

Sensing parasites: Proteomic and advanced bio-detection alternatives



Carlos Sánchez-Ovejero ^a, Fernando Benito-Lopez ^b, Paula Díez ^{c,d}, Adriano Casulli ^e, Mar Siles-Lucas ^a, Manuel Fuentes ^{c,d,*}, Raúl Manzano-Román ^{a,**}

^a Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), 37008 Salamanca, Spain

^b Analytical Chemistry Department, Universidad del País Vasco UPV/EHU, 01006 Vitoria-Gasteiz, Spain

^c Department of Medicine and General Cytometry Service-Nucleus, Cancer Research Centre (IBMCC/CSIC/USAL/IBSAL), 37007 Salamanca, Spain

^d Proteomics Unit, Cancer Research Centre (IBMCC/CSIC/USAL/IBSAL), 37007 Salamanca, Spain

^e Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, - 00161 Rome, Italy

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ABSTRACT

Parasitic diseases have a great impact in human and animal health. The gold standard for the diagnosis of the majority of parasitic infections is still conventional microscopy, which presents important limitations in terms of sensitivity and specificity and commonly requires highly trained technicians. More accurate molecular-based diagnostic tools are needed for the implementation of early detection, effective treatments and massive screenings with high-throughput capacities. In this respect, sensitive and affordable devices could greatly impact on sustainable control programmes which exist against parasitic diseases, especially in low income settings.

Proteomics and nanotechnology approaches are valuable tools for sensing pathogens and host alteration signatures within microfluidic detection platforms. These new devices might provide novel solutions to fight parasitic diseases. Newly described specific parasite derived products with immune-modulatory properties have been postulated as the best candidates for the early and accurate detection of parasitic infections as well as for the blockage of parasite development.

This review provides the most recent methodological and technological advances with great potential for bio-sensing parasites in their hosts, showing the newest opportunities offered by modern “-omics” and platforms for parasite detection and control.

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* Correspondence to: M. Fuentes, Department of Medicine and General Cytometry Service-Nucleus, Cancer Research Centre (IBMCC/CSIC/USAL/IBSAL), Avda. Universidad de Coimbra S/N, 37007 Salamanca, Spain.

** Correspondence to: R. Manzano-Román, Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain.

E-mail addresses: mfuentes@usal.es (M. Fuentes), raul.manzano@irnasa.csic.es (R. Manzano-Román).

1. Introduction

Parasitic diseases represent particular challenges for human and animal health mainly in developing countries. They are strongly associated with poverty causing a considerable health and economic impact, especially when considering co-introduced and co-invading parasites [1,2]. Moreover, parasitic infections can be found worldwide and can be potentially introduced from endemic to non-endemic areas mainly due to human and animal population movements and climate change, resulting in their emergence and re-emergence [3,4]. Neglected parasitic infections are very prevalent especially among children and immunocompromised hosts – even in developed countries –, causing high morbidity and mortality rates [5,6]. The lack of appropriate diagnostic tools for many of these neglected diseases, combined with their lack of appropriate sensitivity and/or specificity, makes the investigation on new type of detection devices a must.

Zoonotic parasites are also the cause of substantive economic losses in livestock populations [2]. In this sense, the interest in understanding disease transmission among wild and domestic animals, and between them and human population has grown, resulting in the emergence of the “one health approach”, which aims to model the transmission of parasitic diseases [7,8]. Detection and transmission control constitute significant components to the overall management of many pathogen infections (including parasites). Moreover, the rapid diagnosis of many complex parasitic diseases with on time treatments and tailored control measures is essential to avoid sequelae, comorbidity and economic losses.

Drug treatment remains as the principal approach for the control of parasites in animals; however, parasites have shown molecular resistance mechanisms hampering this strategy. Successful and sustainable control strategies depend on the development of new tools for targeting both parasites/hosts and vectors [9]. To this end, applying new knowledge and techniques in combination with updated mass drug administration programmes is essential.

The diagnosis of the most health impacting parasites is often cumbersome, where current diagnostic tests for important zoonotic parasitic diseases can provide incorrect results and so lead to unforeseen consequences [10]. Therefore, there is a much-need for rapid, simple, sensitive, and affordable diagnostic tests to improve disease control and patient management, mainly in poor-resource settings where diseases like malaria or human sleeping sickness are endemic. In these scenarios, many people do not have access to laboratory facilities and many barriers technologies, costs and expertise lead to the necessity of incorporate point of care (POC) tests which offer flexibility, reliability and robustness for both case detection and large population screenings [11].

Outside medical or veterinarian laboratories, rapid on-site diagnosis is very important in order to prevent and manage outbreaks and to apply appropriate prophylaxis, treatment and control programmes [12]. In this sense, biosensor-based tools developed for the diagnosis of pathogens are an emerging issue (131 PubMed papers from 2010 when searching “biosensor diagnosis pathogens” on December 2015) and ultimately a growing area of concern when referring to the diagnosis of parasitic infections (38 PubMed papers from 2010 when searching “biosensor diagnosis parasites” on December 2015).

The main goal of this emerging area of knowledge is to develop devices with multiplex capabilities as suitable screening methods for the detection of several parasites and their corresponding vectors. Despite the recent advances in bio-sensor technology for infectious and parasitic diseases, they still remain as one of the major causes of mortality and morbidity throughout the world. This review presents current examples and perspectives for integrating modern sensing technologies for the detection of parasitic diseases.

2. Predictive candidates to detect parasitic diseases

Candidate biomarkers for the diagnosis of parasitic diseases must be identified in differentially expressed molecules between healthy

subjects and infected patients. The first step towards an effective treatment of parasitic infections is an early detection and later differentiation of the disease progression and/or recurrences over time. Having into account the variations of the individual immune responses to the infection, the identification of candidate biomarkers is a more appropriate approach for reliable and specific parasite identifications than defining a single candidate. Thus, a combination of key hosts and parasite molecules is ideal to be included in target panels with the aim of achieving low overlapping between different diseases, and therefore, facilitate an integrated diagnosis.

Protein–protein interactions at the host–parasite interplay are highly relevant in the context of modifying protein expression levels and inducing protein expression. Identifying these alterations in host protein profiles during infection could facilitate the understanding of disease pathogenesis, host immune response, and identification of potential protein markers for the detection and prognosis of the disease. In this sense, parasitic infections may lead to highly relevant alterations on the level and expression of multiple serum proteins involved in essential physiological pathways (*i.e.* lipid-binding proteins) as well as in the change of very specific host molecules (*i.e.* the host erythrocyte membrane proteins during malaria infection) [13,14].

Investigating the metabolic consequences of parasitic infections in the host is now feasible based on modern technological advances. Experimental infections with single or multiple parasite species may allow the discovery of specific or common biomarkers although they should be further validated in free-living populations [15]. To this end, for example, it has been shown by serum proteomics of *Eimeria* sp. infected chickens that proteins usually not detected in the blood like those associated with mitochondrial metabolism are good candidates for time-course studies in coccidiosis. However, the host genetic background leading to different levels of susceptibility is a key factor in the alteration profile of proteins [16].

Regarding parasite derived molecules, the strategy to detect circulating antigens has the potential to discriminate active from past infections. Assessment of antigens during a given infection and specifically the excreted–secreted or surface-exposed parasite proteins should be the best for an accurate immunodiagnostic procedure. The study of the surfome in parasites is revealing novel molecular targets for specific diagnosis. Many of these molecules are represented by glycoproteins located at the extracellular region attached to the plasma membrane, although antigenic variability at this level makes the selection of molecules cumbersome [17,18]. This highlights the importance of comparative surfome analysis to increase the chance to find specific targets in parasites, leading to differential surface markers useful to avoid potential misdiagnosis like for example of Chagas' disease [17]. In addition, the potential detection of fine post-translational modifications creating neo-epitopes during specific parasitic disease pathogenesis could lead to monitor disease activity, like in many human diseases [19].

Epitope mapping studies aimed to identify unique diagnostic molecules from polymorphic immune-dominant antigens in parasites using computational methods are also a promising area [20]. For instance, the identification of many novel epitopes with diagnostic potential has been proved for the protozoan *Trypanosoma cruzi* [21]. Moreover, the detection of new diagnostic epitopes from circulating antigens is also a practical diagnostic strategy.

Some of the putative new protein biomarkers to detect parasites are likely to be present in biological fluids at extremely low concentrations and protected inside secreted microvesicles. Microvesicle-based secretion seems to be a general mechanism for protein secretion by protozoan parasites. It is well reported that the biomolecular cargo (*i.e.* proteins, lipids, nucleic acids) inside these microvesicles is involved in signalling for parasite infection and its survival therefore it could be also exploited in disease treatments [22,23]. Since extracellular vesicles are highly immunogenic, they can be considered as suitable candidates to detect parasitic diseases [23].

3. Proteomic platforms for the identification of parasite biomarkers

Parasites have complex life cycles and redundancy molecules can be found in many infectious processes, posing additional difficulties for their specific identification by classical biochemical approaches. Additionally, parasitic infections may alter the metabolic activities in their host being these alterations the basis of metabolic fingerprint approaches for understanding the metabolic consequences of the infections. Therefore, these could be considered as a source for novel diagnostic or prognostic biomarkers [15]. Nowadays, modern -omic technologies are offering high-throughput strategies for such a difficult system biology exploration, with the essential support of novel bioinformatic tools [24,25].

The identification of biomarkers of parasitic infections by differential protein profiling of specific hosts or parasitic molecules is a promising area; mainly based on induced changes by parasites at post-translational modifications, peptide degradations or protein variants [26]. Tissues (human and animal) and proximal bio-fluids could contain molecular information on the physiological and pathological state of the organism since these tissues and bio-fluids are the route and destination of parasites and/or their secretions. The proteomic characterisation of proximal bio-fluids may provide useful and comprehensive information for diagnostic, prognostic and predictive biomarkers. The challenge is the detection of very low abundant protein circulating biomarkers – this kind of molecules provides a greater amount of downstream information content than nucleic acids – that may be essential for early diagnosis [27].

3.1. The potential of mass spectrometry

Briefly, mass spectrometry (MS) is a robust, versatile and sensitive analytical technology allowing high-throughput detection with mass accuracy, precise quantitation and verification of protein variants, splice isoforms, metabolites and disease-specific post-translational modifications from tissues, body fluids or cell cultures [28,29]. Highly sensitive technologies and high-throughput systems – like MS – are promising tools for novel biomarkers since MS allows characterisation of the fine tune changes in proteins, – including differences at population level – [30].

Commonly, MS-based proteomics approaches are a suitable resource to identify biomarkers for the detection of parasites as the main technique or coupled with other proteomic and conventional biochemical techniques. For instance, this is the basis for the identification of potential malaria markers in patient's sera and recently applied to identify specie-specific proteins in infected patients [31,32].

De Bock and colleagues highlighted the potential of MS-based technologies (*i.e.* surface-enhanced laser desorption/ionisation time of flight-MS, SELDI-TOF-MS) as research tools to interrogate protein-based biomarkers in parasitic diseases. In general, SELDI-TOF determines differential specific protein profiles (in particular, low molecular-weight molecules) in proximal bio-fluids. The above-mentioned technology has also been applied in parasitic diseases by sensing unusually truncated host proteins in response to *T. cruzi* infection with high sensitivity and specificity [33]. Moreover, matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) is a valuable diagnostic technique already applied for detecting and differentiating *Entamoeba* spp or *Babesia canis canis* infections, and becoming a miniaturised bioanalytical tool for detecting and discerning proteins from bio-complex samples [34–36]. MALDI-MS has applications in biomarker discovery, pathogen identification and has great potential for lipid-based biomarker sensing [37,38]. For the identification of vectors like mosquitoes, MALDI-TOF allows accurate identifications which are important to elucidate their role as vectors. Importantly, the technique has also the potential for rapid in one-shot dual identifications (vectors and pathogens) [39,40]. The utility of MS in routine analyses to point out markers of parasitic infections in bio-fluids and tissues was proved to be effective

in many recent publications (Table 1), but still needs to be broadly used for research and clinical diagnosis. Many pre-analytical protocols during parasite sample preparation must focus on maximising the number of protein identifications on perform highly reproducible assays.

Other MS-based techniques, not predominately applied in diagnostic parasitology, but that hold great potential for the development of this area are:

- (i) MS imaging is an analytical tool providing information on the spatial distribution and relative abundance of biomolecules in tissues [37]. Its power depends on an unbiased preliminary knowledge of molecular identities required, its ability to distinguish between diseases with similar histological characteristics, the different type of molecules detected, the quality of tissue preservation/conservation and the unique applications for clinical diagnostics [58]. It would probably be advantageous to correlate molecules and pathogenesis of impacting diseases like severe malaria, severe hepatosplenic schistosomiasis and cutaneous forms of leishmaniasis.
- (ii) An emerging technology for pathogen detection is PCR-electrospray ionisation MS, a versatile technique to characterise multiple strains and organisms having high potential to identify tick-borne pathogens [59,60].
- (iii) Multiple reaction monitoring mass spectrometric assays can be used in order to quantify multiple protein isoforms, specific proteins or modified peptides [61]. This technique requires enriched samples to accurately analyse modified biomolecules like lipopolysaccharides, glyco-sphingolipids and glycoproteins, which are highly relevant antigens of parasites [62].
- (iv) Direct identification of compounds originated from pathogens physiology/metabolism within hosts can be done by metabolomic approaches [63]. Metabolomic profiles based on MS strategies detecting variations in differential metabolic signatures in cells, tissues or body fluids, may open a way for sensing parasites. When trying to detect and understand host or parasite derived metabolites at low concentration, capillary electrophoresis MS with electrospray ionisation and gas-chromatography MS offer valuable discriminatory power [45,64,65]. It could be also applied to time-course and accumulation of drug metabolites after treatment of parasitic diseases.

3.2. Nanoproteomics

Nanoproteomics is a new “-omic” emerging discipline coming from the advances and integration of nanotechnologies and proteomics, useful for rapid diagnostic screening at nanometre scale in the clinical practise. Nanotechnology has the potential to minimise most of the problems related to the proteomic technologies (*i.e.* complexity, protein modifications) providing advantages at the detection level and allowing new methodological approaches such as multiplexing thus opening new biologically relevant insights [66].

Multiplexing is an important feature that may facilitate the simultaneous study of different parameters within a unique platform. Protein and glycan microarrays, bead arrays or 3D printings are some of the methodologies included in this field within on-demand diagnostics platforms. The use of dynamic sensing arrays based on micron and/or nano-sized beads allows increasing the level of multiplexing with the potential to be adaptable in microfluidic devices for immunoassays [67]. Protein microarrays are miniaturised immunoassays in an array format which allows the study of thousands of proteins simultaneously, offering the opportunity to perform high-throughput screening for new biomarkers of infectious and parasitic diseases at the global level [68, 69]. Peptide chips could be a good strategy for the identification of potential immunodominant antigens and for epitopes description [70]. Biochips immuno-like polymer membranes for POC sensing of proteins in serum samples are also an interesting field [71]. In fact, novel single-

Table 1
New potential biomarkers identified in host–parasite models found by mass spectrometry techniques.

Disease/Parasite	Biomarkers	Source	Method	Features/Details	References
<i>Trypanosoma cruzi</i>	C3a anaphylatoxin, apolipoprotein A fibronectin	Serum	Mass spectrometric profiling by SELDI-TOF	Useful for subjects with latent Chagas disease	[33]
<i>T. rangeli</i>	<i>T. rangeli</i> GP63-related protein and an FcBP protein	Serum	MS/MS analysis	Proteins from trypomastigote surform	[17]
<i>T. brucei gambiense</i>	Osteopontin and β -2-microglobulin	Cerebrospinal fluid	MALDI-TOF-TOF MS	Good candidates for the development of a test for staging patients	[41]
<i>Schistosoma mansoni</i>	Fuco-oligosaccharides that are produced by schistosome eggs	Urine	MALDI-TOF	Oligosaccharides detected in infection urine are shown to be excreted by live eggs and not by worms	[42]
	Phenyl acetyl glycine (PAG)		UPLC-MS	PAG is a statistically discriminant metabolite which can provide important information regarding the disease	[43]
<i>Plasmodium falciparum</i>	Metabolites between supernatants from <i>Plasmodium</i> : 3-methylindole, succinylacetone, S-methyl-L-thiocitrulline, O-arachidonoyl glycidol	In vitro cultured samples	High-resolution metabolomics (HRM): C18 liquid chromatography coupled with Fourier-Transform Mass Spectrometry (FTMS)	Low molecular-weight metabolites for future development of non-invasive malaria diagnostic tools	[44]
	Urinary metabolites: UK1, UK	Urine	Nuclear magnetic resonance (NMR) profiling followed by LC-MS	Two unique structurally related urinary candidate biomarkers have not been described so far in the eukaryotic organism.	[45]
<i>P. berghei</i>	Proteins: cE5, B3VDI9_ANOGA, and AGAP008216-PA	Salivary gland samples from <i>Anopheles gambiae</i>	SELDI-TOF-MS	Markers involved in blood feeding	[46]
<i>Echinococcus</i> spp.	Antigen B-related molecules (EgAgB; EgAgB1-5)	Sera	MALDI-TOF-MS	Immunodominant epitopes changed as the disease progresses	[47]
<i>Giardia lamblia</i>	Encystation-specific vesicles (ESVs) and endocytic organelles termed peripheral vesicles (PVs)	Microsome fractions derived from trophozoites	Combining flow cytometry-based organelle sorting with In silico filtration of mass spectrometry data	Proteins from secretory and endocytic organelles	[48]
<i>Entamoeba histolytica</i>	Cyst-wall specific glycoproteins Jacob, Jessie and chitinase	Faecal specimens	LC-MS/MS mass spectrometer	Promise as diagnostic targets	[49]
Eimeria/Coccidiosis	Proteins from the neck region (rhoptry neck proteins, RON): RON2L1, RON2L2	Serum	Gel LC-MS/MS	From enriched rhoptry fractions isolated from the sporozoite stage	[50]
<i>Toxoplasma gondii</i>	Excretory–secretory antigen (ESA): microneme protein 10 and dense granule protein 7, phosphoglycerate mutase 1	Serum	MALDI-TOF-TOF	From tachyzoites	[51]
<i>Trichinella spiralis</i>	Excretory–secretory (ES) proteins of muscle larvae (ML)	Serum	MALDI-TOF	Come mainly from the excretory granules of the stichosome and the cuticles membrane	[52]
Porcine cysticercosis (<i>Taenia solium</i>)	Clusterin, lecithin-cholesterol acyltransferase, vitronectin, haptoglobin and apolipoprotein A-I	Serum	SELDI-TOF technology	Detection of viable cysts (active disease)	[53]
<i>Leishmania infantum</i>	Profile of the volatile organic (VOCs) emitted	Hair sample	Solid-phase microextraction (SPME) combined with gas chromatography–mass spectrometry (GC-MS)	Significant variations between healthy dogs (G1) and infected dogs (G2 + G3).	[54]
	Proteins Li-isd1, Li-txn1, Li-ntf2	Urine	RP-HPLC-MS	To distinguish active visceral leishmaniasis from asymptomatic infection	[55]
<i>Trichomonas vaginalis</i>	Papain-like (TvCP2, TvCP4, TvCP4-like, TvCPT), and one legumain-like (TvLEGU-1) cysteine proteinases (CPs)	Serum	MALDI-MS and ESI-LC-MS/MS	Antigen cocktail of recombinant proteinases that could increase the sensitivity and specificity for the immunodiagnosis	[56]
<i>Teladorsagia circumcincta</i> (parasitic nematode)	Gelsolin, α -1 b glycoprotein and haemopexin	Lymph formed from the interstitial fluid (sheeps)	MALDI-TOF and MS/MS analyses	The proteomic study of lymph has the potential to give new insights into local responses to infection	[57]

domain antibodies (*i.e.* scFvs, nanobodies), may offer interesting advantages over monoclonal antibodies if applied to these methodologies [72,73].

Glycan microarrays are also powerful tools for biomarker discovery. These microarrays have the potential to identify carbohydrate antigens and improve the serodiagnosis of different parasitic infections, although advanced methods for the synthesis, isolation, and characterisation of glycans are still required [74–76]. The diagnostic potential of glycans found in *Leishmania*, *Schistosoma* and *Trichinella* parasites points out their relevance.

3.3. Subcellular proteomics

The analysis of the composition of particular purified cellular compartments is gaining importance in diagnostic research. Extracellular vesicles represent a promising source of circulating biomarker cargo encapsulated in a lipid coat. Among them, exosomes, 40–100 nm in size vesicles, which are important in inter-cellular communications, play critical roles in many biological functions. Exosome surface protein contents can be a rich source of biomarkers in blood samples and proximal fluids. These vesicles could have a high potential as optimal diagnostic

targets based on the differential display of specific exosomal protein markers. Exosome-MS protein libraries prepared by either the shave – for surface protein exosomal markers – or complete exosome material, produce ideal peptide libraries for MS/MS analysis.

There are accumulating evidences of the release of intercommunicating extracellular vesicles in parasitic diseases, being an integral part of the parasite's infectious life cycle [77,78]. The potential that proteomics, especially MS, shows for sensing and deciphering the cargo of these parasite or host secreted biomarker enriched microvesicles is very high in the field of parasitic diseases diagnosis [79]. The detection of parasitic derived microvesicles would be highly valuable for example to diagnose and guide management of chronic and complex asymptomatic diseases, such as cystic echinococcosis or in vector transmitted infections like Leishmaniasis, in which exosomes are newly identified virulence factors [78].

Molecules in the exosome cargo like the microRNAs have also great potential for the diagnosis of parasitic diseases [80]. Another type of analytes that could be studied in the exosome space, e.g. lipids, might represent good biomarkers to investigate in the near future. All these provide an important base to continue researching in parasite derived exosomes as diagnostic targets and demonstrating their utility as clinical biomarkers.

4. Parasite testing: biosensor-based platforms

There is an important demand in parasitic disease diagnostics for portable and highly sensitive systems. Novel detection platforms have the potential to develop robust, multiplexed ultrasensitive protein detection devices with high efficiency, high data quality, and cost-effectiveness for the identification of pathogens and disease biomarkers in both well-equipped and/or limited clinical facilities [81].

Biosensors are also new molecular technologies that attempt to overcome many of the detection limits due to the low abundance of key biomarkers [82]. Some of these technologies have impressively improved sensitivity compared to conventional immunoassay approaches [83]. However, few biosensors have been developed and commercialised for the detection of infectious or non-infectious diseases, but none of them for parasitic diseases.

Biosensors can be used as POC devices to detect host and parasite virulence and specific biomarkers, being good alternatives to current standard methods [84]. These biomarkers can be transferred to a biosensor format for multiplexed ultrasensitive sensing directly from proximal body fluids, enabling simultaneous detection of specific, to several evolutive forms, antibodies. This biomarker serves as revolutionary POC diagnostic technology to improve the prevention, control, and treatment of parasitic diseases. The inclusion of species-specific markers for differential diagnosis and polymorphism detections would directly impact sensing and help to understand parasites. The integration of high-throughput -omic data, which is relevant to essential host-parasite interaction networks, within these biomarkers appears as the most accurate scenario to understand and guarantee infection status.

These devices have the potential to identify single molecules without the use of microscopes and moreover the same device could potentially be used for several different tests for biomarkers and bio-particles, providing an interesting alternative to standard tests like PCR [84]. This could be very advantageous in low-resource settings where people may not be well trained even in standard test and few technologies are well-established at a POC level, or where the conditions are not appropriated for analysis. These devices will be particularly useful to detect invasive infections or to prevent further disease spread in populations that still rely in conventional parasitological techniques.

Advanced developments for onsite diagnosis could come from single cell proteomic studies with a microfluidic antibody capture chip platform, able to detect target markers in real clinical practise [85]. However, the impact of microfluidics on interesting markers like for example

exosomes is still small. Therefore, interfacing biosensors with MS is leading to the high-resolution identification of macromolecules and thus, search for parasitic targets [86]. The interrogation of protein biomarkers in specific cell types and during defined periods in hosts constitutes a potential emerging area of research to provide key advances in the field of parasitology. This could be especially relevant for understanding (i) anti-parasite immune responses in different clinical forms of parasitic diseases (i.e. Chagas disease) and their role driving the development of this disease and (ii) the systemic impact of parasitic infections.

The development of specific methodologies is still required to accomplish and integrate all the above technologies in a high quality and robust sensing device. The ultimate advances in bio-fabrication techniques allow creating biosensors with living cells in 3D to more closely model the *in vivo* cell environment, having these devices the potential to combine bio-sensing and therapeutic treatments [87]. The implementation of these tests on clinical applications might positively influence animal and human clinical management as well as significantly reduce costs.

4.1. The potential of biosensors to detect and diagnose parasites

The diagnosis of many parasitic diseases relies on showing parasites in tissue samples and on standard tests, nevertheless there are still gaps in the diagnosis of well know parasites or even in distinguishing between species and subspecies [88] (Table 2). The lack of diagnostic tests influences decisively health care decisions. In general, parasitological techniques oriented to detection by microscopic examination have many disadvantages such as: they are mainly invasive, leads to misdiagnosis, require expert microscopists, they are time consuming and lacking of accuracy [109]. Moreover, many rapid diagnostic tests lack accuracy, validation, or both. At the hospital level, but even more in the field, there is an increasing demand for better diagnostic tests to detect parasitic diseases at an early stage.

The pathogenicity and life cycles of parasites are highly complex and governed by the parasite–host interactions. The trend is, instead of testing for a single biomarker, to identify the panel of biomarkers that together may be better predictors of clinically relevant parasites towards efficient decision-making in individual POC settings. As commented previously, MS and proteomic techniques can assist as standard tools for the identification of infection induced up and down regulations that may serve as specific protein biomarkers. Moreover, host-derived metabolic and specific pathogenicity sensors would also play a role modulating parasitic disease progression to ensure survival and long-term persistence [110,111].

In this sense, the infection and persistence by protozoan parasites is associated with changes in host tissue protein composition, highlighting that both parasite and host-derived molecules modulate disease progression [110,112]. For instance, these kinds of bidirectional protein signatures have been identified by proteomics in *Plasmodium falciparum* [113]. Also, in the management of the complex Echinococcosis, was recently suggested that a combination of markers would be highly desirable for the follow-up of treated patients to avoid recurrence [107]. Moreover, an integrative approach that looks for an effective diagnosis was also suggested for Schistosomiasis [114].

4.2. The critical role of biosensors in important parasitic diseases

The broad technologies underlying biosensors have experienced many developments, with the goal to enable small, sensitive and easy-to-use devices. As mentioned in previous points, micro- and nanotechnologies can offer new technological tools and potential applications for developing bio-sensing devices for infection's biomarkers. Many of these biosensors have proven to be useful for the detection of pathogen signatures and circulating proteins in patients [115]. The access to

Table 2
Some key problems to diagnose parasitic diseases and encountered in existing diagnostics for a range of important parasitic species.

Parasitic disease	Problems related to their diagnosis	Species	diagnostic method	Deficiencies/Inadequacies	References
Malaria	<ul style="list-style-type: none"> –Conventional microscopy is the gold standard for malaria diagnosis. 50–100 parasites/μl of blood can be detected in a good lab. Sensitivity is only 500 parasites/μl in non-specialised labs. Requires expertise. –Great difficulty of establishing quality-assured microscopy in rural and resource-poor settings. –Field implementation of many techniques remains a problem. –Rapid diagnostic tests have good sensitivity for densities of <i>P. falciparum</i> greater than 500 parasites/μL. However, the World Health Organisation recommends a lower sensitivity limit of detection for rapid diagnostic test for <i>P. falciparum</i> of 95% at a parasitaemia of 100 P/μL. –Current tests are essentially qualitative and do not quantitate the risk of developing severe complication. –Most RDTs that detect multiple species do not differentiate non-<i>P. falciparum</i> species from each other, nor do they differentiate mixed infections of <i>P. falciparum</i> and non-<i>P. falciparum</i> from <i>P. falciparum</i> mono-infection. –Placental malaria poses a great diagnostic challenge, lack of accurate and sensitive diagnostic tool for malaria infections in pregnancy. –Heterogeneity of <i>Leishmania</i> parasites complicates the diagnosis. –Proteins stage-specifically expressed and associated with virulence have a high antigenicity during the active disease phase. –Antigens used in serology show a large number of cross reactions with other trypanosomatids. –Invasive parasitological methods currently used to identify infected individuals. –No symptoms in acute or chronic phase and once the immune response is established, parasite detection is very difficult. –Misdiagnosis between species. –There is currently no single reference standard test. –Methodological limitations, especially in sensitivity and specificity. The direct or parasitological tests have unacceptably low sensitivity in the chronic phase. –There is a need for tools that can identify patients cured shortly after specific treatment. Other needs include a marker for prognosis and early diagnosis of congenital transmission. –Failure to detect parasites in infected newborns at one month of age due to low sensitivity of the assays. –Detection of oocysts is of little significance owing to short patency. The serological diagnosis of prenatal infection is difficult. –Low and focal distribution of parasites in the tissues or to the presence of non viable parasites. –Diagnosis of acute infection in human pregnancy is difficult since antibodies can be detected for a very long time after the acute phase. –Microscopic examination of excreta is the gold-standard test albeit with some limitations like decrease of sensitivity in low-endemicity areas. –Indirect methods using clinical, subclinical, or biochemical morbidity markers are not specific. –Considerable phenotypic variability between isolates of <i>Echinococcus granulosus sensu lato</i> –Diagnosis is mostly based on imaging techniques but sometimes they are inconclusive. –The diagnostic sensitivity of the methods can strongly depend on the stage of infection. –Low specificity and sensitivity of the currently available commercial tools. –Prognosis-associated follow-up parameters are still lacking. Invasive procedures following therapeutic interventions in AE patients. –There is a need to develop reliable tools for improved viability assessment. –There is an urgent need for well-validated non-invasive markers. 	<i>Plasmodium falciparum</i>	Haematological parameters (analytical biochemistry)	Unreliable laboratory indicators in acute uncomplicated malaria	[89]
			Nested PCR	Time consuming, open to considerable risk of contamination, low cost-efficiency and low sensitivity and specificity in certain cases.	[90]
			Light microscopy	Their efficacy is affected by several key factors such as the level of parasitemia, among others.	[91]
			Histopathology	Frequently not available in most settings, relatively costly and labour intensive.	[92]
			Rapid diagnostic tests	The specificities, sensitivities, numbers of false positives, numbers of false negatives and temperature tolerances of these tests vary considerably	[93]
				Performance varies between lots and widely between similar products. Also varies the concentration in the blood of the protein to be detected.	[94]
Leishmaniasis		<i>Leishmania infantum/donovani</i>	Quantitative PCR	Invasive samples for accurate detection	[95]
			Serology	The specifications for VL diagnostic tests vary among the different endemic regions.	[96]
		<i>Leishmania braziliensis</i>		No gold-standard test for tegumentary leishmaniasis, a combination of different diagnostic techniques is often necessary.	[97]
			Serology (ELISA)	Potential as an alternative method for confirmation.	[98]
Trypanosomiasis		<i>Trypanosoma cruzi</i>	INP micromethod	Parasite burden in some patients is below the detection limit	[99]
			Serological methods	Frequently display cross-reactivity against other pathogens and long term required for host seroreversion after the etiologic treatment of <i>T. cruzi</i> infection.	[100,101]
		PCR	Sensitivity and specificity have low accuracy. Controversial for chronic phase disease diagnosis.	[102]	
Toxoplasmosis		<i>Toxoplasma gondii</i>	Serological methods	Equivocal results with conventional serological techniques are not uncommon when IgG titers are close to the cut-off value of the test	[103]
Schistosomiasis		<i>Schistosoma</i> spp.	Serology	Antibody cross-reactivity with antigens from other helminths.	[104]
			PCR	Discrepancies among study findings regarding test sensitivity as a result of technical problems.	[105]
Echinococcosis		<i>Echinococcus granulosus</i>	ELISA	Sensitivity depends on the localization, size, number and stage of cysts. Several other factors could also affect the results of the tests. Cross reactions with other parasites are common. Antibody persistence.	[106]
			Imaging follow-up	None of the available imaging procedures are currently able to accurately assess <i>E. granulosus</i> viability and/or predict cyst progression/abortion	[107]
		<i>Echinococcus multilocularis</i>	Serology	No single accurate assays for the follow-up: need for combining cytokine and chemokine levels with other circulating markers. Poor correlation between the presence of antibodies in animal serum and worms in the intestine	[107,108]

Table 3
Microfluidic devices for the diagnosis of malaria infected cells.

Usage	Device/Platform	Highlights	References
Characterisation of disease states of single cells	Electric impedance microflow cytometry	Allows differentiation of infected from uninfected RBCs as well as among different <i>P. falciparum</i> intraerythrocytic asexual stages	[116]
	A polydimethylsiloxane microfluidic channel	Potential tool for studying the invasion mechanism as well as performing antimalarial drug assays	[117]
Malaria diagnosis	A cell microarray chip	Offers higher sensitivity in the detection of malaria infected erythrocytes than conventional light microscopy	[118]
	A lab-on-chip	Capable of detecting all <i>Plasmodium</i> sp. with a DL for <i>Plasmodium falciparum</i> of 2 parasites/ μ L of blood	[119]
	Polydimethylsiloxane microfluidic channels	Indicate that surface morphologies can serve as an alternative biomarker for malaria diagnosis	[120,121]
Separation of infected and non-infected cells	Dielectrophoresis based continuous separation	Higher inter-particle distance between red blood cells and <i>Plasmodium falciparum</i> -infected red blood cells	[122]
Detection of <i>Plasmodium berghei</i> in blood	Acoustic fields to lyse cells	DL of 30 parasites in a microliter-sized blood sample	[123]
Estimation of physical splenic filtration of infected cells	A MCD filtration model	Identify rheological diversity in RBC populations	[124]
Separation of infected cells based on their deformability	A microfluidic device precisely controlling pressure	Potential to study the pathophysiology and the effect of drugs	[125]
	A hyperbolic converging microchannel for continuously monitoring cell deformation in the extensional flow region	Overcomes the limitation of conventional methods by reducing experiment time	[126]
	An automated microfabricated deformability cytometer	Measure mechanical deformability and biomechanical properties of cells. Especially applicable to heterogeneous cell populations	[127]
	A simple long straight channel microfluidic device	An ideal technique for on-site iRBCs enrichment in resource-limited settings	[128]
	A 2-microm microfluidic channel	In contrast to <i>P. falciparum</i> -infected RBCs, mature <i>P. vivax</i> -infected RBCs readily became deformed through 2-microm constrictions	[129]
Measurement of the density of single living cells	A microfluidic mass sensor	Identifying <i>Plasmodium falciparum</i> malaria infected erythrocytes	[130]
Imaging malaria parasites	A lensfree on-chip microscope	Imaging in thin blood smears	[131]
Monitoring heme dynamics and/or detecting hemozoin in malaria infected cells	A RALS approach	Utility of the technique as a diagnostic and monitoring tool for minute sample volumes	[132]
	A magneto-optical method	Detect parasites at very low densities at the ring stage and in the case of the later stages	[133]

timely and accurate diagnostic tests has a significant impact in the management of many parasitic diseases like malaria.

At present, many microfluidic platforms are under development in order to address the main disadvantages presented above. Microfluidics provides an ideal interface for the manipulation of cells (*i.e.* red blood cells) or even the microorganisms in a completely integrated system that can be fabricated in mass production at low costs (Table 3). These devices have the potential of contributing to the diagnosis, control and treatment of malaria. In the near future, by using a drop of blood obtained from a finger pick, these assays could be automated to reduce human interventions in sample analysis for easy and massive large population screenings.

Sissel Juul and colleagues developed an impressive device, based on droplet microfluidics, in 2012 [134]. The microfluidic device is able to specifically and sensitively detect malaria-causing *Plasmodium* parasites employing isothermal conversion of single DNA cleavage-ligation events catalysed specifically by the *Plasmodium* enzyme topoisomerase I and detectable at the single-molecule level. This device allows for sensitive, specific, and quantitative detection of all human-malaria-caused by *Plasmodium* species in single drops from whole blood with a detection limit of less than one parasite/ μ L.

Very recently Warkiani and colleagues [135] developed a highly integrated system that allows enrichment and purification of malaria parasites from whole blood using a label-free, shear-modulated inertial microfluidic device. From 2 to 10 parasites were separated per millilitre and quantified using qPCR. This technique is approximately 100-fold more sensitive than conventional microscopy analysis of thick blood smears and ideal for further integration into an automatic system with downstream detection for POC diagnostic devices.

Red blood cells (RBCs) infected with malaria can be easily detected using microfluidic devices by looking at the morphological changes on the surface of the RBCs. Using controlled surface roughness and shear forces in a microfluidic channel malaria infected RBCs can be slowed and eventually immobilised on the roughened surface from whole blood. Although not well optimised for being used as POC device, it is ambitious to be an alternative biomarker for malaria diagnosis [121]. Following the same concept of looking at RBCs, Quan Guo and co-workers [125] developed a microfluidic technique for measuring the deformability of single RBCs based on their ability to deform through micrometre-scale constrictions. Although slow and tedious to extract information from the device is able to distinguish among uninfected RBCs and RBCs with various stages of *P. falciparum* infection.

Other ways of parasite detection are the fabrication of special flow-through separator structures inside the microfluidic channel for trapping parasites [136] or the apertures designed by Chunxiao Hu and collaborators [137] that form a trap where the parasite are trapped around a mid-point of its body. These types of traps have been used for differentiating plant parasitic nematodes by their stylet activity through integrated electrodes that record electrical signals.

An interesting review reported some relevant biosensors developed in POC system for an important vector-borne infection as dengue, highlighting their enormous potential in this field [138]. In general, technologies based on biosensors have been applied in diagnostic investigations of a wide range of parasites as can be seen in Table 4. In this way, advanced research through the integration of different techniques for multiplexing and high-throughput analysis on a chip might lead to the development of multi-parasite detection devices highly advantageous for tropical parasitic diseases [158,159]. Moreover, microfluidic devices open new avenues to investigate full parasite behaviour [160,

Table 4
List of some successfully applied sensors of parasites reported in the literature.

Parasite	Bio-sensing approach	Highlights	Targets and/or detection limit	References
<i>Leishmania</i> sp.	Immunosensor-based assay (monoclonal antibody coupled to a bioelectronic device) for detecting antigens quickly	Quantify amastigotes in organs for studies on pathogenesis and immunity	Amastigotes	[139]
<i>Plasmodium</i> sp.	Aptasensor device based on cationic polymers and gold nanoparticles	Allows detection of the two main species of malaria (<i>P. vivax</i> and <i>P. falciparum</i>)	Lactate dehydrogenase	[140]
	Rolling-Circle-Enhance-Enzyme-Activity-Detection system (REAAD)	Detection of malaria parasites in crude blood samples with a colorimetric detection system	Topoisomerase I activity	[141]
	A label-free DNA biosensor based on quartz crystal microbalance (QCM)	Specific for <i>P. falciparum</i> detection	Merozoite surface protein 2	[142]
	A miniaturised imaging system: sub-pixel resolving optofluidic microscope	Combination of microfluidics and inexpensive image sensors on an on-chip device	RBCs infected with <i>P. falciparum</i>	[143]
	A disposable plastic chip and a low-cost, portable, real-time PCR machine	Containing a Peltier element for thermal cycling and a laser/camera setup for amplicon detection.	DL of 2 parasites/ μL^{-1} of blood	[119]
	A microfluidic device to measure red blood cell deformability (infected cells)	Combination of microfluidic and controlled pressures	Deformability values of uninfected and parasitized cells	[129]
<i>Cryptosporidium parvum</i>	A droplet microfluidics platform	DL of less than one parasite/ μL^{-1} in single drops of unprocessed blood or saline	Micrometre-sized products derived from the action of topoisomerase	[138]
	A microbead immunoagglutination assay combined with Mie scatter detection in a microfluidic device	DL of ≤ 1 oocyst per large volume of water with the potential to be used in field situations	Cryptosporidium oocyst wall proteins (COWP)	[144]
	A polydiacetylene (PDA) liposome chip based on fluorescence	Non-labelling detection of the waterborne pathogen	DL of 1×10^3 oocysts/ mL^{-1}	[145]
	A microfluidic device equipped with a micromesh and FITC-labelled antibody.	Combination of micromesh for entrapment of oocysts with fluorescence immunoassay	High-throughput counting of oocysts	[146]
<i>Schistosoma japonicum</i>	A piezoelectric immunosensor	Mixed self-assembled monolayer membrane (mixed SAM) technology	<i>S. japonicum</i> antibody (SjAb) directly detected in the serum	[147]
	Glutaraldehyde or chitosan cross-linked electrochemical immunosensor	High sensitivity and broad linear range response	<i>S. japonicum</i> antigen ($50 \mu\text{g/L}^{-1}$ Optimal concentration)	[148]
	A liquid-phase piezoelectric immunosensor (LP-PEIS)	Detect antigens in patients' sera as well as ELISA but in a simple and quick operation	<i>S. japonicum</i> circulating antigen (SjCAg)	[149]
<i>Schistosoma haematobium</i>	On-chip imaging: mini-microscopes constructed from webcams and mobile phone cameras	Low-cost diagnostics of urogenital schistosomiasis.	Fields images of <i>S. haematobium</i> eggs	[150]
<i>Giardia lamblia</i>	A PEMC biosensor immobilised with a monoclonal antibody	The cysts bind to the antibody on the sensor changing the resonant frequency	DL of 1–10 cysts/ mL without a preconcentration step	[151]
	Optofluidic microscopy imaging	Successful microscopy imaging by flowing/scanning the target objects across a slanted hole array	Imaging of both <i>Giardia</i> trophozoites and cysts	[152]
<i>Trypanosoma</i> sp.	Gold electrode modified with a thiol sensitised with parasite proteins	100% specificity for the samples studied	Human anti- <i>T. cruzi</i> IgG	[153]
<i>Strongyloides stercoralis</i>	An immunological assay for diagnosing strongyloidiasis based on a novel diffraction-based optical biosensor technology	Serological assay based on real-time optical diffraction (NIE dot)	Antigen called NIE derived from <i>Strongyloides stercoralis</i> L3-stage larvae	[154]
<i>Entamoeba histolytica</i>	Portable screen-printed electrodes for the label-free electrochemical detection	Nano-yeast-scFv probes	<i>E. histolytica</i> cyst antigens	[155]
<i>Babesia bovis</i>	A device based on impedance spectroscopy combined with microfluidic	Sorting based on microfabricated cell cytometer	Allows single cell analysis of normal and <i>B. bovis</i> infected red blood cells	[156]
<i>Lucilia cuprina</i> (Cutaneous myiasis)	Electronic nose technology	Array of sensors that react to volatile chemical compounds	Discriminate infection measuring parasite odour on the day of larval implantation	[157]

PEMC, piezoelectric-excited millimetre-sized cantilever; DL, Detection Limit.

[161] and parasite drug response [162] in order to design new strategies to fight them. These technologies are also applicable in toxicology and drug discovery programmes for human metabolic studies and degenerative diseases.

In particular, rapid developments are occurring in the field of paper-based analytical devices (μPADs). μPADs are a new type of analytical platforms for ASSURED diagnostic tests (Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment free, Deliverable to end users, *World Health Organisation*). These devices should be simple to use, portable, inexpensive able to detect multiple analytes, and usable in using small volumes of sample. Therefore, the perfect tools for parasite detection in developing countries are coming. For instance, Horning and co-workers [163] developed a paper microfluidic cartridge for the automated staining of malaria parasites with acridine orange prior to microscopy. The cartridge enables simultaneous, sub-minute generation of both thin and thick smears of acridine orange-stained parasites, and has the potential to be used in limited-resources settings (Fig. 1).

In the tropics and in the resource-limited settings, water-borne parasites are among the most important parasitic diseases and many obstacles prevent their detection [164]. There is a need for rapid and simple screening of water sources to protect human health from water-related diseases. Currently, information about compact imaging systems incorporating a chip-scale microscope brings light to the diagnosis of major enteric parasites to save many lives [165]. Also, devices can track infectivity as early as 12 h post-infection, faster than other state of the art techniques [166]. Therefore, efforts are needed to facilitate market entry of these new technologies and facilitate mechanisms for their implementation. It is believed that PADs represent a realistic alternative for low cost, mass production and marketable devices. Moreover, the transition of paper-based microfluidic devices from the laboratory into the market need to be accomplished by providing the effective fluid flow control on paper and developing paper compatible easy and cheap sensing mechanisms.

Other important parasites are those food-transmitted, which are generally under-detected. These food-borne parasites have complex

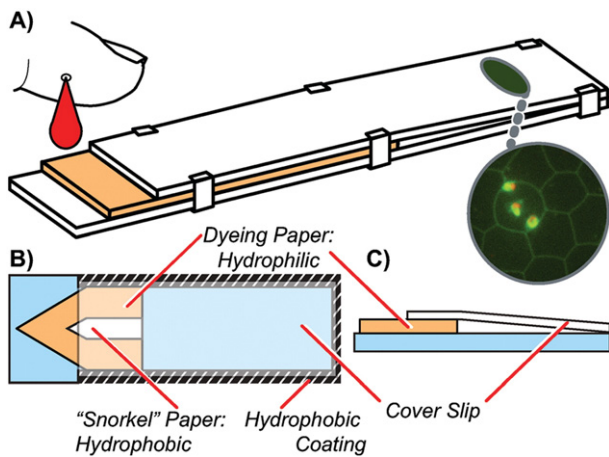


Fig. 1. New type of analytical paper microfluidics for sensing parasites. A) A 3D representation of the cartridge. B) A top-down view of the cartridge. C) A cross-section of the cartridge, emphasising the slanted nature of the coverslip during use, which permits imaging in both thick (many cells) and thin (single cell) regions. Reproduced with permission from Matthew P. Horning et al. 2014 © 2014, The Royal Society of Chemistry.

life cycles, which made difficult their control. In addition, few food-borne parasitic pathogens are effectively monitored in food [167]. Actually, there is a tremendous need to track back food-borne infections using new technologies such as biosensors due to their high specificity and potential to decrease the detection times [168].

5. Conclusions and perspectives

An efficient diagnosis is very important for the prevention and treatment of infectious and parasitic diseases. Bio-sensing devices based on a high-throughput format could have a favourable impact on disease screening and control implementation. New proteomics developments and their adaptation to POC may allow in a near future to produce low cost devices, increasing the sensitivity and shortening the time of detection compared to conventional tools.

In this sense, there is an important need to start developing and commercialising devices focusing on POC diagnostics applications in human and veterinary parasitology. These devices would favour the overall health of people in developing countries by taking broad and on time prevention and control measures and by assessing the treatment efficacy and facilitating the understanding the distribution patterns of hosts and vectors of important zoonotic parasitic diseases. Moreover, key biosensors might allow the on-site molecular characterisation of multi-parasite species bringing important information on the complex host–parasite interplay.

The convergence of proteomics and nanotechnology more likely will provide specific immune-sensors and immune-assays for detecting biomarkers related to infections. In this regards, the development of portable POC diagnostic tools for detecting circulating exosomes as biomarkers, therapeutic targets and signalling molecules of parasitic origin is a feasible and important goal in parasitology research. The integration of data from detected parasitic molecules and changes detected in host immune and metabolic responses to the infection might provide detailed sensory information for precise molecular-level diagnostics and monitoring tools.

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