUR version of this assay is more consistent, especially at low analyte levels, because the fluorescent product of AUR is liberated into bulk solution and readily detectable while fluorophores attached to the reporter aptamer might occasionally be hidden behind magnetic beads near the detection limit. Conversely, fluorophores could be quenched by nearby beads or other proximal fluorophores on the high end of analyte concentration, if packed into a small area after magnetic collection when an enzyme-linked system is not used. A highly portable and rechargeable battery-operated fluorometer with on board computer and color touchscreen is also described which can be used for rapid (<1 h) and sensitive detection of *Leishmania* promastigote protein extracts (~100 ng per sample) in buffer or sandfly homogenates for mapping of *L. major* parasite geographic distributions in wild sandfly populations.

Keywords Amplex Red · Aptamer · Assay · Leishmania · Magnetic bead · Portable fluorometer · Resazurin · Resorufin · SELEX

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Introduction

Leishmaniasis is a serious parasitic disease affecting approximately 12 million people worldwide, primarily in the Middle East, Central and South America, Africa and Asia. It has been estimated that between 350 million and 700 million people are at risk for *Leishmania* infection with 500,000 to 1.5 million new cases around the globe each year and at least 80,000 deaths per year [1–4]. Depending on the species, leishmaniasis can manifest as mild and self-healing lesions to severe cutaneous or mucocutaneous ulcers or the potentially lethal visceral form (also known as kala-azar, black fever, and dum-dum fever), all of which result from the bite of infected female phlebotomine sandflies. To date, there is no vaccine available for leishmaniasis [1] and rapid, but accurate diagnosis is key to effective treatment of the disease [5]. Those who develop ulcerated lesions are often diagnosed by examination of painful skin or bone marrow biopsies. There are also a variety of time-consuming immunoassays such as ELISAs [4, 6] or molecular diagnostics such as PCR-based assays [4, 7, 8] which are typically sent off-site, but can be field-portable

[7]. Although field diagnosis has been enhanced by immunochromatographic or lateral flow test strips that range in reported sensitivity from 60 % to 100 % with high specificity >95 % [4], there still appear to be antigenic species and life-cycle stage issues as well as undetected asymptomatic or low-level infection cases that speak to a need for greater sensitivity, reliability, and rapid diagnosis [4, 5].

Clearly, most of the diagnostic development community is focused on clinical detection of *Leishmania* parasites in humans [1–6]. While the assay strategy reported herein could be adapted to human clinical leishmaniasis diagnostics, if amastigotes or their soluble antigens were targeted in the future, we have instead focused on a highly portable method of detection of *L. major* promastigotes in sandfly populations for military and civilian environmental distribution mapping [7, 8]. Our system is based on a DNA aptamer-coated magnetic bead (MB) enzyme-linked fluorescent sandwich assay (Fig. 1) which is assessed by a sophisticated, yet handheld, fluorometer. While not a clinical assay, this field-portable assay system could help to prevent the spread of potentially lethal leishmaniasis in humans by enabling better understanding of parasite distribution patterns in nature.

In the 4 years since development and publication of our initial *Campylobacter* capture aptamer-MB plus reporter aptamer-quantum dot (QD) sandwich assay in this journal [9], we have learned many valuable lessons about the best ways to detect foodborne bacteria in complex food matrices. This acquired experiential knowledge has helped us to evolve the current *L. major* assay for use in sandfly homogenates and other biological matrices. While it will be apparent that some aspects of our aptamer MB-based sandwich assay format have changed, such as the use of an enzyme-linked fluorescence system [10, 11], the capture and reporter DNA aptamer components have persisted and other groups have begun reporting similar sensitive fluorescent aptamer-MB sandwich assays [12, 13] which speaks to the utility of this diagnostic strategy and platform.

The most fundamental change in our magnetic aptamer sandwich assay approach has been a shift away from the use of commercially available quantum dots and towards enzyme-linked systems to release fluorescent products such as resorufin from Amplex Ultra Red (AUR) into bulk solution (Fig. 1). Although QDs present numerous advantages such as high quantum efficiency, long Stoke's shifts, the potential to develop multiplexed assays with a single light source in conjunction with aptamers [13, 14], QDs also possess some key shortcomings such as blue shifting and dramatic loss of fluorescence in some biochemical environments or when oxidized or otherwise experiencing damage to the Zn/S or other coating materials [15–25]. Hence, while our *Campylobacter* [9] and other assays [14] have exhibited exceptional sensitivity with aptamer-QD conjugate reporting schemes, their reliability from batch to batch, especially in some biological matrices, was dubious and caused us to explore new avenues for sensitive detection. We

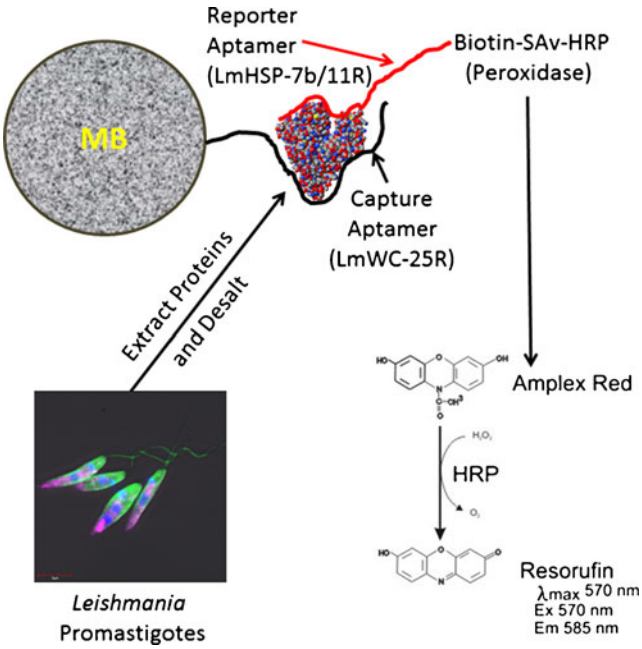

Fig. 1 Conceptual diagram showing the assay system from extraction of promastigote proteins with 1.5 M $MgCl_2$ and desalting to capture proteins on aptamer-coated magnetic beads (MBs) and sandwich assay-based reporting of the proteins using peroxidase and Amplex Ultra Red (AUR)

replaced QDs with the red-emitting TYE 665 dye in a number of our aptamer-MB sandwich assays, but found that detection on both the very low and high ends of analyte concentration was not very linear or consistent (data not shown). On the low end (e.g., ≤100 cells/ml), the fluorescence from a small number of captured bacteria or other cells could be obscured by the MBs themselves, if target cells were captured on the far side of the MBs or their photon emissions were absorbed by neighboring beads. On the high end of our titration curves (e.g., $\geq 10^6$ cells/ml), we often observed "hook effect" and other non-linear phenomena which may be attributable to self-quenching of QDs or traditional fluorescent dyes when packed together in a small area and in close proximity to one another.

Regardless of the fluorescence damping mechanisms, it was clear that reproducibility and consistent linearity were difficult to achieve with QDs or fluorescent dyes in a variety of biologically relevant matrices. These limitations prompted us to explore an enzyme-linked aptamer-MB sandwich assay strategy which resembles fluorescent ELISA approaches which are decades old [11], but have the distinct advantage of enhancing signal-to-noise ratios by continually pumping fluorescent product (resorufin, Fig. 1) into bulk solution where it cannot be obscured by magnetic beads and will not self-quench as easily because of the increased volume factor and distance between fluorophores in solution.

For the current assay, we also chose to detect 1.5 M $MgCl_2$ chaotropic salt-extracted soluble proteins [26] of *L. major* promastigotes alone or in sandfly homogenates so as to avoid

capture of flagellated parasites which could break away from aptamer-coated MBs. We have utilized 1.5 M $MgCl_2$ for bacterial protein extraction in the past with good assay linearity and detection limits [9, 27]. However, excess Mg^{2+} must be removed or diluted as it can affect aptamer conformations and binding to the intended target [28].

Aptamers are DNA or RNA oligonucleotides which are affinity selected from a large, highly diverse randomized, DNA or RNA sequence library (~10^{15} different sequences in the starting pool) and PCR-amplified repeatedly to evolve a small set of oligonucleotides which emulate antibody binding [29]. Aptamers can exhibit superior affinity and specificity versus their closest antibody counterparts [29], if properly selected under stringent conditions. Aptamers do not require host animals for production, leading to lower cost and labor burdens to produce high-affinity and potentially very specific binding reagents useful for diagnostics and therapeutics [29]. Because of these advantages, there is significant interest in the development of aptamers against parasites [30–37] which have evolved mechanisms to avoid many aspects of the host's immune system. In particular, aptamers that target unique histones, and other antigens from *Leishmania* parasites have been reported [31, 34–36], but few *Leishmania* surface-binding aptamer DNA sequences have yet been documented [30].

In the present report, we describe the pairing of two aptamers developed against whole *L. major* promastigotes and a specific recombinant surface protein in our MB sandwich assay with linkage to streptavidin-peroxidase and AUR for sensitive (~100 ng of extracted protein) and rapid (<1 h) detection of *L. major* promastigote extracts in buffer and homogenized sandflies. We also describe a new custom handheld fluorometric reader designated as the **FL**uorescent **A**ssay **S**ensor **H**andheld or "FLASH" reader which rivals the commercially available Picofluor™ or Quantifluor™ [9] and possesses the advantages of a photomultiplier tube (PMT), optional laser excitation source, on board computer, and color touchscreen with a menu of assays and reader control parameters.

Materials and Methods

Materials

Recombinant *L. major* hydrophilic surface protein (rHSP; amino acids 18–177), Swiss-Protein No. Q4QB56 having the following amino acid sequence:

MGSSCTKDSA KEPQKSADKI KSTNETNQGG NASGSRKSAG GRTNEYDPKD DGFTPNNEDR CPKEDGHTGK NDDGGPKEDG HAPKNDDHAP KEDGHAPKND DHAPKEDGHA PKNDDHAPKE DGHAPKNDDH APKEDGHAPK NDGDVQKKSE DGDNVGEGGK

was purchased from Bioclone, Inc. (San Diego, CA; Cat. No. PL-0342). Kinesin peptides derived from the repetitive regions of *Leishmania* kinesins as described by Burns et al. [38] and having the following amino acid sequences:

Kinesin peptide 1: LEQQLRDSEERAAELASQLEA TAAAKMSAEQDRENTRAT
Kinesin peptide 2: LEQQLRESEARAAELASQLESTT AAKSSAEQDRESTRAA

were synthesized and purified to >98 % purity by GenScript Corp. (Piscataway, NJ). Human N-terminal procollagen Type III peptide (PIIINP) having the following amino acid sequence [39]: QEAVEGGCSHLGQSYADRDVWKPEPCQICV was also synthesized and purified to >98 % purity by GenScript Corp. *E. coli* intimin-gamma was obtained from Dr. Allison O'Brien of the Uniformed Services University of the Health Sciences (USUHS, Bethesda, MD). Certified disease-free human serum was obtained from Lonza BioWhittaker, Inc. and stored at −20 °C until thawed, diluted and used as an assay matrix. Sandflies were obtained from Dr. Edgar Rowton's laboratory at the Walter Reed Army Institute of Research (WRAIR, Silver, Spring, MD).

Leishmania Promastigote Culture, $MgCl_2$ Chaotropic Protein Extraction, Desalting and Storage

Frozen vials of *Leishmania infantum* Nicolle strain (ATCC No. 50134) and *Leishmania major* Jericho II strain (ATCC No. 30882) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Vials were thawed briefly in a 37 °C water bath without shaking and 0.5 ml of Brain Heart Infusion (BHI) medium was added to each vial. Contents of the vials were gently transferred to 10 % sheep blood agar slants. Slants were incubated at room temperature, vertically, and allowed to grow in a dark humidified chamber. After 3 days, 0.1 ml of culture was transferred to 5 ml of 20 % heat-inactivated fetal bovine serum (FBS) plus 10 U penicillin and 10 μg streptomycin in Schneider's *Drosophila* media in a T-25 cell culture flask and cultures were incubated vertically at room temperature in the humidified incubator. Cultures were split in log phase by a 1:100 dilution in fresh medium (Schneider's *Drosophila* medium with 20 % heat-inactivated FBS) every 2–3 days. Based on cell counts using a hemocytometer, it was determined that *Leishmania* promastigotes doubled every 7.5 to 7.6 h.

Approximately 10^9 *L. major* promastigotes were pelleted by low-speed centrifugation and washed in sterile phosphate buffered saline (PBS, pH 7.2) and pelleted again. The pellet was resuspended in 1 ml of 1.5 M $MgCl_2$ for removal of proteins by the chaotropic action of $MgCl_2$ overnight at 4 °C [9, 26, 27]. For use in the aptamer-MB sandwich assays, it was necessary to remove the excess $MgCl_2$, because high levels of $MgCl_2$ are known to disrupt aptamer conformations and

binding [28]. Therefore, the 1 ml sample was split into two 500 μl aliquots and added to 2 Amicon® Ultra 3kD molecular weight cut off (MWCO) spin columns in microfuge tubes and centrifuged at 14,100×G for 1 min to desalt the sample. Then the spin columns were inverted and placed into sterile microfuge tubes and overlaid with 500 μl of sterile PBS and centrifuged as before to force *Leishmania* proteins off the membrane surface and into microfuge tubes for use in experiments or long-term storage at −20 °C. Protein concentrations were estimated by BCA (bicinchoninic acid) protein assay (Thermo Fisher Scientific Corp., Pittsburgh, PA) according to the manufacturer's instructions.

Electrophoretic Characterization of 1.5 M $MgCl_2$ L. Major Promastigote Extracts

Ten μl of cold overnight 1.5 M $MgCl_2$-treated *L. major* promastigote extract was mixed 1:1 with 2× Laemmeli sample buffer (Bio-Rad) and loaded into the wells of a 4–20 % SDS-HEPES Precise™ mini polyacrylamide gel alongside 5 μl of SigmaMarker™ wide range molecular weight markers (Sigma Chemical Co., Cat. No. S8445) and electrophoresed at 100–200 V for ~1 h in cold SDS-HEPES running buffer. The gel was briefly rinsed in deionized water and then stained in Simply Blue™ (Invitrogen/Life Technologies) Coomassie blue stain according to the manufacturer's instructions, destained and photographed. The gel was then fixed overnight in 40 % ethanol and 10 % aqueous acetic acid solution with gentle mixing and silver stained using a SilverQuest™ (Invitrogen/Life Technologies) kit according to the manufacturer's instructions and photographed again.

DNA Aptamer Development, Cloning and Sequencing

Approximately 3.5×10^7 fresh log-phase *L. major* promastigotes were used per round of whole cell (WC)-SELEX (Systematic Evolution of Ligands by EXponential enrichment) to develop the capture aptamers (14). Approximately 900 μg of rHSP protein was immobilized onto 2×10^7 Dynal® M280 (2.8 μm diameter) tosyl-coated MBs (Life Technologies Inc., Carlsbad, CA) for 1 h at room temperature on a rotating mixer. Recombinant HSP-conjugated MBs were collected using a Dynal® DynaMag-2 magnetic rack and washed in 1 ml of PBS three times before storage at 4 °C.

All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). MB-based SELEX was performed using 160 nmoles of 72 base SELEX template library sequence: 5′-ATCCGTCACACCTGCTCT-N_{36}-TGGTGTTGGCTCCCGTAT-3′, where N_{36} represents the randomized 36-base region of the DNA library. Primer sequences were: 5′-ATACGGGAGCCAACACCA-3′ (designated forward, or F) and 5′-ATCCGTCACACCTGCTCT-3′ (designated reverse, or R) to prime the template and nascent strands, respectively. The lyophilized 160 nmole random library template was rehydrated in 500 μl of sterile TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) buffer, mixed with 500 μl of PBS and heated to 95 °C for 5 min to ensure that the DNA library was completely single-stranded and linearized. After cooling, the 1 ml of DNA template solution was added to 2×10^7 rHSP-coated MBs or whole promastigotes (in 100 μl of PBS) and mixed at room temperature for 1 h.

Following interaction with the randomized DNA library template, DNA-rHSP-MB complexes or whole promastigotes were separated from unbound DNA by collection on the DynaMag-2 magnetic rack and the supernatant was aspirated and discarded. DNA-target-MBs were then washed three times in 100 μl of PBS with vigorous vortex mixing followed by magnetic collection. Following the third wash, DNA-rHSP-MBs or whole promastigotes were resuspended in 150 μl of sterile, nuclease-free water and heated at 95 °C for 5 min to release bound DNA aptamers. The hot supernatant was collected and 5 μl aliquots of eluted DNA were PCR-amplified in 100 μl reaction volumes using a SpeedStar® (hot start) PCR kit (Takara Bio Inc., Shiga, Japan). PCR was conducted as follows: an initial 94 °C phase for 5 min, followed by at least 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 15 s at 72 °C followed by a 72 °C completion stage for 5 min, and refrigeration at 4 °C.

PCR amplicon bands were verified to be 72 bp after each round of SELEX by electrophoresis in 2 % TAE (Tris-Acetate EDTA) agarose gels with ethidium bromide staining. If more than one band emerged, the 72 bp band was excised on a UV transilluminator with a sterile razor blade and aptamers from the gel slice were eluted into 50 μl of Qiagen elution buffer using a Qiagen Gel Purification spin column (Germantown, MD). If the aptamer amplicon was faint or not visible in the gel, the number of rounds of PCR was increased. Negative control PCR reactions without the SELEX template were run to ensure that nonspecific DNA was not amplified. For the second and subsequent rounds of SELEX, the 50 μl of elution buffer were diluted in 50 μl of PBS, followed by dilution in 800 μl of PBS and addition of 100 μl of fresh rHSP-MB conjugate (~2×10^7 MBs) or ~3.5×10^7 whole promastigotes. This constituted the first of 10 rounds of rHSP-MB-SELEX or whole promastigote SELEX.

Following round 10, aptamers were cloned into chemically competent *E. coli* using a Lucigen GC cloning kit (Middleton, WI) according to the manufacturer's protocol and clones were sent to Sequetech, Inc. (Mountain View, CA) for proprietary GC-rich DNA sequencing. Sequence names are coded throughout, indicating that particular sequences derived from selection against whole cell (WC) promastigote or rHSP targets along with F for forward and R for reverse clone designations as indicated in Supplemental Tables 1 and 2. Slash marks in the aptamer names indicate multiple clones containing identical aptamer sequences.

ELASA Screening of Candidate Aptamers

To evaluate relative affinity rankings for each of the candidate WC or rHSP aptamers, an enzyme-linked aptamer sorbent assay (ELASA) were conducted similar to previously reported ELASA screenings [27, 30, 40] by first immobilizing 500 ng of either *L. major* WC promastigote protein extract or rHSP in 100 μl of 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4 °C in covered flat-bottom polystyrene 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). The plates were decanted and washed three times in 200 μl of PBS. Wells were then blocked with 150 μl of 10 % ethanolamine in 0.1 M $NaHCO_3$ for 1 h at 37 °C followed by three more washes with 200 μl of PBS as before. A total of 30 different *L. major* WC promastigote and 34 unique rHSP 5′-biotinylated aptamers were synthesized in 96 well plates by IDT and rehydrated in 100 μl of PBS for 1 h with gentle mixing on an rotary mixer and applied to their corresponding microplate wells at 1 nanomole per well for 1 h at room temperature with gentle mixing. The plates were decanted and washed three times in 200 μl of PBS for at least 5 min per wash with gentle mixing. One hundred μl of a 1:5,000 dilution of streptavidin-peroxidase from a 1 mg/ml stock solution (Thermo Fisher Scientific, Product No. 21126) in PBS was added per well for 30 min at room temperature with gentle mixing. The plates were decanted and washed three times with 200 μl of PBS per well as before. One hundred μl of One-Component® ABTS substrate (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD) which had been equilibrated to room temperature was added to each well and incubated for 15 min at room temperature. Reactions were halted by addition of 100 μl of 1 % SDS as the strongest reactions approached an absorbance of 2.0 at 405 nm using a Thermo Electron MultiSkan™ microplate reader (Thermo Fisher Scientific; Waltham, MA).

Secondary Structural Analyses

Secondary stem-loop structures in the Supplemental figures were determined using UNAFold software on Integrated DNA Technologies, Inc.'s website (http://www.idtdna.com/Unafold/). In particular, DNA parameters at 25 °C, 0 mM Mg^{2+}, and physiological (154 mM) Na^{+} concentrations were used.

Fluorescence Staining and Laser Scanning Confocal Microscopy

Fresh log-phase *L. major* promastigotes were fixed in 4 % formaldehyde freshly prepared from depolymerized paraformaldehyde in PBS at 25 °C for 30 min or at 4 °C overnight. Promastigotes were pelleted by centrifugation at 7,519×G in a Sorvall® tabletop centrifuge and washed by resuspending and re-pelleting twice for 10–15 min each in PBS. Pelleted promastigotes were resuspended in 5 μl (~7.5 μg/ml) of 5′-biotinylated aptamers in 95 μl of PBS plus 0.05 % Tween® 20 for 1 h with gentle vortex mixing followed by two 10 min washes in PBS. Aptamer-bound promastigotes were then exposed to Cy5-streptavidin (diluted 1:100 from stock; Invitrogen; Frederick, MD) with gentle vortex mixing for 30 min. Promastigotes were washed in PBS two more times for 10 min per wash and pelleted by centrifugation. The pellet was then placed on a gelatin-coated microscope slide containing Vectashield® (low fluorescence mounting medium) with DAPI (4′, 6-diamidino-2-phenylindole; Vector Laboratories, Inc., Burlingame, CA) for rapid localization of promastigotes during fluorescence microscopy by painting their genomic and kinetoplast DNA blue with DAPI. Slides were then topped with a coverslip and sealed with nail polish prior to microscopic observation.

Fluorescently labeled promastigotes were analyzed using an Olympus FV1000 laser scanning confocal microscope (Olympus America, Center Valley, PA) equipped with a 60× oil objective (NA1.4) with a z-step size of 0.44 μm at 40 μs/pixel scan speed. The fluorophores used in this study included DAPI (ex/em=358/461 nm) and Cy5 (ex/em=650/667 nm) with look-up tables (LUTs) applied such that DAPI appears blue, and surface-bound aptamers appear pink to magenta. Laser power and other confocal settings were initially optimized on a test sample and fixed so that test and control samples are directly comparable to each other in their intensity. Images were initially processed using Olympus FluoView™ software, and each figure shown is a flattened Z-stack of multiple image planes. Assembly of figures for publication was done with Adobe Photoshop® (V12.1, Adobe Systems Incorporated, San Jose, CA).

Handheld Fluorometer (FLASH Reader)

A custom handheld fluorometer referred to as the FLASH reader was designed and built by Nanohmics, Inc. (Austin, TX) to provide very sensitive and quantitative fluorescence measurements. The core functionality of the FLASH reader is provided by a computerized numerical control (CNC)-machined aluminum optomechanical assembly, featuring an integral epifluorescence optical configuration. The instrument features a sample receptacle enabling the measurement within standard cuvettes, with a primary focus within the bulk solution. The system has been optimized for the Amplex Ultra Red (AUR) excitation and emission spectra—fluorescence excitation is provided by a 2.6 mW epoxy-encased LED (LED525E, Thorlabs Inc.) with center wavelength of 525 nm. The excitation source is passed through a single bandpass filter (FF01-525-15, Semrock Inc.), centered at 525 nm with 15 nm bandwidth FWHM. A 45-degree dichroic beam-splitter with a cut-on frequency of 565 nm (565DRLPXR, Omega Optical) enables separation of the excitation and emission bands, while a 575 nm cut-on longpass filter (575ALP, Omega Optical Inc.)

provides rejection of out-of-band light within the collection optics chain. A photomultiplier tube (PMT; Cat. No. H10722-01, Hamamatsu Corp.) provides signal transduction of the collected optical signal.

A custom fluorescence measurement printed circuit board was designed to provide control of the excitation light-emitting diode (LED) source, the PMT gain, and 16-bit ADC conversion of the PMT signal. The LED source is modulated with a 5 kHz sinusoidal signal, and the resulting PMT signal is passed through a 10 kHz anti-aliasing filter. This signal is deconvolved via a Fast-Fourier Transform (FFT), which provides enhanced signal to noise ratio (SNR) via synchronous detection of the signal component at the modulation frequency. Control of these electrical subsystems has been achieved through use of an integrated single board computer (SBC), running custom written software that enables the adjustment of both PMT and excitation sinusoid gain settings.

Enzyme-Linked Fluorescent Aptamer-Magnetic Bead Sandwich Assay

Twenty μl of 5′-biotinylated LmWC-25R capture aptamer-streptavidin-Dynal/Life Technologies M280 (2.8 μm) magnetic beads (~4×10^7 aptamer-MBs) were added to 500 μl of PBS, diluted human serum, or sandfly homogenate as indicated in the figure legends. For sandfly experiments, generally two uninfected or *L. major*-infected sandflies per ml of PBS were homogenized in a GentleMACs™ tissue homogenizer (Miltenyi, GmBH, Germany) using the "protein" setting. The number of *Leishmania* promastigotes per sandfly in the infected population was not known and in some experiments, sandfly homogenization was conducted in 1.5 M $MgCl_2$ and left overnight at 4 °C followed by desalting in a spin column and back flushing of extracted proteins as previously described. Tubes were mixed gently on a rotating mixer for 15 min at RT. A Dynal MPC-S® or comparable magnetic rack was used to collect MBs in microcentrifuge tubes for 1 min. The supernatant devoid of MBs was carefully aspirated and discarded in 5 % bleach water solution. Five hundred picomoles of 5′-biotinylated reporter aptamer (LmHSP-7b/11R) in PBS was added to each tube and tubes were gently mixed again for 10 min at RT. MBs were again collected on the magnetic rack for 1 min. MBs were washed 3 times for 1 min per wash in 1 ml of PBS and resuspended by gentle pipetting 3 times with magnetic collection for 1 min between each wash. The supernatant was removed and the MBs with aptamer-captured *Leishmania* proteins were resupended in 500 μl of 0.25 μg/ml of streptavidin-horseradish peroxidase (Sav-HRP) in PBS per sample for 10 min at RT with gentle mixing. MBs were again collected using the magnetic rack for 1 min per sample and washed 3 times in PBS with resuspension in fresh 1 ml volumes of PBS and magnetic collection as before. Amplex® Ultra Red (AUR; 1 mg, Life Technologies Inc.) was stored at −20 °C, thawed just prior to use and dissolved in 100 μl of pure DMSO by brief vortex mixing. Stock AUR solution was diluted 1:1,000 in PBS prior to use along with 25 μl of 3 % H_2O_2 per ml of diluted AUR. MBs were collected using the magnetic rack and resuspended in 1 ml of diluted AUR solution with 0.075 % H_2O_2, vortex mixed on the lowest mixer setting for 5 s and transferred to polystyrene cuvettes (Thermo Fisher Scientific No. 14-955-129) containing an additional 1 ml of diluted AUR plus 0.075 % H_2O_2 solution. Fluorescence was assessed within the first 1 min of development using the FLASH reader with PMT settings between 20 % and 30 % of maximum voltage.

Results

Protein extraction can be achieved by several means including detergents and high molarity chaotropic salts [26]. For this and other projects, we have chosen to use 1.5 M $MgCl_2$ extraction overnight at 4 °C [9, 27], because it appears to yield a multitude of intact proteins against which our aptamers can bind. Figure 2 illustrates the entire array of protein bands that can be extracted from *L. major* promastigotes from 6.5 kD to 200 kD when stained with Coomassie blue or silver stain.

The complete body of candidate aptamer DNA sequences derived for both the capture (WC) and reporter (rHSP) aptamer families are presented in Supplemental Tables 1 and 2. Supplemental Figs. S1–S3 document the various

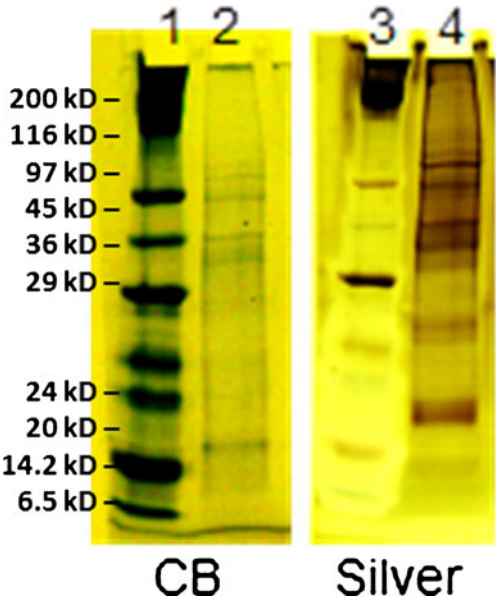

Fig. 2 Extracted soluble proteins from ~10^7 *L. major* promastigotes (lanes 2 and 4) after overnight exposure of promastigotes to cold 1.5 $MgCl_2$ and electrophoretic separation. A Coomassie blue (CB) stained 4–20 % gradient SDS-polyacrylamide gel (*left side*) was later fixed and silver stained (*right*). Molecular weight markers from 6.5–200 kD are shown in lanes 1 and 3

ELASA screening trials used to rank the relative affinity of the capture and reporter aptamers. While the ultimately successful capture (LmWC-25R) and reporter (LmHSP-7b/11R) aptamers did not always rank first in the ELASA screens for each trial, they were at or near the top in each case (Figs. S1–S3) and performed best in numerous sandwich assay pairings (data not shown for brevity) which led to their selection for the final assay formulation. When the primary nucleotide sequences of the selected capture and reporter aptamers are compared to the sequences of other aptamer candidates from the final pool, several guanine (G)-rich segments emerge (Supplemental Tables 1 and 2). The LmWC-25R capture aptamer contains three such segments, while the LmHSP-7b/11R reporter aptamer does not contain these segments and may be more specific for the HSP protein for that reason.

Confocal fluorescence microscopy images shown in Fig. 3 verify that both the capture (LmWC-25R) and reporter (LmHSP-7b/11R) Cy5-SAv-biotin-aptamers (pink/magenta) strongly bind surface moieties on *L. major* promastigotes (panels b, c, f, and g). Moreover, Fig. 3 illustrates that in the absence of a biotinylated-aptamer, pink/magenta Cy5 staining is not observed (panels a and e), and in the presence of an unrelated Cy5-SAv-biotin-aptamer for *Salmonella* (StW-19 F), no pink/magenta staining is observed (panels d and h). The blue DAPI staining serves as a useful marker to quickly locate and identify promastigotes by their genomic and kinetoplastid DNA in both the upper and lower rows of Fig. 3 while the upper row uses a mixture of brightfield and confocal fluorescence microscopy to reveal flagella on the promastigotes (indicated by the arrows) and confirm that the objects are not simply debris.

Figure 4 illustrates the secondary stem-loop structures of the selected capture and reporter aptamers as determined by UNAFold software. These two aptamers seem to share some commonality in the boxed loop structures, but are otherwise distinct. The loop structures on the reporter aptamer (LmHSP-7b/11R) are highlighted in Supplemental Table 2 and are unique in the pool of capture and reporter aptamer candidates which may contribute to the reporter aptamer's specificity for *L. major* promastigotes and the overall specificity of the assay.

Images of the portable second generation (G2) FLASH reader are presented in Fig. 5, panels a–c which show a transparent design graphic, actual appearance of a unit and its color touchscreen showing the main assay menu. Other screens which are not show, but can be selected by the user, control the instrument PMT and other parameters including filenames and output from the onboard computer and Windows® interface. Data can be transferred via a USB port on the rear of the instrument (not shown). Figure 5d illustrates the very linear performance of the FLASH reader versus the commercially available Picofluor™ or Quantifluor™ handheld fluorometers (Promega Corp.) using a different, but similar AUR-based aptamer-MB sandwich assay. The correlation coefficient

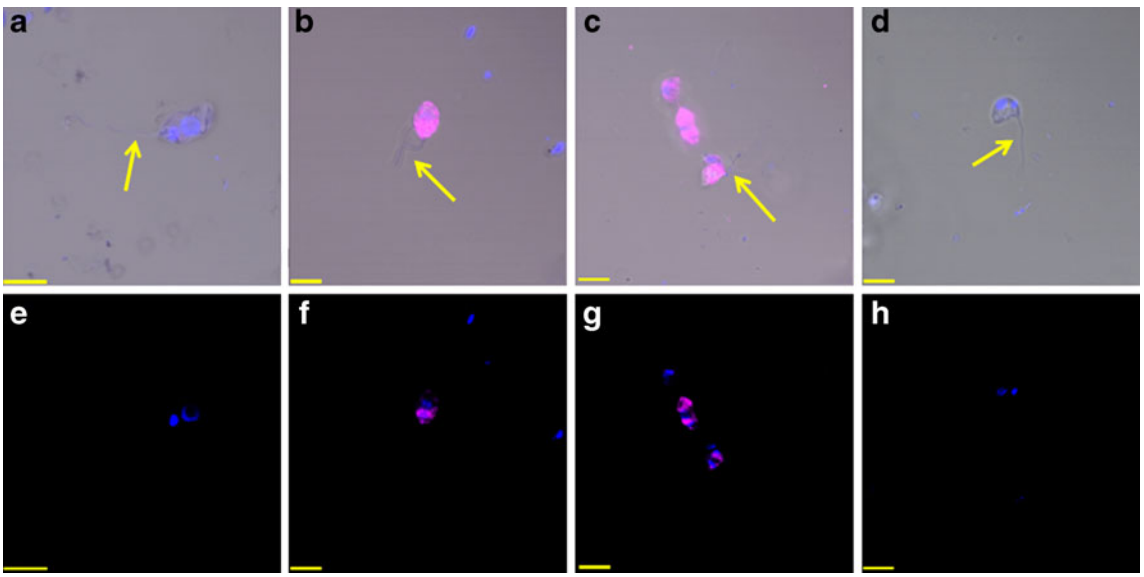

Fig. 3 Montage of *L. major* promastigote confocal fluorescence microscopy images after staining with Cy5-streptavidin-5′-biotin labeled LmHSP-7b/11R reporter aptamer (panels **b** and **f**, showing a flattened z-projection containing four slices) and LmWC-25R capture aptamer (panels **c** and **g**, showing a flattened z-projection containing ten slices) to prove that the aptamers bound the promastigote surface by yielding pink/magenta fluorescence. Also shown are negative control results without biotinylated aptamers (panels **a** and **e**, a flattened z-projection containing five slices) and controls with an unrelated aptamer (designated StW-19 F, panels **d** and **h**, showing a single slice image) which showed no pink/magenta Cy5 staining. Blue staining of DNA by DAPI was used to aid in rapidly locating all promastigotes. The top row of panels presents a combination of confocal fluorescence microscopy and brightfield microscopy to reveal flagella (indicted by *arrows*) and prove that the detected bodies were in fact promastigotes and not cellular debris. The bottom row of panels shows the same images without the brightfield component. For all images, the scale bar represents 5 μm

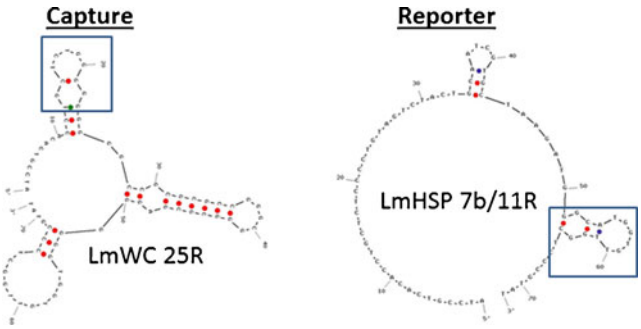

Fig. 4 Secondary stem-loop structures of the chosen capture (LmWC-25R) and reporter (LmHSP 7b/11R) aptamers according to UNAFold software analyses at 25 °C in physiologic salt concentration. Boxed loop structures demonstrate some similarity in structure despite not emanating from the same exact DNA sequence

(r^2 value) between the Picoflour™ and the custom G2 FLASH reader was 0.9792 for the >300 samples analyzed by both systems side-by-side and mere seconds apart. This high correlation coefficient testifies to the quality and linearity of the new G2 FLASH reader versus a well-established commercial fluoromter (i.e., the Picofluor™).

Figure 6a supports the linearity of the *L. major* assay as assessed by the G2 FLASH reader and its specificity versus $MgCl_2$-extracted *L. infantum* proteins and a variety of other related (e.g., *Leishmania* kinesin peptides [38]) and unrelated bacterial (*E. coli* intimin-gamma protein) or collagen (PIIINP peptide [39]) targets in Fig. 6b. Figure 6a also supports the limit of detection being ~100 ng of promastigote protein extract since each data point and error bar represents the mean and 2× standard deviations which do not overlap between the blank and the 100 ng data points for *L. major* protein analysis.

We also tested the *L. major* assay in 50 % human serum which despite the relatively low autofluorescence of serum above 580 nm, required us to increase the PMT setting to 30 % of maximum (thus accounting for the greater y-axis values in Fig. 7) to observe the damped 50 % serum assay curve versus the very linear result produced in PBS as shown in Fig. 7. The gray bars in Fig. 7 demonstrate a weak assay with hook effect on the high end (at 1,000 ng of promastigote proteins) in 50 % human serum. The same assay was much stronger and more linear when conducted in PBS (black bars in Fig. 7).

While strong detection in serum is desirable for clinical diagnostics, it is not relevant for our current promastigote-targeted field assay, because amastigote forms of *Leishmania* dominate in the human host and promastigote forms are found in sandflies. Therefore, our promastigote assay is actually more germane to some military field applications [7, 8]. By aiding detection of *Leishmania* promastigotes in homogenized sandflies captured at various locations, the present assay system could help to limit the spread of this parasitic disease in human populations. Unfortunately, when we first attempted the assay in infected sandfly homogenates, we were not able to detect *L. major* (Fig. 8, left side) even in PBS until we extracted the promastigote-infected homogenate with 1.5 M $MgCl_2$ overnight at 4 °C and then desalted the samples in a spin column as previously described. As the right side of

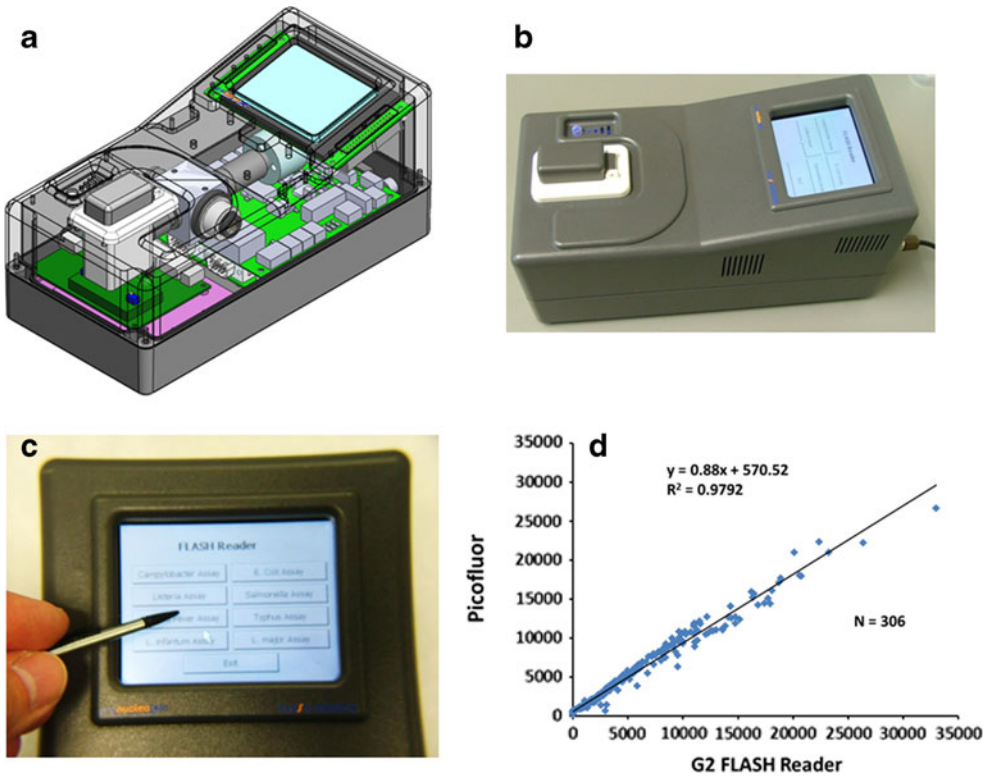

Fig. 5 Collage of images of the second generation (G2) FLASH reader and its color touchscreen (panels **a**–**c**) including a transparent engineering design (panel **a**). Panel **d** illustrates the very linear performance of the G2 FLASH reader from 306 runs of a similar AUR-based aptamer-magnetic bead-based assay and its very favorable comparison to the commercially available Picofluor™ or Quantifluor™ (Promega Corp.) with a correlation coefficient (r^2) value of 0.9792

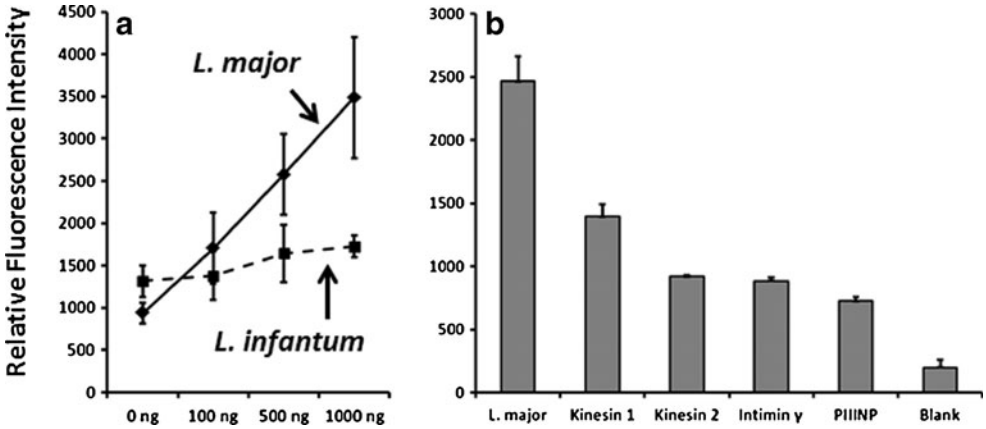

Fig. 6 Panel **a** illustrates the linear fluorescence response of the *L. major* AUR-aptamer MB assay versus nanograms of promastigote soluble protein extract (*solid line*) versus the same assay conducted with protein extracts from the *L. infantum* species of promastigotes (*dashed line*). Data points and error bars represent the means and 2× standard deviations of three independent trials per data point. Panel **b** summarizes cross-reactivity data for the *L. major* AUR-aptamer-MB sandwich assay tested against 500 ng of *L. major* protein extract and 500 ng each of *Leishmania* kinesin peptides 1 and 2 as well as *E. coli* intimin-gamma, a completely unrelated human collagen peptide (PIIINP) and blank samples without a target analyte. Again, means (represented by *bar heights*) and 2× standard deviation error bars of three independent measurements are shown

Fig. 8 demonstrates, $MgCl_2$ extraction dramatically increased the detectability of *L. major* promastigote proteins in sandfly homogenates.

Discussion

We elected not to attempt to detect live promastigotes because they have powerful flagella and could break free of capture aptamer-MBs and swim away. Heat killing of the promastigotes would denature and alter their surface epitopes, thereby possibly affecting assay performance since the aptamers were selected against live promastigotes or intact rHSP. In addition, homogenizing sandflies is an integral part of the standard procedure for detecting promastigotes from sandfly samples. Therefore, we wished to conform as closely as possible to standard methods, but eventually needed to extract *Leishmania* proteins even in sandfly homogenates to achieve reliable detection.

The fact that our sandwich assay detects extracted proteins is interesting in that two epitopes on the same target protein are needed to form a sandwich assay, but the capture and reporter aptamers were developed against two different target materials (whole cells and recombinant surface protein). Therefore, either the LmWC-25R capture aptamer raised against *L. major* whole cells is detecting the HSP protein, which is a surface marker (Swiss-Protein No. Q4QB56) [41] or a complex of HSP with some other *L. major* proteins has formed and we are detecting the protein complex. It is possible too that the sandwich assay is detecting promastigote membrane fragments that result from chaotropic disruption or lysis of the plasma membrane.

Regardless of what is actually being detected, we have demonstrated a robust, sensitive (LOD of ~100 ng per 2 ml sample) sandwich assay which is relatively facile and rapid (i.e., completed in ≤1 h after sample extraction in $MgCl_2$) and

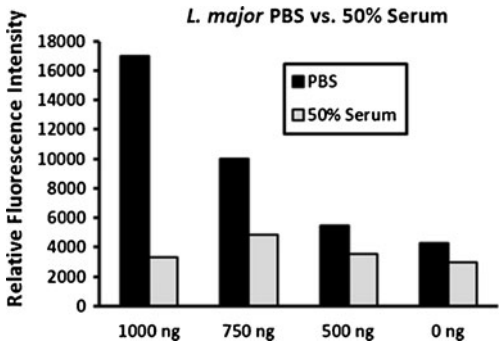

Fig. 7 Comparison of the *L. major* assay's performance in phosphate buffered saline (PBS) versus 50 % human serum and PBS as a function of protein extract level in nanograms per sample

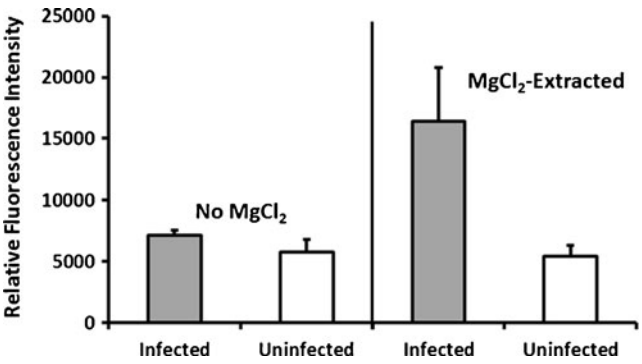

Fig. 8 Comparison of the assay's detection of uninfected and *L. major* promastigote-infected sandfly homogenates (2 flies per ml of PBS) without (*left side*) and with (*right side*) 1.5 M $MgCl_2$ extraction of proteins after homogenization and subsequent desalting versus uninfected sandfly homogenate with no known presence of promastigotes. The number of promastigotes per sandfly in the infected groups was not known. Means and 2× standard deviation error bars are shown for three independent measurements

highly portable for field work in austere environments which compares favorably with the ~2 h PCR method [7]. The assay also appears to be reasonably specific for *L. major* promastigotes. Since promastigotes are the predominant form of *Leishmania* parasites in infected sandflies, their detection in sandflies is more important than in human serum where the amastigote form would dominate and very few, if any, promastigotes would be expected. Thus, the assay's lack of performance of our promastigote assay in human serum is not of much concern since this was not its intended purpose. However, the assay's performance in sandfly homogenates was important and was greatly enhanced by extraction of the promastigote proteins with 1.5 M $MgCl_2$ followed by desalting in sandfly homogenate as illustrated in Fig. 8. The presently described assay was designed for mapping areas of high *L. major* incidence in sandflies in the field for the Defense Department so as to aid in prevention of leishmaniasis in soldiers and civilians [7, 8], and as such it clearly serves its purpose in sandfly homogenates.

We have not yet tested the assay for detection of amastigotes in suspicious cutaneous lesions or mucocutaneous ulcer exudates, because we have not had access to such clinical samples. However, there is reason to hypothesize that our promastigote assay might also detect *L. major* amastigotes in lesions since very few genes (e.g., <3.5 % of all *L. mexicana* gene) appear to be up or down regulated during differentiation of the promastigote into the amastigote form [42].

In a broad sense, the methodology presented here also suggests that rapid, sensitive, and highly portable detection of *Leishmania* infections by numerous species including *L. donovani, L. infantum* or *L. chagasi,* which cause the more lethal visceral form of leishmaniasis, should be possible using aptamer-based MB enzyme-linked fluorescent sandwich assays or other similar aptamer assay formats. Such new technology could be used to avoid misdiagnoses and initiate beneficial early treatment [4–6] of this dreaded parasitic disease.

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