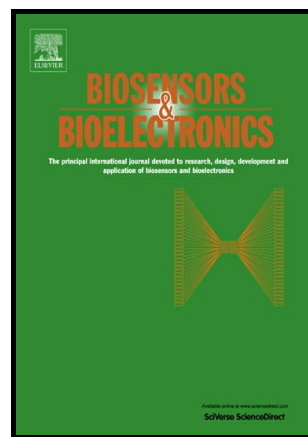


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Electrochemical Magnetic Microbeads-based Biosensor for Point-of-Care Serodiagnosis of Infectious Diseases

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Short Title: Magnetic beads-based electrochemical biosensor for infectious diseases

Abstract

Access to appropriate diagnostic tools is an essential component in the evaluation and improvement of global health. Additionally, timely detection of infectious agents is critical in early diagnosis and treatment of infectious diseases. Conventional pathogen detection methods such as culturing, enzyme linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) require long assay times, and complex and expensive instruments making them not adaptable to point-of-care (PoC) needs at resource-constrained places and primary care settings. Therefore, there is an unmet need to develop portable, simple, rapid, and accurate methods for PoC detection of infections. Here, we present the development and validation of a portable, robust and inexpensive electrochemical magnetic microbeads-based biosensor (EMBIA) platform for PoC serodiagnosis of infectious diseases caused by different types of microorganisms (parasitic protozoa, bacteria and viruses). We demonstrate the potential use of the EMBIA platform for *in situ* diagnosis of human (Chagas disease and human brucellosis) and animal (bovine brucellosis and foot-and-mouth disease) infections clearly differentiating infected from non-infected individuals or animals. For Chagas disease, a more extensive validation of the test was performed showing that the EMBIA platform displayed an excellent diagnostic performance almost indistinguishable, in terms of specificity and sensitivity, from a fluorescent immunomagnetic assay and the conventional ELISA using the same combination of antigens. This platform technology could potentially be applicable to diagnose other infectious and non-infectious diseases as well as detection and/or quantification of biomarkers at the POC and primary care settings.

Keywords: Point-of-Care diagnosis; electrochemical biosensor; magnetic-beads; recombinant antigens; infectious diseases.

1. Introduction

Access to appropriate diagnostic tools is an essential component in the evaluation and improvement of global health (Giroi et al. 2006; Hay Burgess et al. 2006). Diagnostics are crucial for identifying the presence and cause of disease at the individual and population levels, both in humans and animals. In addition, timely detection of infectious agents is critical in early diagnosis and treatment of infectious diseases. Conventional pathogen detection methods such as culturing, enzyme linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) require relatively sophisticated infrastructure and well-equipped laboratories, and must be performed by highly trained staff, conditions that make them not adaptable to point-of-care (PoC) needs at resource-constrained and primary care settings (Song et al. 2014; Urdea et al. 2006). Additionally, many current diagnostic tools require more than one visit of the patients to the health care units, which is complicated or not possible for many people in developing countries, especially for those living far away from the health centers. Therefore, there is an increasing interest in developing portable, simple, rapid, and accurate methods for detection of pathogens at the PoC. The implementation of rapid, specific and reliable PoC devices for the in situ diagnosis, mass-screening surveys and/or intervention campaigns (e.g., during the onset of outbreaks) in endemic areas could significantly improve the diagnostic and clinical management fields, particularly for tropical and/or neglected diseases (Peeling and Mabey 2010).

The biosensors are the ideal systems to address all those requirements and frequently offer a good response in terms of reliability, analytical performance and mass-screening. Furthermore, biosensors with high degree of integration are more amenable for mass production offering a better and more reproducible response. In the last years, optical, electrochemical and piezoelectric biosensors devices have emerged as important tools capable of improving detection of a variety of analytes (Bănică 2012; Holford et al. 2012). In particular, electrochemical biosensors can be developed with a higher degree of integration than optical biosensors, which require the use of light sources and detectors. Electrochemical transducers have been widely used in biosensors due to their high sensitivity, low cost, simple design and small size making them excellent candidates for the development of portable biosensors (Dorothee Grieshaber 2008).

Most of the biosensing techniques are carried out either on solid-phase supports or biological membranes. Magnetic microspheres have been widely used as a solid-phase support for the detection of biomolecules such as nucleic acids, polypeptides and/or antibodies (Kuramitz 2009). When coated with antigens, magnetic beads provide an ideal support for serodiagnosis allowing the removal of unwanted sample constituents by a simple magnetic

separation step with minimal loss of the target material (Ciocchini et al. 2013; Ciocchini et al. 2014; Iwashkiw et al. 2012). Additionally, magnetic microspheres confer a number of other benefits compared to other solid supports, including relatively large surface-to-volume ratio, high throughput capacity and reproducibility, reduced assay time and small sample volume requirements.

In this work, we have developed and evaluated an electrochemical magnetic microbeads-based biosensor (EMBIA) for PoC serodiagnosis of infection diseases caused by different types of microorganisms (parasitic protozoa, bacteria and viruses) using previously validated recombinant antigens. The EMBIA biosensor or platform includes three main components: antigen-functionalized magnetic microbeads, disposable electrochemical cells-electrode cartridges and a portable potentiostat. In this platform, superparamagnetic microbeads functionalized with the corresponding antigens are sequentially incubated with the samples and the HRP-conjugated antibodies, and the electrochemical readings performed on the 8-channel-electrochemical cells-electrode cartridges using the portable potentiostat. Our data support the potential use of the EMBIA platform for the *in situ* diagnosis of human and animal infectious diseases with an excellent signal-to-noise ratio. In the particular case of Chagas disease, a more extensive validation was performed showing that the EMBIA platform displayed an excellent diagnostic performance, in terms of specificity and sensitivity, almost indistinguishable from a fluorescent immunomagnetic assay and the conventional ELISA using the same combination of recombinant antigens.

2. Material and methods

2.1. Serum samples

Human serum samples. The institutional review board of the corresponding institutions approved the use of serum samples in the current study. Written informed consent was obtained from all individuals. Serum samples were collected from clotted blood obtained by venipuncture and stored at -20 °C, and were decoded and de-identified before they were provided for research purposes. Therefore, no information (e.g., name, age, sex, etc.) regarding the patients was available.

A total of 78 positive samples obtained from Chagas disease patients coursing the chronic phase of the disease (positive reference samples) were analyzed. Of the 78 positive samples 46 were obtained from our own collection and were described elsewhere (Balouz et al. 2015; Di Noia et al. 2002). These samples were positive by three independent assays: ELISA using total parasite homogenate, Indirect Hemagglutination Assay (IHA) and Indirect Immunofluorescence (IIF). The remaining 32 samples were provided by the Laboratorio de Enfermedad de Chagas, Hospital de Niños "Dr. Ricardo Gutierrez" (Buenos Aires, Argentina). These samples were positive by ELISA using total parasite homogenate (Wiener lab, Argentina) and IHA (Polychaco, Argentina).

For diagnosis of human brucellosis, 17 serum samples, from a previously characterized sera collection provided by the Brucellosis Division from the Hospital de Enfermedades Infecciosas Francisco Javier Muñiz (Buenos Aires, Argentina), were analyzed (Ciocchini et al. 2013). These samples were obtained from patients with positive blood culture for *Brucella abortus*, *Brucella melitensis* or *Brucella suis* and serologically-positive by the following serological tests currently used for diagnosis of the disease: Rose Bengal Test (RBT), Serum Agglutination Test (SAT; Huddleson), Tube Agglutination Test (TAT; Wright), TAT-2ME (2-mercaptoethanol), Competitive ELISA (CELISA) and Complement Fixation Test (CFT).

A total of 122 negative samples were obtained from healthy blood donors. These samples, considered as negative reference samples for Chagas disease and human brucellosis, were negative in routine screening tests for both diseases. Of the 122 negative samples, 59 were obtained from the "Fundación Hemocentro Buenos Aires" (Buenos Aires, Argentina), 27 from the Hospital de Enfermedades Infecciosas "Dr. Francisco Javier Muñiz" (Buenos Aires, Argentina), 16 from Hospital Italiano de Buenos Aires (Buenos Aires, Argentina) and 20 from the Hospital Municipal "Dr. Diego E. Thompson" (San Martín, Buenos Aires, Argentina).

Bovine serum samples. All animal serum samples were provided by the National Brucellosis Reference Laboratory (DILAB-SENASA) from Argentina. Twelve serum samples

obtained from naturally *B. abortus*-infected bovines were analyzed (Ciocchini et al. 2014). These samples were serologically positive by the serological tests currently used for diagnosis of the disease: Buffer Plate Agglutination Test (BPAT), SAT, CELISA, indirect ELISA (iELISA), Fluorescent Polarization Assay (FPA) and CFT. Fifteen positive serum samples obtained from cows with clinical signs of foot-and-mouth disease (FMD) and serologically positive by 3ABC-iELISA and confirmed by Electro-ImmunoTransfer Blotting technique (EITB) were analyzed. Twenty-three serum samples obtained from officially certified brucellosis and FMD-free herds were used as negative reference samples. These samples resulted negative in routine screening tests for both diseases (BPAT for bovine brucellosis, and 3ABC-iELISA for FMD).

2.2. Production and purification of recombinant antigens

Plasmids and bacterial strains used in this work are listed in Table S1.

T. cruzi antigens (*GST-Ag1*, *GST-Ag36*, *GST-SAPA* and *GST-TSSA*). A panel of validated *T. cruzi* antigens, for which the performance in Chagas disease serodiagnosis has been already reported, was used (see supplemental material). Ag1, Ag36 and SAPA constructs were subcloned in pGEX1 λ T expression vector (GE Healthcare Life Sciences), which provides translational fusion to Glutathione S-transferase (GST) protein, from the plasmids pTrcHis-TS-Ag1, pTrcHis-TS-Ag36 and pTrcHis-TS-SAPA, respectively (Buscaglia et al. 1999). Briefly, these constructs were excised from pTrcHis vector (Invitrogen) by *Eco*RI digestion and subsequently ligated into pGEX1 λ T. Cloning of the predicted mature product (i.e., without most of the endoplasmic reticulum and GPI anchor signals) of the TSSA gene from the CL Brener clone (GenBank accession number ACY54510) into the plasmid pGEX2T has been previously described (De Marchi et al. 2011). *Escherichia coli* DH5 α F'IQ™ (Life Technologies) strain was used for all the cloning procedures. All constructs were further analyzed by DNA sequencing (Applied Biosystems ABI3130 capillary sequencer). For expression and purification of the antigens, the *E. coli* BL21-CodonPlus (DE3)-RP strain was transformed with the plasmids pGEX1 λ T-Ag1, pGEX1 λ T-Ag36, pGEX1 λ T-SAPA or pGEX2T-TSSA. The resulting strains were grown overnight at 37 °C with agitation in Luria-Bertani (LB) broth supplemented with 20 g/mL chloramphenicol and 100 g/mL ampicillin, and re-inoculated at 1:100 dilution into fresh LB medium. After incubation for 3 h at 37°C (OD₆₀₀ $\approx$ 0.5), protein expression was induced by addition of isopropyl- α -D thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Following a 3 h-induction period, cells were harvested by centrifugation and cytoplasmic extracts were obtained by lysing the cells with lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 0.5 mg/mL lysozyme, 10 mM MgCl₂, 2 mM PMSF, 2 mM EDTA and 10 μ g/mL DNase) and sonication. After centrifugation, the soluble fraction was subjected to

glutathione-Sepharose affinity chromatography following the manufacturer's instructions (GE Healthcare Life Sciences).

Brucella antigen (OAg-AcrA). Production and purification of the OAg-AcrA glycoconjugate was performed as previously described (Ciocchini et al. 2013; Ciocchini et al. 2014; Iwashkiw et al. 2012). Briefly, *Yersinia enterocolitica* O:9 wild type strain transformed with the plasmids pMAF10 (encoding the *Campylobacter jejuni* oligosaccharyltransferase PglB) and pMH5 (encoding the *C. jejuni* carrier protein AcrA fused to a 6x histidine tag) was grown overnight at 37 °C. Cultures were re-inoculated at 1:100 dilution into fresh LB medium and grown at 37 °C for 2.5 h ($OD_{600} \approx 0.5$) and PglB expression was induced by addition of arabinose to a final concentration of 0.2% (w/v). Four hours after induction at 37 °C, PglB was re-induced by a second addition of arabinose to maximize glycosylation of AcrA. Cells were harvested by centrifugation after a 20 h induction period and periplasmic extracts were prepared by lysozyme treatment and the glycoprotein purified by Immobilized Metal Affinity Chromatography (IMAC) following the manufacturer's instructions (GE Healthcare Life Sciences).

FMDV antigen (3ABC). Total RNA of the strain FMDV 01 Campos was extracted using the Trizol® reagent (Life Technologies) according to the manufacturer's instructions. cDNA synthesis was performed using the ThermoScript RT-PCR System (Life Technologies) and the 3ABC gene was amplified by PCR using Pfx DNA polymerase (Life Technologies) and the oligonucleotides *Nde*I-3ABC (CGCATATGATCTCAATTCCTTCCAA, underlined sequence indicates the restriction site) and 3ABC-*Hind*III (CCAAGCTTCTCGTGGTGTGGTGGTTCGGG). The amplified DNA fragment was digested with *Nde*I and *Hind*III and cloned into the plasmid pET22b (+) (Novagen) previously digested with the same enzymes. The resulting plasmid was named pET22b-3ABC. Subsequently *E. coli* BL21-CodonPlus (DE3)-RP strain was transformed with the plasmid pET22b-3ABC and the resulting strain was grown overnight at 37 °C with agitation in LB medium supplemented with 20 g/mL chloramphenicol and 100 g/mL ampicillin, and re-inoculated at 1:100 dilution into fresh LB medium. After incubation for 3 h at 37 °C ($OD_{600} \approx 0.5$), protein expression was induced by addition of IPTG to a final concentration of 1 mM. After induction for 18 h at 18 °C, cells were harvested by centrifugation and the cytoplasmic extracts were obtained by incubating the cells with lysis buffer (see above) and sonication. After centrifugation, the soluble fraction was subjected to Immobilized Metal Affinity Chromatography (IMAC) following the manufacturer's instructions (GE Healthcare Life Sciences).

All the purified antigens were dialyzed against 0.01 M phosphate-buffered saline pH 7.2 (PBS), quantified by Bradford's protein assay and analyzed by Coomassie brilliant blue-stained SDS-PAGE and Western blotting (data not shown).

2.3. Coating of superparamagnetic microbeads

Superparamagnetic COOH-modified microbeads (5% solids, 7.9 μm , Bangs Laboratories) were activated in one step with NHS [N-hydroxysuccinimide] in 100 mM MES [2-(N-Morpholino) ethanesulfonic acid] pH 5.5 buffer and EDAC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride]. Activated beads were washed with PBS and incubated for three hours with different amounts of GST-Ag1, GST-Ag36, GST-SAPA, GST-TSSA, OAg-AcrA or 3ABC. One mL of antigen solution (0.05 to 0.2 mg/mL) was incubated with 0.25 mL of beads suspension, thus yielding enough material for 50 reactions (see below). Coated magnetic beads were washed with quenching buffer (35 mM glycine, 1% gelatin from cold-water fish skin) and incubated overnight at 4 °C with the same buffer and orbital agitation. Finally, coated beads were washed with storage buffer (1% gelatin from cold-water fish skin, 2.25% Tween 20, 0.01% sodium azide), resuspended in 1 mL of the same buffer and stored at 4 °C until use.

2.4. Electrochemical enzyme-linked magnetic beads immunoassay (EMBIA): principle of the assay and optimization

Antigen-coated magnetic beads were incubated with human or bovine serum samples, washed three times with PBS containing 0.2% Tween 20 (PBST) and bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma-Aldrich) or HRP-conjugated goat anti-bovine IgG (H+L, Jackson ImmunoResearch Laboratories) antibodies. After washing three times with PBST, the beads were resuspended in PBS and transferred to the electrochemical cells (disposable cartridges with eight electrodes and electrochemical cells, see below). In the cartridge, the particles are magnetically collected and placed onto the surface of the electrode. After adding the substrate (1.5 mM H_2O_2) and 4 mM hydroquinone in PBS, peroxidase activity was amperometrically recorded using a mini-portable potentiostat. The electrode potential was set at -0.23 V and the resulting current recorded for 20 s. The instant limit current at 20 s was used as a measure of the reactivity. Potentials were measured and referred against an Ag|AgCl|0.1 M KCl reference electrode. Washes between incubation steps were performed using a magnetic rack without the need of centrifugation (Fig. 1). Optimization of the assay was performed by a checkerboard titration analysis. Based on these analyses, the optimal conditions for the EMBIA were: a) 2 μg of antigen per reaction (in the case of *T. cruzi* antigens 0.5 μg each of the four antigens); b) 20 μL of coated beads per

reaction (in the case of *T. cruzi* antigens, 5 μ l of microbeads functionalized with each antigen were mixed); c) 1:100 dilution of the serum samples; d) 1:5,000 dilution of the secondary antibodies and e) 5 min of incubation (at room temperature without agitation) of coated beads with serum samples and conjugate antibodies. These conditions were used to test all the samples.

2.5. Hardware design and development

An 8-channel portable potentiostat was developed to carry out measurements of potentiostatic steps and cyclic voltammetry (Fig. 2A). The device can be controlled via Bluetooth using a set of basic commands. The instrument can control the working electrode voltage in the -2.5 V to +2.5 V range. The valid working electrode current is in the -10 μ A and 10 μ A range, and the design can be easily adapted for different current ranges. The device is powered by a rechargeable battery and the housing was printed in ABS and designed for electronic protection and error-free cartridges insertion.

Power. A single-cell Li-ion battery (3.7 V) with a charge of 2600 mAh was used which provides more than 9 hours of autonomy continuous measurements. Battery charging control and voltage stepping up to 5 V was implemented using the integrated standalone switching charger ACT2801 which includes an internal boost, protection circuits for high and low voltage, protection against over temperature and short circuit. Due to the bipolar voltage range required, a dual output DC/DC converter module NMA0509SC was used to power analog circuitry. This module provides -9 V and +9 V outputs from 5 V provided by the ACT2801.

Analog Circuitry. Electrodes control and signal conditioning for a single electrochemical cell were carried out using two different types of precision operational amplifier (OPAM). A low offset OPAM OP07 was used to control the counter electrode. To avoid loading reference electrode, an LT1056 JFET input OPAM in buffer configuration was selected, providing an input impedance of (10^{12} Ohms). The current to voltage conversion, carried out in the working electrode circuitry, was also implemented using an LT1056 OPAM in transimpedance amplifier (TIA) configuration. This OPAM introduces a very low current error, typically in the range of 10 pA and guaranteed to be under 0.34 nA for the full operation range. External offset correction was not necessary in any of the cases.

Multiplexing. The eight channels were multiplexed by using two CMOS analog multiplexers DG408 for counter electrode voltage and working electrode current. Reference electrode voltage was not multiplexed due to its floating condition in unused channels and its very high input impedance. Also a circuit with two cheaper analog multiplexer CD4051 was successfully tested.

Control and Analog to Digital Conversion. The potentiostat was controlled by a microcontroller connected to a PC or mobile device via Bluetooth using a HC-06 module. This wireless module provides a serial port profile over Bluetooth V2.0 and was directly connected to the microcontroller to receive serial commands and send acknowledges and data. Checksum error checking was added by firmware. These commands, described in a complete firmware user manual, allows to turn on analog circuitry, channel selection, to configure potential step or voltammetry parameters and limits, start and interrupt assays and others.

Device acknowledge includes error information, and received and transmitted checksum. Microcontroller has a 10 bits A/D converter used to measure the working electrode current and the reference voltage. Counter electrode voltage was controlled with a 12 bits PWM output.

2.6. Electrodes and electrochemical cells

Thick film electrodes were printed onto 0.5 mm acrylic substrates by screen-printing technology. Disposable acrylic cartridges with eight electrochemical cells were designed and manufactured with dimensions fitting an 8-channel micropipette (Fig. 2B). Each cell contained two carbon electrodes (working electrode and counter electrode), and one Ag|AgCl reference electrode (Fig. 2C). Commercial carbon ink (Dupont BQ242), Ag ink (Dupont 5025) and Ag/AgCl ink (Dupont 5870) were used. Electrode layout was transferred by means of photolithography to a stainless steel mesh 200 (wires per inch) with a negative photosensitive film (Ulano CDF-4). All inks printing were performed using an EKRA Microtronic-II printer and dried in a box oven at 70 °C during 20 min. The central working electrodes were designed to be aligned with neodymium magnets to concentrate the magnetic particles. To build the eight electrochemical cells on the same piece where the electrodes were printed, each one with a volume of 40 µl, an acrylic top piece was added.

2.7. Software development

The electronic instrumentation of 8-channel portable potentiostat was developed to communicate with a host device using a serial communication protocol (Fig. S1). The host device can be a PC (e.g., a notebook) or an Android device (e.g., a smartphone or tablet). In order to simplify the validation of this protocol, we implemented a plain text set of commands. In this way, the device could be controlled using a simple terminal emulator software. Wireless Bluetooth communication was chosen to avoid the need of a physical connection with the host and to make possible the use of small host devices such as smartphones. Since Android is the

most popular operating system used in small devices (about 80% of market share), an Android application was developed with an easy-to-use user interface.

Test Software: A test software for GNU/Linux distributions was developed. It allowed carrying out amperometric records for potential steps and cyclic voltammetry. In both cases, filtering and an optional and configurable preconditioning step was added. The user can select measurement parameters and visualize its evolution in real time. The software can display simultaneously up to eight recorded assays. Back end was developed in C++ language. A full set of classes was written for an object-oriented device management. It manages the equipment connection, sends low level commands, process acknowledges and data, reads calibration constants and others. Perl scripting language and Gtk2 were used for front-end development and communicates with the back end through pipes. This software was useful for general testing, electrodes characterization and particles validation.

Android App: An Android application was also developed in Java language, with complete classes and associated documentation, compatible with API 10 (Android 2.3.3) or greater. Eclipse IDE (Integrated Development Environment) using the ADT (Android Development Tools) plug-in was used to develop the Android application. The app allowed to carry out potential step measurements, setting assay options and threshold presets for different diseases. Furthermore, an Android library was written to provide specific methods related to the device including a specific class for device management that implements the singleton design pattern, ensuring only one instance of the class across the application. The class also allows to register handler objects to receive and process its status connection messages and others. This implementation simplified the software maintenance and future updates, and permits making different applications that manage this device.

When the user starts the application, it searches and connects to a nearby device through an RFCOMM socket by using a secure connection. Once connected, the user triggers an assay and the app sends commands to the device to start a potential step measurement, applying configured voltage in the electrochemical cell and reading work current. The progress of the measurement is displayed on the screen. When the measurement is finished, the app calculates the average value of the work current and indicates the result of the measurement. Additionally, it saves a record for the entire measurement. For the average calculation, only a certain percentage of last samples is considered. A menu button, located at the top right corner, allows the selection of the measurement channels and threshold values.

2.8. Other assays

Fluorescent immunomagnetic assay for Chagas disease. Functionalized microbeads were incubated with human serum samples, washed three times with PBST and bound antibodies were detected with DyLight 650-conjugated goat anti-human IgG antibodies (Jackson ImmunoResearch Laboratories). After washing three times with PBST, fluorescence was directly determined using a plate fluorometer (DTX 880 Multimode Detector, BeckmanCoulter). Washes between incubation steps were performed using a magnetic rack without the need of centrifugation. In order to determine the optimal conditions for the assay (antigen concentration, sample dilution, secondary antibody dilution, and incubation times), a checkerboard titration analysis was carried out using selected positive (displaying high-, intermediate- and low-reactivity to our panel of recombinant antigens) and negative serum samples. Best results were obtained when using: a) 0.5 µg of each antigen (GST-Ag1, GST-Ag36, GST-SAPA or GST-TSSA) per reaction (total: 2 µg of antigen per reaction); b) 20 µL of coated beads per reaction (5 µL of microbeads functionalized with each antigen were mixed); c) 1:100 dilution of the serum samples; d) 1:1,000 dilution of the secondary antibodies and e) 5 min of incubation of coated beads with serum samples and conjugate antibodies (at room temperature without agitation).

Enzyme-linked Immunosorbent Assay (ELISA) for Chagas disease. Polystyrene ELISA high binding microplates (Costar, Corning Incorporated) were coated with 200 ng of a mixture of 50 ng of each antigen (GST-Ag1, GST-Ag36, GST-SAPA and GST-TSSA) in 50 µL of 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) and incubated at 4 °C overnight. After washing four times with PBST, plates were blocked with PBST supplemented with 5% bovine skim milk (blocking buffer) and incubated for 1 h at 37 °C. Serum samples were diluted 1:200 in blocking buffer and incubated for 1 h at room temperature. After four washes, bound antibodies were detected by the addition of HRP-conjugated goat anti-human IgG antibodies (Sigma-Aldrich) at 1:4,000 dilution in blocking buffer and incubating for 1 h at room temperature. Plates were washed four times with PBST and developed by addition of the substrate-chromogen solution (0.36% H₂O₂ and 0.01% 3,3',5,5'-Tetramethylbenzidine [TMB] in citrate-phosphate buffer pH 5.0). After 5 min, the reaction was stopped with 2N H₂SO₄ and the absorbance at 450 nanometers was determined using a plate reader (FilterMax Technologies).

2.9. Data analysis

Results of the EMBIA were determined as the average of the last 100 measured values of current (I). Percentage of reactivity was calculated as follows: % of reactivity = (I of the test

sample -I of PBST/I of the positive control - I of PBST) x 100. Results of the fluorescent immunomagnetic assay were expressed as percentage of reactivity of the mean Fluorescence Units (FU) of the positive control serum included in each assay run. Percentage of reactivity was calculated as follows: % of reactivity = (FU of the test sample/mean FU of the positive control) x 100. ELISA results were expressed as percentage of reactivity of the mean absorbance at 450 nanometers (Abs450) of the positive control serum included in each assay run. Percentage of reactivity was calculated as follows: % of reactivity = (Abs450 of the test sample/mean Abs450 of the positive control) x 100. Dot and receiver-operating characteristic (ROC) analyses (Greiner and Gardner 2000; Greiner et al. 1995; Swets 1988), as well as Mann-Whitney tests were performed using the GraphPad Prism software [version 5.01 for Windows, San Diego California USA, (<http://www.graphpad.com>)]. ROC analysis allows to determine for each assay the area under the ROC curve (AUC), the cut-off value that concurrently optimizes Se and Sp, the cut-off value for which the diagnostic Se is 100% and the cut-off value that gives a 100% Sp. These latter two cut-off values represent the boundaries of an intermediate range (IR) of reactivity values for definition of indeterminate test results (i.e., those that cannot be conclusively considered either negative or positive under the experimental conditions). The Youden's index (*J*) was calculated as $Se + Sp - 1$; the value of *J* index is maximal at the cut-off value that concurrently optimizes sensitivity and specificity. Pairwise comparisons of AUCs were performed using the MedCalc Statistical Software version 13.0.6 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2014) (Hanley and McNeil, 1983), and *P* values < 0.05 were considered significant.

3. Results and Discussion

3.1. Characterization of the electrochemical system of the EMBIA platform

The EMBIA platform contains three main components including antigen-functionalized magnetic microbeads, disposable electrochemical cells-electrode cartridges and a portable potentiostat. The detection system of the proposed platform for PoC diagnosis of infectious diseases is based on the capability to detect electrochemically the catalytic activity of enzymes immobilized on the surface of magnetic beads, which are in turn brought onto the electrode surface by means of magnets. In order to assess the response of the electrochemical instrumentation as well as the efficiency of the magnetic collection of microbeads, the HRP enzyme was covalently immobilized directly to the surface of magnetic microbeads following the same procedure described for the immobilization of the antigens. HRP-microbeads were placed into the electrochemical cell and different concentrations of hydrogen peroxide were added using hydroquinone as a redox mediator. As the hydrogen peroxide concentration was increased, it was also observed an increase in the measured current indicating that the kinetics for the enzymatic oxidation of hydrogen peroxide could be followed amperometrically with the electrochemical instrumentation of the EMBIA platform (Fig. 3). HRP enzyme immobilized onto microbeads showed a Michaelis-Menten kinetics, as can be observed in the linearized Lineweaver-Burk plot shown in the inset of Fig. 3. These results indicate that the enzyme immobilized onto the surface of magnetic microbeads exhibits a catalytic activity, which can be amperometrically measured and explained in terms of the Michaelis-Menten kinetics.

3.2. EMBIA platform for POC diagnosis of infectious diseases

As a proof of concept, the EMBIA platform was evaluated for the diagnosis of four infectious diseases caused by different types of microorganisms; Chagas disease caused by the parasitic protozoa *Trypanosoma cruzi*, human brucellosis caused by bacteria of the genus *Brucella* (mainly *B. melitensis*, *B. abortus* and *B. suis*), bovine brucellosis caused by *B. abortus* and foot-and-mouth disease (FMD) caused by the foot-and-mouth disease virus (FMDV). Magnetic beads coated with the corresponding recombinant antigens were incubated with a set of negative and positive control serum samples and bound antibodies were detected by the addition of anti-human or anti-bovine IgG HRP-conjugated antibodies. Peroxidase activity was electrochemically detected with the electrochemical cells-electrode cartridges and the portable potentiostat using H_2O_2 and hydroquinone as the substrate and redox mediator, respectively. The electrode potential was set at -0.23 V and the resulting current was recorded

during 20s. As shown in Fig. 4, characteristic time-current curves for positive and negative control sera were obtained. The current values for negative sera were typically around 900 nA, similar to the values obtained with PBST used as diluent of the samples, while the current values for positive sera were around 3000 and 4000 nA. These results indicate that the differences in current values at the limit current obtained at 20 s can be used to clearly differentiate positive from negative sera.

In order to demonstrate the usefulness of the EMBIA platform for diagnosis of infectious diseases, a panel of previously characterized human and bovine serum samples were analyzed (see Materials and methods). The obtained reactivity values were outlined in dotplot diagrams (Fig. 5). For the negative and positive samples the reactivity values showed no or a minimal overlap between the two sets of samples ($P < 0.0001$). In the case of human and bovine brucellosis, the results were in complete agreement with those obtained using the same antigen (OAg-AcrA) but in different platforms such as the fluorescent immunomagnetic assay and the indirect ELISA (glyco-iELISA) (Ciocchini et al. 2013; Ciocchini et al. 2014).

Taken together, these results demonstrate the potential of the EMBIA platform for PoC diagnosis of human and animal infectious diseases with an excellent signal-to-noise ratio and allowing to clearly differentiating infected from non-infected individuals or animals.

3.3. Validation of the EMBIA platform for diagnosis of Chagas disease

To validate the EMBIA platform for the diagnosis of Chagas disease, a large panel of previously characterized serum samples was tested and its diagnostic performance was compared with the fluorescent immunomagnetic and indirect ELISA assays using the same combination of antigens. The reactivity values obtained with the EMBIA, fluorescent immunomagnetic assay and iELISA are outlined in dotplot diagrams shown in Fig. 6 A, B and C (upper panel). For the negative and positive samples, the reactivity values of all the assays showed a minimal overlap between the two sets of samples ($P < 0.0001$). These results indicate that using the same combination of antigens and different diagnostic platforms (EMBIA, fluorescent immunomagnetic assay and iELISA) it is possible to discriminate between chronic Chagas disease patients and healthy individuals.

In order to evaluate the diagnostic performance of the EMBIA, a receiver-operating characteristic (ROC) analysis was performed. Based on the ROC results, the area under the ROC curve (AUC) values were 0.9864 (95% CI, 0.9691 to 1.000), 0.9991 (95% CI, 0.9975 to 1.000) and 0.9997 (95% CI, 0.9988 to 1.000) for the EMBIA, fluorescent immunomagnetic assay and iELISA, respectively (Fig. 6 A, B and C, middle panel; and Table 1). These results indicate that the EMBIA platform is highly accurate for the diagnosis of Chagas disease with a diagnostic

performance similar to that of the fluorescent immunomagnetic assay and the iELISA since no significant difference between the AUC values were observed (EMBIA vs. fluorescent immunomagnetic assay, $P = 0.152$; EMBIA vs. iELISA, $P = 0.132$, fluorescent immunomagnetic assay vs. iELISA, $P = 0.523$). Furthermore, a plot of the diagnostic sensitivity (Se) and specificity (Sp) as a function of the cut-off values (TG-ROC plot) was performed for each assay in order to select different cut-off points so that the desired operating characteristics of the test in terms of diagnostic Se and Sp can be adjusted. This analysis allowed us to determine for each assay the cut-off value that concurrently maximizes Se and Sp, the cut-off value for which the diagnostic Se is 100% and the cut-off value that allows a 100% Sp (Fig. 6 A, B and C, lower panel; and Table 1). With the EMBIA platform, high values of diagnostic Se and Sp at the selected cut-off values were obtained. A cut-off value of 25.9% resulted in a diagnostic Se of 100% (95% CI, 92.6 to 100) and a Sp of 71.7% (95% CI, 56.5 to 84.0). At the cut-off value of 37.5%, for which the J index was maximal ($J = 0.94$), the diagnostic Se was 93.7% (95%CI, 82.8 to 98.7) and the Sp 100% (95% CI, 92.3 to 100). These values were similar to those obtain with the fluorescent immunomagnetic and iELISA assays (Table 1). Taken together, our results demonstrate that the EMBIA platform is highly accurate for the *in situ* diagnosis of Chagas disease displaying a diagnostic performance similar to that obtained with the fluorescent immunomagnetic assay and the classical ELISA platform using the same combination of recombinant antigens.

Different technologies were combined and integrated in the EMBIA platform including microelectronics, electrochemistry, the use of magnetic beads and biotechnology, among others. Microelectronics and electrochemical transducers allow the development of small size, low cost and robust biosensors with a high degree of integration. Additionally, magnetic microbeads were used in this platform as a solid-phase support for the antigens, which simplifies the assay since the incubations and removal of unwanted sample constituents can be performed by a simple magnetic separation step without the need of centrifugation and with a minimal loss of the target material. Furthermore, the use of microbeads allowed the reduction of the time of the assay and the volume of sample required for the analysis and even more importantly, to combine different antigens in a controlled and standardized manner as in the case for the diagnosis of Chagas disease. Finally, biotechnology, in particular genetic engineering and bacterial glycoengineering, has allowed us to produce and purify large amounts of highly specific recombinant antigens at relatively low cost. By combining these technologies, we were able to develop a portable, robust and low cost device for PoC diagnosis of multiple human and animal infectious diseases caused by different etiological agents such as parasitic protozoa, bacteria and viruses. Furthermore, we have performed a complete

validation of the EMBIA platform for the diagnosis of Chagas disease displaying an excellent diagnostic performance. Further work will be required to validate the EMBIA platform in the field and for the diagnosis of other infectious or non-infectious diseases as well as for the detection and/or quantification of biomarkers.

4. Conclusions

We have developed and evaluated an electrochemical magnetic beads-based biosensor (EMBIA platform) for PoC serodiagnosis of human (Chagas disease and human brucellosis) and animal (bovine brucellosis and FMD) infectious diseases caused by different types of microorganisms. The diagnostic performance of the EMBIA platform was compared to the well-established iELISA and a fluorescent immunomagnetic assay, displaying excellent results. Due to its portability, short incubation times, the possibility to perform 8 readings in less than 3 minutes (20 s per reading), simplicity and high accuracy, we propose that the EMBIA platform technology can be applied to PoC diagnosis of these and other infectious diseases at resource-constrained and primary care settings.

Supplementary data

Supplementary data associated with this article can be found in the online version.

Acknowledgements

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Figure legends

Figure 1. Schematic representation of the electrochemical enzyme-linked magnetic beads immunoassay and detection principle. Antigen-coated magnetic particles are incubated with the serum samples, washed and then incubated with HRP-conjugated secondary antibodies. After washing, the particles are magnetically collected and placed onto the surface of the electrode. Peroxidase activity is amperometrically recorded using an 8-channel portable potentiostat after adding H_2O_2 and hydroquinone as the substrate and redox mediator, respectively. All incubation and washing steps are performed in 8-tube strips using a magnetic rack without the need of centrifugation.

Figure 2. Portable electrochemical magnetic beads-based platform (EMBIA platform) for PoC diagnosis. (A) Portable 8-channel potentiostat device (nanoPoC). The device, developed to carry out measurements of potentiostatic steps and cyclic voltammetry, can be controlled via Bluetooth using a set of basic commands and is powered by a rechargeable battery. A special designed housing allows error-free cartridge insertion. (B) Acrylic disposable electrochemical cells-electrode cartridge. The disposable acrylic cartridges with eight electrochemical cells were designed and manufactured with dimensions fitting an 8-channel micropipette. (C) Electrodes. Each cell contained two carbon electrodes (working electrode and counter

electrode), and one Ag|AgCl reference electrode. The central working electrodes were designed to be aligned with neodymium magnets to allow the concentration of the magnetic particles on the surface of the electrode.

Figure 3. Current-potential curves obtained on screen-printed electrodes using magnetic particles with immobilized HRP at a scan rate of 25 mV s^{-1} . Different concentrations of hydrogen peroxide have been used (from top to bottom: 0, 0.5, 1, 1.5 and 2 mM), while the concentration of the redox mediator hydroquinone was kept constant at 4 mM.

Figure 4. EMBIA platform current transients of positive and negative sera. Representative time-current curves for positive and negative serum samples used as control sera for diagnosis of Chagas disease (A), human brucellosis (B), bovine brucellosis (C) and foot-and-mouth disease (FMD) (D). The electrode potential was set at -0.23 V and the resulting current recorded for 20 s. Current values for negative sera were typically around 900 nA similar to the values obtained with 0.01 M phosphate-buffered saline pH 7.2, 0.2% Tween 20 (PBST) used to dilute the samples (1:100 dilution).

Figure 5. Dot plot analysis of the results obtained with the EMBIA platform for diagnosis of Chagas disease, human and bovine brucellosis and foot-and-mouth disease (FMD). Magnetic beads coated with the corresponding antigens were incubated with negative and positive sera for the diagnosis of Chagas disease (A), human brucellosis (B), bovine brucellosis (C) and FMD (D). Bound antibodies were detected using HRP-conjugated goat anti-human or anti-bovine IgG antibodies. The mean and standard deviation for each group are indicated. ***, $P < 0.0001$, Mann-Whitney test.

Figure 6. Validation of the EMBIA platform for diagnosis of Chagas disease and comparison with the fluorescent immunomagnetic assay and iELISA. A) Dot plot, ROC and TG-ROC analysis of the results obtained with the EMBIA platform. Magnetic beads coated with Ag1, Ag36, SAPA or TSSA (Chagas antigens) were mixed in equal parts and 20 μl of the mixture were incubated with the indicated human serum samples (46 negative and 48 positive sera). Bound antibodies were detected using HRP conjugated goat anti-human IgG antibodies and measuring the electric current generated after adding the substrate. B) Dot plot, ROC and TG-ROC analysis of the results obtained with the fluorescent immunomagnetic assay. Magnetic beads coated with the Chagas antigens were incubated with the indicated serum samples (122 negative and 74 positive sera). Bound antibodies were detected using DyLight 650-conjugated goat anti-human

IgG antibodies and directly reading the fluorescence. C) Dot plot, ROC and TG-ROC analysis of the results obtained with the iELISA. ELISA plates coated with a mixture of Ag1, Ag36, SAPA and TSSA were incubated with the indicated serum samples (122 negative and 72 positive serum samples). Bound antibodies were detected using HRP-conjugated goat anti-human IgG antibodies. Results are expressed as percentage of the reactivity of the positive control serum included in each assay. The mean and standard deviation for each group are indicated. ***, $P < 0.0001$, Mann-Whitney test. AUC, area under the ROC curve; values in parentheses indicate the 95% confidence interval. The dash vertical lines in the TG-ROC curves indicate the cut-off values for which maximal Se or Sp is achieved.

Supplementary figure legends

Figure S1. Developed software diagram: the host and the PoC device communicates wirelessly using Bluetooth. The host uses Android OS, where the nanoPOC application is running. The User Interface Activity communicates with the user and translates its commands into a plain text protocol. The Communication Class sends and receives commands to/from the PoC device using Android's Bluetooth API.

Highlights

- Development of an electrochemical magnetic beads-based biosensor (EMBIA) for PoC diagnosis.
- EMBIA platform combines microelectronics, electrochemistry, the use of magnetic beads and biotechnology.
- Validated for serodiagnosis of human and animals infections caused by parasitic protozoa, bacteria and virus.
- Displayed an excellent diagnostic performance comparable to the well-established iELISA and a fluorescent immunomagnetic assay.

Tables

Table 1. AUC values, sensitivity, specificity and Youden's index of the tests calculated for different cut-off values^a.

Assay ^b	AUC ^c	Cut-off (%)	Se (%) ^d	Sp (%) ^d	J ^e
EMBIA	0.9864	> 25.9	100 ^f (92.6-100)	71.7 (56.5-84.0)	0.72
	(0.9691-1.000)	> 37.5	93.7 (82.8-98.7)	100 (92.3-100)	0.94
Fluorescent immunomagnetic assay	0.9991	> 24.9	100 (95.1-100)	95.1 (89.6-98.2)	0.95
	(0.9975-1.000)	> 29.9	97.3(90.6-99.7)	100 (97.0-100)	0.97
iELISA	0.9997	> 17.9	100 (95.0-100)	98.3 (94.1-99.8)	0.98
	(0.9989-1.000)	> 28.4	97.2 (90.3-99.7)	100 (96.9-100)	0.97

^aThe analysis was performed using serum samples obtained from patients with Chagas disease and healthy blood donors (see Materials and methods).

^bEMBIA, electrochemical enzyme-linked magnetic beads immunoassay.

^cAUC, area under the ROC curve. Values in parentheses indicate the 95% confidence interval.

^dSe (%), sensitivity (TP/TP+FN) x 100; Sp (%), specificity (TN/TN+FP) x 100. Values in parentheses indicate the 95% confidence interval.

^eJ, Youden's index (Se + Sp – 1).

^fMaximum values for Se, Sp and J are indicated in bold.

Figure 1

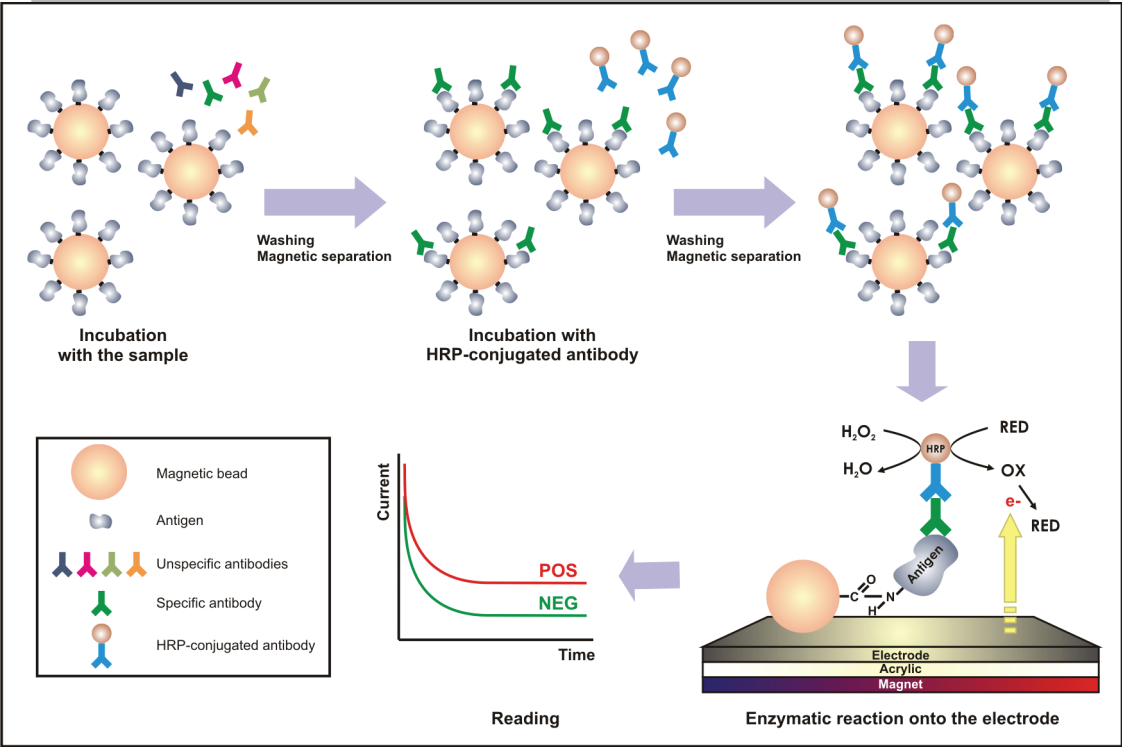


Figure 1

Figure 2

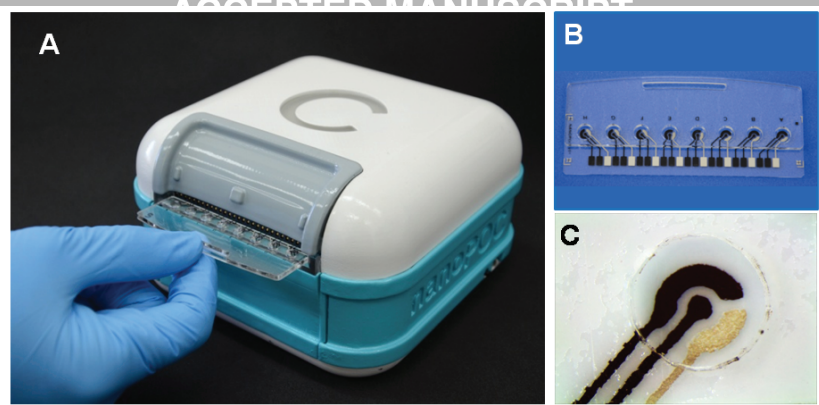


Figure 2

Figure 3

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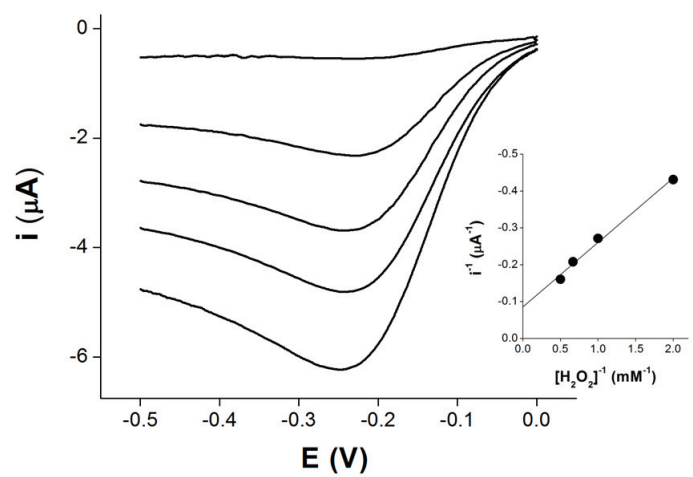


Figure 3

Figure 4

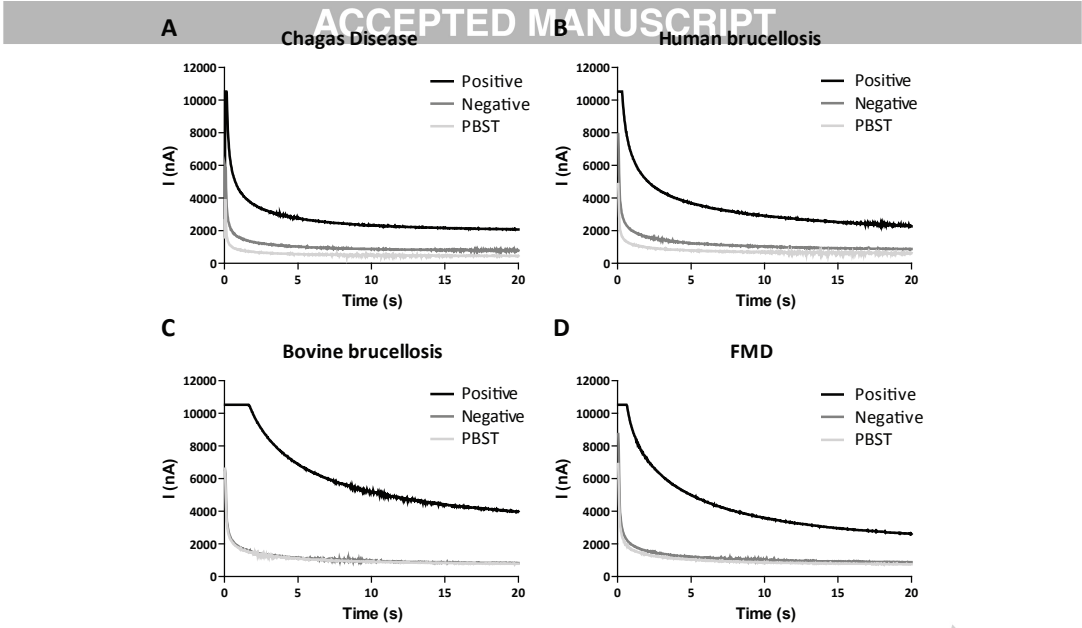


Figure 4

Figure 5

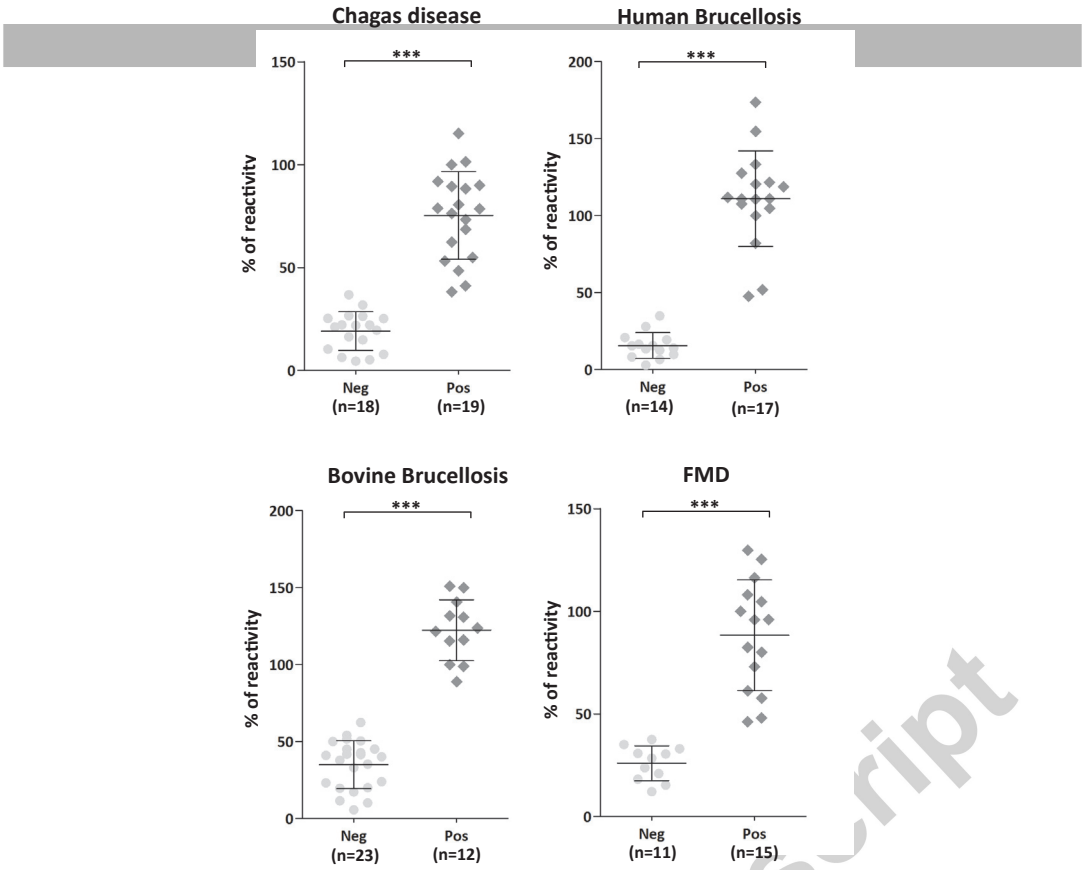


Figure 5

Figure 6

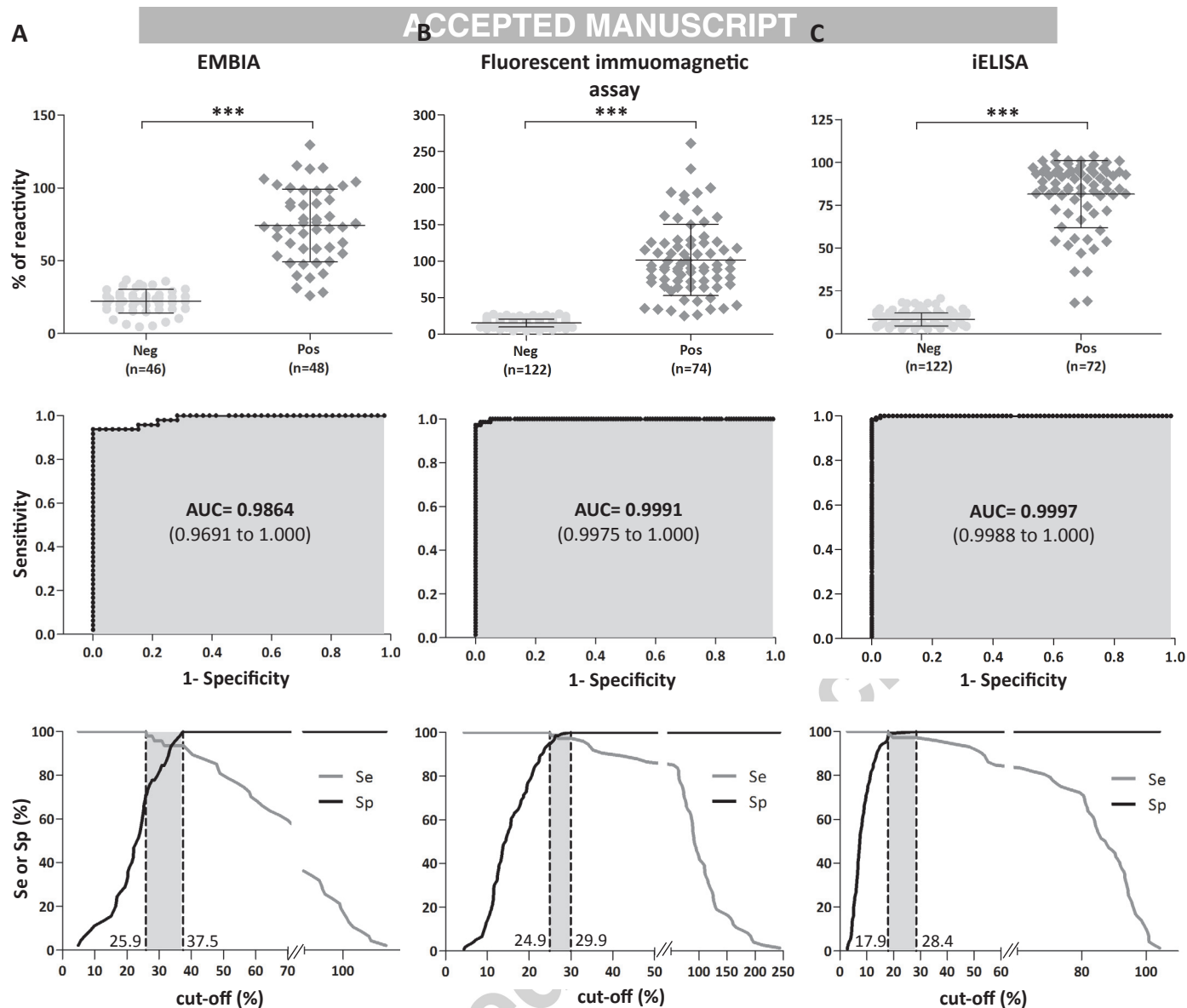


Figure 6