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Recent advances in nucleic acid-based methods for detection of helminth infections and the perspective of biosensors for future development

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Review

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Abstract

Pathogenic helminth infections are responsible for severe health problems and economic losses worldwide. Timely and accurate diagnosis of helminth infections is critical for adopting suitable strategies for pathogen control. Here, we review recent advances in nucleic acid-based diagnostic methods, including polymerase chain reaction, quantitative qPCR, loop-mediated isothermal amplification and recombinase polymerase amplification, and discuss their advantages and disadvantages for diagnosing helminth infections. In addition, we highlight recent advances in biosensors for the detection of nucleic acid biomarkers that can potentially be used for the diagnosis of helminth infection.

Introduction

Helminths are invertebrates morphologically characterized by elongated, flat or round bodies, and include trematodes, cestodes and nematodes (Kaliappan *et al.*, 2013). Among them, soil-transmitted helminths (STHs) and schistosomes are responsible for the most prevalent parasitic infection of humans living in resource-limited areas of developing countries. Studies indicate that STHs, such as *Ascaris lumbricoides* and *Trichuris trichiura*, as well as hookworms, affect over 1.5 billion people worldwide (WHO, 2019*a*). Schistosomiasis caused by the genus *Schistosoma* affects at least 220.8 million people worldwide (WHO, 2019*b*). Currently, it is still prevalent in 78 countries where 700 million people are at the risk of contracting schistosomiasis. The World Health Organization has already developed a roadmap to control and eliminate the related neglected tropical diseases caused by these helminths. The major control strategy against these helminth infections is mass drug administration (Warren *et al.*, 2001; Minetti *et al.*, 2016). Consequently, it is critical to timely and accurately identify helminth infections to determine whether drug treatment is working efficiently and then deploy appropriate strategies for disease intervention. Currently, several 'gold standard' techniques are used for diagnosing human infection including the identification of parasites in blood (lymphatic filariasis (LF)) or skin snip biopsy (onchocerciasis), eggs or larvae in stools or urine (STHs and schistosomiasis) by microscopy and the detection of antibodies or pathogen antigens circulating in blood (onchocerciasis, LF and schistosomiasis) or urine (schistosomiasis) by immunological methods. However, these approaches usually have some limitations such as relatively low sensitivity (particularly for low and mid intensity infections), relatively low-throughput, increased time-consumption, skilled personnel being required for operation and lack of onsite applicability. In addition, serological methods for helminth diagnosis have limited ability to distinguish between ongoing and previous infections (Hinz *et al.*, 2017).

It is essential to develop rapid and sensitive techniques with high-throughput for diagnosing helminth infections. Immunodiagnostic techniques such as the detection of antibodies and antigens, and molecular diagnoses such as polymerase chain reaction (PCR), quantitative qPCR (qPCR), loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) can fulfil these requirements. In addition, nucleic acid-based methods also offer the most rapid and sensitive detection with the potential to distinguish different species and to monitor potential genetic variation in pathogen populations owing to selection pressure caused by drug usage. Although the broad application of current nucleic acid-based detection methods for surveying, monitoring and evaluating helminth control programs have so far been hindered by some obvious flaws such as lack of standards of sample preparation and nucleic acid isolation, lack of suitable internal control for target deoxyribonucleic acid (DNA)/(ribonucleic acid) RNA amplification, potentially high background noise, potential amplification inhibition and lack of accurate data interpretation for intensity of infection,

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these techniques may best guide decision making for controlling helminth diseases because of their higher sensitivity, rapid and high-throughput (Corrado *et al.*, 2016). Hence, we mainly focused on nucleic acid-based diagnostic methods including PCR, qPCR, LAMP and RPA, reviewed their recent advances and highlighted the potential usage of biosensors, a modern technique, for the diagnosis of helminth infection.

Nucleic acid-based methods for detection of helminth infections

To date, several different nucleic acid-based methods have been available for the detection of helminth infections such as PCR, multiplex PCR, qPCR, LAMP and RPA. These methods offer considerable advantages such as precision, timeliness and sensitivity of parasite detection. We have briefly reviewed recent advances in each method and then discussed their utility in the diagnosis of helminth infections in the following sections.

PCR

The PCR is based on using the ability of DNA polymerase to synthesize a new strand of DNA complementary to the template strand. The technique has been successfully used for detecting schistosome DNA from host serum, urine and faeces as well as water containing cercaria and infected snails (Enk *et al.*, 2012; Sarhan *et al.*, 2015; Sengupta *et al.*, 2019). A new PCR-based approach has been shown to identify different *Schistosoma* species (*S. mansoni*, *S. japonicum*, *S. haematobium*, *S. intercalatum* and *S. bovis*) and is applicable to human urine samples with high specificity and sensitivity (Sandoval *et al.*, 2006). Recently, a study documented that PCR assay targeting the 121-bp tandem repeat sequence sensitive for *S. mansoni* and the *Dra1* tandem repeat sequence specific for *S. haematobium* can distinguish *Schistosoma* species for diagnosis of early schistosomiasis (Bonnefond *et al.*, 2019). For filarial pathogens, the amplification of genomic DNA repeat sequences (188 bp DNA repeat sequence) *Ssp*I from *Wuchereria bancrofti* (Zhong *et al.*, 1996), *Hha* I repeat region from *Brugia malayi* (Fischer *et al.*, 2000), LLMF72 or a repeat region (15r3) from *Loa loa* (Fink *et al.*, 2011) and the O-150 repeat sequence from *Onchocerca volvulus* (Toé *et al.*, 1998) by the PCR has been used to detect pathogen infection. In addition, the PCR can also amplify *W. bancrofti* DNA from host sputum, serum and urine with high diagnostic accuracy (Kagai *et al.*, 2008; Ximenes *et al.*, 2014). Moreover, the detection of *Taenia solium* DNA from cerebrospinal fluid (CSF) samples by the PCR has diagnostic potential for neurocysticercosis, which cannot be detected using radiology or serology (Michelet *et al.*, 2011; Chaya and Parija, 2014). Furthermore, the PCR was used to amplify pathogen-specific DNA in host saliva and urine, which may become a new type of non-invasive biomarker for the diagnosis of schistosomiasis japonica (Weerakoon and McManus, 2016). In addition, this has been shown to have good potential for detecting early stage of schistosome infection by amplifying schistosome specific DNA from host sera using the PCR (Xia *et al.*, 2009; Kato-Hayashi *et al.*, 2010).

The traditional PCR can be modified by incorporating additional primers and amplification to increase the sensitivity for detecting helminth infection. For example, a two-step semi-nested PCR for amplifying *Oesophagostomum bifurcum* egg DNA in human faecal samples was shown to achieve a specificity of 100% (Verweij *et al.*, 2000). In addition, the semi-nested PCR technique showed a 1 000 000-fold increase to the detection limit for *W. bancrofti,* compared with that of the conventional PCR (Ximenes *et al.*, 2014).

By incorporating fluorescent dyes or fluorescently labelled oligonucleotide probes, the amplified DNA can be quantified. This is called a real-time qPCR, which is now one of the most widely used tools in research and clinical diagnosis. The qPCR has been used to detect schistosomes, STHs (e.g. *S. stercoralis, T. trichiura, A. duodenale, N. americanus and A. lumbricoides*) and *O. volvulus* infection (Morales-Hojas *et al.*, 2001; Lier *et al.*, 2009; O'Connell and Nutman, 2016*a*; Prince-Guerra *et al.*, 2018). In schistosomes, the qPCR was shown to identify considerably more positive cases than the Kato–Katz method and could be adopted as a suitable strategy for schistosomiasis elimination (He *et al.*, 2018).

Recently, microRNAs (miRNAs) have been considered as a new class of biomarkers for diagnosing many diseases, particularly for circulating miRNAs (Cheng, 2015). For example, Cheng and coworkers (2013) identified five schistosome-specific miRNAs (*Bantam*, *miR-3479*, *sja-miR-8185*, *miR-10* and *miR-3096*) in the plasma of rabbits infected with *S. japonicum*. Four out of these five miRNAs were shown to be significantly abundant in plasma of *S. japonicum*-infected mice, suggesting these miRNAs can serve as a potential biomarker for schistosomiasis diagnosis. More interestingly, several further studies demonstrated that parasite-derived miRNAs (*miR-277*, *miR-3479-3p* and *bantam*) could detect individuals infected with *S. mansoni* or *S. haematobium* from low and high-infection intensity sites by the real time qPCR (Hoy *et al.*, 2014; Meningher *et al.*, 2017).

Apart from diagnosis, the PCR and qPCR can also be used to distinguish between species of parasites. For example, a copro-PCR method targeting a segment of the internal transcribed spacer (ITS) region and *COX1* gene was shown to distinguish between *O. viverrini* and *C. sinensis*; results suggested that *C. sinensis* was present in an area in central Thailand where it had not been previously detected (Traub *et al.*, 2009; Cai *et al.*, 2014). In addition, the PCR and qPCR for diagnosis of STH infections and for monitoring genetic variation of helminths under mass drug administration have been elegantly reviewed (Corrado *et al.*, 2016; O'Connell and Nutman, 2016*b*; Papaiakovou *et al.*, 2019). The qPCR was able to detect and distinguish filarial infection in domestic cats among *B. malayi, D. immitis* and *D. repens* as well as *A.* (*Dipetalonema*) *reconditum*, showing more sensitivity than microfilariae detection (Wongkamchai *et al.*, 2014).

Unlike the conventional qPCR, the Droplet Digital™ PCR (ddPCR) can perform absolute quantification of PCR products without the need for generating standard curves (Elmahalawy *et al.*, 2018). In principle, the ddPCR can fractionate a loading sample into thousands to millions of droplets, and PCR amplification of the template molecules occurs in each individual droplet, followed by a real time or endpoint detection of the amplification. The distribution of target sequences into droplets is described by the Poisson distribution, thus allowing accurate and absolute quantification of the target without using reference materials with known target concentrations (Gutierrez-Aguirre *et al.*, 2015). The ddPCR has been successfully used to detect *W. bancrofti* DNA (targeting the long DNA repeat (LDR) element) and *B. malayi* DNA (targeting the Hha I element) in host blood samples and mosquito vectors with high sensitivity and specificity (Jongthawin *et al.*, 2016). In gastrointestinal (GI) nematode parasitic infection, the ddPCR can detect and distinguish major GI nematodes by targeting the internal transcribed spacer region 2 (ITS2) of the ribosomal RNA gene in ovine parasites such as *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus species* (Elmahalawy *et al.*, 2018). In addition, the ddPCR assay exhibited a high level of diagnostic performance compared with Kato–Katz and antibody-based ELISA for diagnosis of *Schistosoma* infection although it is expensive

and requires specialized equipment (Weerakoon *et al.*, 2017*a*,*b*; Cai *et al.*, 2019).

Multiplex PCR

The multiplex PCR can amplify several different DNA sequences simultaneously using multiple primers (Touron *et al.*, 2005; Hawkins and Guest, 2017). Using selected target sequences for amplification, this PCR approach can not only detect the pathogen's genotype, but also distinguish among species. For example, *S. mansoni* and *S. haematobium* from a set of duplicate stool samples can be distinguished using primers for species-specific amplification of *cytochrome C oxidase* genes by the multiplex PCR, and the results showed a significant correlation with microscopic egg counts for *S. mansoni* in stool and *S. haematobium* in urine (ten Hove *et al.*, 2008). In addition, the multiplex PCR was also shown to distinguish between differential infections by *T. solium* and *T. asiatica* (Wicht *et al.*, 2010; Jeon and Eom, 2013). Moreover, the multiplex PCR has been shown to discriminate *N. americanus, A lumbricoides* and *T. trichiura* infections from faecal samples (Phuphisut *et al.*, 2014). In the Philippines, a study indicated that the multiplex PCR could simultaneously detect two cestode parasites *T. saginata* and *T. solium* alongside *A. lumbricoides* and hookworms in stool samples (Gordon *et al.*, 2015). Moreover, a recent study indicated that a multiplex single-tube PCR assay can specifically discriminate trematode, cestode and nematode species by targeting the 28S rRNA gene (trematode), *COX1* gene (cestode) and *COX1* gene (nematode) (Wong *et al.*, 2019).

By incorporating Luminex beads or fluorescent dye, the multiplex PCR can be modified as qPCR to improve its sensitivity and specificity. For example, several helminths including *A. lumbricoides, A. duodenale, N. americanus* and *S. stercoralis* can be quantitatively co-detected using a Luminex bead-based multiplex qPCR, showing high sensitivity (90–100%) and specificity (95–100%) from 319 stool samples (Taniuchi *et al.*, 2011). In addition, a multiplex qPCR for detecting *N. americanus* and *A. duodenale* also showed 100% specificity and 98.5 to 100% sensitivity (Verweij *et al.*, 2007). Moreover, a pentaplex qPCR was not only able to simultaneously detect *A. duodenale*, *N. americanus*, *A. lumbricoides* and *S. stercoralis* but also to show significantly increased sensitivity of detection (up to 62.3% compared with the detection of microscopic examination of 7.8%) for one or more helminth species (Basuni *et al.*, 2011). Recent studies also indicated that a multiplex TaqMan qPCR assay can specifically identify encapsulated *Trichinella* species in North America (Almeida *et al.*, 2018), and a multiplex qPCR can detect and quantify DNA from *F. hepatica* and the intermediate snail host, *A. tomentosa*, in water samples (Rathinasamy *et al.*, 2018).

Loop-mediated isothermal amplification (LAMP)

Although the PCR and multiplex PCR offer significant advantages for detecting helminth infections such as more sensitivity and specificity, high-throughput and cost-effectiveness, both methods are heavily dependent on a particular instrument or elaborate detection methods. Alternatively, LAMP can amplify target DNA by DNA polymerase based on a set of specific primers under a constant temperature of 60–65°C without a thermocycler and accumulated 10^9 copies of target DNA in less than an hour, with a detection limit of a few copies. More importantly, the amplification endpoint could be observed using agarose gel electrophoresis for visual observation of DNA under UV light and using turbidity determination that is more suitable for field research. As a pyrophosphate ion is released once a nucleotide is added to the DNA strands, a visible white precipitate of magnesium pyrophosphate is formed and used to determine whether the target nucleic acid was amplified or not.

The use of LAMP for helminth detection has been recently summarized and reviewed (Amoah *et al.*, 2017; Deng *et al.*, 2019). Here, we highlight only the studies related to schistosomes. LAMP has been successfully used to amplify the *SjR2* gene of *S. japonicum* from the blood samples of infected rabbits at 3rd day post-infection, indicating the potential of early detection of *S. japonicum* infection (Xu *et al.*, 2015). In addition, by targeting *S. haematobium* ribosomal intergenic spacer DNA, LAMP was shown to detect *S. haematobium* DNA in heated pellets from patients' urine samples without requiring a complicated procedure for DNA extraction with 100% sensitivity and 86.67% specificity (Gandasegui *et al.*, 2015). Moreover, LAMP can detect miracidium DNA at the first day of snail exposure (intermediate hosts) to *Schistosoma* (Abbasi *et al.*, 2010) and also detect infection in pooled samples of field-collected snails (Tong *et al.*, 2015). Furthermore, LAMP was shown to be an effective means to detect low-intensity infection of schistosomiasis (Lodh *et al.*, 2017). Overall, these studies suggested that LAMP might become a new surveillance and response tool for schistosomiasis control.

Compared to the PCR, LAMP has better sensitivity that could enhance the effectiveness of surveillance and mass drug administration of control programs for helminth diseases. However, primer design is a critical part of LAMP assay, because the use of multiple primers for LAMP assay may increase the risk of primer–primer hybridization, resulting in template-free amplification that needs to further evaluate false-positive outcomes (Hsieh *et al.*, 2014). Similar to the PCR, LAMP cannot be used for detecting unknown or unsequenced targets of pathogens since specific DNA sequences are required for primer design (Mori and Notomi, 2020).

Recombinase polymerase amplification (RPA)

RPA is another fast and sensitive isothermal amplification-based method for amplifying target DNA or RNA using a bacteriophage derived recombinase in combination with a polymerase (Piepenburg *et al.*, 2006; Euler *et al.*, 2012). RPA comprises three core enzymes including a recombinase, a single-stranded DNA-binding protein (SSB) and a strand-displacing polymerase. Recombinases pair oligonucleotide primers with a homologous sequence, and the SSB binds to displaced strands to prevent primer displacement (Piepenburg *et al.*, 2006). Finally, the strand displacing polymerase begins DNA synthesis where the primer has bound to the target DNA (Euler *et al.*, 2012).

Studies have demonstrated that RPA can detect small amounts of viral, bacterial and parasitic DNA with high sensitivity and specificity (Shahin *et al.*, 2018). In helminths, most of these studies focused on schistosomes. For example, RPA was used to amplify the *SjR2* gene of *S. japonicum,* and the result indicated that this assay could detect 0.9 fg *S. japonicum* DNA within 15 min and distinguish *S. japonicum* from other worm species (Xing *et al.*, 2017). In addition, RPA combined with oligochromatographic lateral flow strips was shown to detect low levels of *S. haematobium* DNA (Dra1) from crude urine (Rosser *et al.*, 2015). Similarly, an RPA-based lateral flow was also developed in *S. mansoni* by amplifying the 28S rDNA region and the result indicated that the lower limit of detection for this assay was 10 pg DNA with the lower test parameters permitting sufficient amplification being 6 min and 25°C (Poulton and Webster, 2018).

RPA can amplify as low as 1–10 DNA/RNA target copies with a rapid turnaround time (less than 20 min) and simple set-up at a single low temperature (37–42°C). Consequently, it provides significant advantages compared with other isothermal methods such as LAMP. In addition, RPA has been shown to amplify

diverse targets, including RNA, miRNA, ssDNA and dsDNA from a wide variety of organisms and samples (Euler *et al.*, 2012). It may be necessary to develop an RPA assay to detect pathogen-specific small RNAs such as miRNAs in future for diagnosing helminth infections. Moreover, RPA has good potential for field application and the use of this method can reduce diagnostic expenses in resource-limited countries (Ajibola *et al.*, 2018). Although PCR, LAMP and RPA methods can also be 'sensed' by incorporating a fluorescent dye or probe and the amplified signal can be detected and presented by an electrical device, these methods usually carry disadvantages such as requiring a multi-step set up, being time-consuming, requiring a trained operator for use and being difficult to apply in the field.

Biosensors for nucleic acid biomarker detection

With the development of modern electrical and chemical techniques, biosensors, a powerful and innovative analytical device shows excellent characterizations, being highly sensitive, rapid, simple, low-cost and field-applicable that can be widely used for drug discovery, diagnosis, biomedicine, food safety, processing and environmental monitoring.

The first biosensor invented by Clark and Lyons (1962); Cheng and coworkers (2013) for measuring glucose levels in biological samples was based on electrochemical detection of oxygen or hydrogen peroxide (Fracchiolla *et al.*, 2013). Since then, incredible progress has been made in terms of both the technology and applications of biosensors. Generally, biosensors consist of at least three elements, namely, a biological capture molecule (bioreceptor), a method of converting capture molecule-target interactions into a signal (transducer) and a data output system. A bioreceptor is an immobilized sensitive biological element (antibody, DNA probe or enzyme) recognizing the analytic (antigen, complementary DNA or enzyme substrate). A transducer is used to convert the (bio) chemical signal resulting from the interaction of the analytic with the bioreceptor into an electronic signal. The intensity of signals generated corresponds to the analyte concentration.

Currently, most reported biosensors have been designed to detect nucleic acid biomarkers and protein biomarkers related to pathogen infection. Nucleic acid biomarker-based biosensors show good potential for the detection and diagnosis of various diseases such as cancer, diabetes, cardiovascular diseases, tuberculosis, hepatitis, dengue and food-borne diseases such as diarrhea, cholera and salmonellosis (Bora *et al.*, 2013). Based on transducer methods, these biosensors can be divided into electrochemical biosensors and optical biosensors.

Electrochemical detection techniques

Various electrochemical biosensors have been reported to detect nucleic acid biomarkers during parasite infection, particularly protozoan infections. For example, Mohan and coworkers used a kind of sol–gel synthesized nickel oxide (NiO) film deposited onto an indium tin oxide (ITO) coated glass plate to immobilize a 23-mer DNA sequence (oligonucleotide) from 18S rRNA gene sequences from *L. donovani* for the development of a DNA biosensor. Response studies of the ss-DNA/NiO/ITO bioelectrode in the presence of methylene blue redox dye as a redox mediator indicated a linear response in the wide concentration range of 2 pg/mL to 2 μg/mL of complementary target genomic DNA (parasite DNA), suggesting the potential of this biosensor for diagnosis of Visceral leishmaniasis (Mohan *et al.*, 2011). In addition, a gold nanoleaf-based biosensor was constructed by immobilizing *Leishmania major*-specific DNA for detecting parasite infection. Further analysis indicated that the biosensor could detect a synthetic DNA target in the range of 1.0×10^{-10} to 1.0×10^{-19} mol L^{-1} with a detection limit of 1.8×10^{-20} mol L^{-1}, and genomic DNA in the range of 0.5–20 ng μL^{-1} with a detection limit of 0.07 ng μL^{-1}. More importantly, the biosensor could not only detect *L. major* in patient samples but also distinguish *L. major* from a non-complementary-sequence oligonucleotide and *L. tropica* with high selectivity (Moradi *et al.*, 2016).

Recently, several electrochemical biosensors were also developed for detecting nucleic acid biomarkers for helminth infection. For example, Santos *et al.* used AuNPs that are the most commonly used nanomaterial in biosensor development through biomolecule conjugation and then obtained compelling sol–gel derivative films to immobilize AuNPs using the organosilane, 3-mercaptopropyltrimethoxysilane. Further analyses indicated that the biosensor could recognize the *S. mansoni* genome sequence at different concentrations in samples of urine, CSF and serum with the limit detection of 0.6 pg μL^{-1} (Santos *et al.*, 2019). The authors also reported the development of another biosensor by using a self-assembled monolayer of mercaptobenzoic acid immobilizing nanostructures composed of gold nanoparticles (AuNPs) and magnetite nanoparticles (Fe_3O_4_NPs) to recognize specific nucleotide sequence of *S. mansoni* present in CSF and serum samples, and this biosensor showed a DNA detection limit of 0.781 and 0.685 pg μL^{-1} (Santos *et al.*, 2017).

Apart from the detection of nuclear acid biomarkers, some biosensors were also developed to detect specific antibodies/antigens related to helminth infection. For example, a piezoelectric immunosensor assay was developed with immobilizing immunoglobulin G (IgG) as a probe to detect *S. japonicum* circulating antigens. By immobilizing purified IgG from infected rabbit's sera with *S. japonicum*, the developed biosensor was shown to specifically detect *S. japonicum* circulating antigens (Cheng *et al.*, 2008). In addition, a superhydrophobic surface-based magnetic electrochemical immunoassay for the detection of *S. japonicum* antibodies was also developed and the results indicated that the biosensor is quantitatively sensitive to SjAb concentrations ranging from 2 ng mL^{-1} to 15 μg mL^{-1}, with a detection limit of ~1.3 ng mL^{-1} (Nie *et al.*, 2012). Moreover, an electrochemical immunosensor was also developed by immobilizing *W. bancrofti* epitope antigens (Wb/ALT2-A5) on a screen-printed carbon electrode by their amine groups *via* chitosan film by coupling with glutaraldehyde as the crosslinker. The detection limitation of this biosensor was 0.002 μg mL^{-1} for human serum with a sensitivity of 1.86 μA (Prado *et al.*, 2018).

Optical detection techniques

Optical detection techniques are those that can sense phenomena related to the interaction of target compounds with the analytes. Usually, these biosensors can measure luminescence, fluorescence or colour changes. Several biosensors have used optical detection techniques to detect nucleic acid biomarkers for parasite infection, particularly for malaria. For example, Ittarat and coworkers described a genosensor based on quartz crystal microbalance (QCM) to detect malaria infection. In this method, the QCM surface was immobilized with a malaria biotinylated probe. Specific DNA fragments of malaria-infected blood were amplified. Hybridization between the amplified products and the immobilized probe resulted in quartz frequency shifts that were measured by an in-house frequency counter. Further analysis indicated that this biosensor can differentially diagnose blood infected with *P. falciparum* from that infected with *P. vivax* and no cross reaction with human DNA (Ittarat *et al.*, 2013). In helminths, a diffraction-based optical biosensor was developed although it detects a protein biomarker (a 31-kDa recombinant antigen called NIE derived from *S. stercoralis*) to diagnose strongyloidiasis. The

Table 1. Nucleic acid-based methods for the detection of helminth infections

Pathogens	Target genes	Detection methods	Types of sample	References
S. japonicum	*SjR2*	PCR	Faeces	(Fung *et al.*, 2012)
S. mansoni	121-bp tandem repeat sequence	PCR	Sera	(Hussein *et al.*, 2012)
S. mansoni *S. haematobium*	*28S rDNA*	PCR	Urine	(Sandoval *et al.*, 2006)
S. haematobium	*Dra* 1	PCR	Urine	(Ibironke *et al.*, 2011; Lodh *et al.*, 2014)
S. japonicum	*COX1*	PCR	Sera and urine	(Kato-Hayashi *et al.*, 2015)
S. japonicum	Repetitive retrotransposon SjCHGCS19	PCR	Serum	(Xu *et al.*, 2017)
T. saginata *T. solium*	*HDP2*	qPCR	Faeces	(Flores *et al.*, 2018)
W. bancrofti	*LDR*	qPCR	Blood	(Plichart and Lemoine, 2013)
A. lumbricoides *A. duodenale* *N. americanus* *S. stercoralis* *T. trichiur*	*ITS1, ITS2*	qPCR	Faeces	(Mejia *et al.*, 2013)
S. japonicum	*NAD6* and *NAD1*	PCR and qPCR	Faeces	(Gordon *et al.*, 2012)
S. mansoni *S. haematobium*	*ITS2*	qPCR	Faeces	(Meurs et al., 2015)
N. americanus *A. duodenale* *A. ceylanicum* *A. caninum* *A. braziliense*	*ITS2*	qPCR	Faeces	(Ngui *et al.*, 2012)
T. trichiura *A. lumbricoides*	*18S rDNA* and *ITS1*	qPCR	Faeces	(Liu *et al.*, 2013)
A. lumbricoides *A. duodenale* *N. americanus* *S. stercoralis*	*ITS1, ITS2* and 18S rDNA	Multiplex qPCR	Faeces	(Basuni *et al.*, 2011)
T. solium *T. saginata*	*COX1*	Multiplex PCR	Faeces	(Jeon *et al.*, 2011)
S. japonicum *S. mansoni*	*NAD1, 2, 6* and *COX2* *NAD5, 6* and *COX2*	Multiplex PCR	Faeces	(Gobert *et al.*, 2005)
F. hepatica *A. tomentosa*	*ITS-2*	Multiplex PCR	Water	(Rathinasamy *et al.*, 2018)
Trichinella Spp.	*ITS1* and *ITS2*	Multiplex qPCR	Meat	(Almeida *et al.*, 2018)
W. bancrofti	Repeated DNA sequence	Semi nested PCR	Urine and sera	(Ximenes *et al.*, 2014)
W. bancrofti	*Long direct repeat element (LDR)*	ddPCR	Blood, vectors	(Jongthawin *et al.*, 2016)
B. malayi	HhaI element	ddPCR	Blood, vectors	(Jongthawin *et al.*, 2016)
S. japonicum	*NAD 1* and *SjR2*	ddPCR	Urine, faeces, salivary gland and serum	(Weerakoon *et al.*, 2017*a*; Weerakoon *et al.*, 2017*b*; Cai *et al.*, 2019)
H. contortus, T. circumcincta and Trichostrongylus spp.	*ITS2*	ddPCR	Parasite DNA	(Elmahalawy *et al.*, 2018)
T. solium	*HDP2*	Semi nested PCR	Cerebrospinal fluid	(Hernández *et al.*, 2008)
S. japonicum	*SjR2*	LAMP	Serum	(Xu *et al.*, 2010)
S. mansoni	Mitochondrial minisatellite DNA	LAMP	Faeces	(Fernández-Soto *et al.*, 2014)
S. stercoralis	*28S rDNA*	LAMP	Faeces	(Watts *et al.*, 2014)
C. sinensis	*COX1*	LAMP	Faeces	(Rahman *et al.*, 2017)

(*Continued*)

Table 1. (*Continued.*)

Pathogens	Target genes	Detection methods	Types of sample	References
T. trichiura *N. americanus* *A. lumbricoides*	*β-Tubulin*	LAMP	Faeces	(Rashwan *et al.*, 2017)
T. canis	*ITS2*	LAMP	Faeces	(Khoshakhlagh *et al.*, 2017)
E. multilocularis	*COX1*	LAMP	Faeces	(Feng *et al.*, 2017)
N. americanus	*ITS2*	LAMP	Faeces	(Mugambi *et al.*, 2015)
B. malayi *B. timori*	*Hha* I	LAMP	Blood	(Poole *et al.*, 2012)
P. westermani	*ITS2*	LAMP	Sputum and pleural fluid	(Chen *et al.*, 2011)
S. haematobium	*Dra* 1	RPA	Urine	(Rosser *et al.*, 2015)
S. mansoni	*28S rDNA* and *ITS*	RPA	Genomic DNA	(Poulton and Webster, 2018)
S. japonicum	*Sj*Ab	Immunosensor	Sera	(Nie *et al.*, 2012)
S. mansoni	*Genomic DNA*	Genosensor	Urine, cerebrospinal fluid and serum	(Santos *et al.*, 2019)
S. mansoni	*Genomic DNA*	Genosensor	Cerebrospinal fluid and sera	(Santos *et al.*, 2017)
S. japonicum	*S. japonicum* circulating antigens	Immunosensor	Sera	(Cheng *et al.*, 2008)
W. bancrofti	*W. bancrofti epitope antigens*	Immunosensor	Sera	(Prado *et al.*, 2018)

biosensor showed excellent agreement ($\kappa = 0.98$) with results obtained by an NIE-based ELISA method (Pak *et al.*, 2014).

In addition, as stated above, miRNAs are recognized a novel kind of biomarker for disease diagnosis, particularly for cancer, for example in early diagnostics, predicting responses to therapy, monitoring relapse and molecular classification of tumours. A recent study documented that circulating miRNAs associated with cancer derived from serum can be detected with a biosensor using DNA concatemer amplification (Hong *et al.*, 2013). If sensitive and effective nucleic acid biomarkers can be identified during helminth infection, and suitable and effective probe layers can be immobilized on a transducer surface, we believe that electrochemical biosensors can become a next generation technique for diagnosis of helminth infections.

All the nucleic acid-based methods discussed and the related targets for detecting helminth infections are summarized and listed in Table 1.

Advantages and limitations of nucleic acid-based methods for detection of helminth infections

Each nucleic acid-based method has its own advantages and limitations. We have summarized them as follows. The PCR is one of the most promising approaches to detect parasite DNA or RNA in human, animal or vector samples owing to its higher diagnostic sensitivity and specificity compared to microscopic examination or immunodiagnostics. However, it has limitations, such as the high cost of the instrumentation required for thermal cycling and signal detection, and the need for well-equipped laboratories (Verweij and Stensvold, 2014). Both qPCR and ddPCR methods can quantitatively determine helminth infection as they are amenable for automation and have relatively high-throughput, whereas the ddPCR can increase the accuracy of quantification at low-target concentrations. Unlike the PCR, LAMP offers sensitivity, high efficiency, rapidity and simplicity for identifying certain infectious diseases, although the specificity is yet to be validated. The main shortcoming of LAMP is the false-positive production caused by non-specific amplification resulting from primers, which could be improved by optimization of the reaction conditions, primer design, detection approaches, etc. RPA offers several advantages such as operation at a low and constant temperature, without the need for an initial denaturation step or the use of multiple primers. Biosensor-based methods have the potential to deliver rapid, accurate and affordable diagnosis of parasite infection. In addition, biosensors can improve diagnosis and follow-up treatment in terms of rapidity, real-time performance and expense compared to current detection technologies such as PCR and ELISA. Furthermore, the use of biosensors offers significant advantages such as small fluid volume manipulation and high-integration capability that facilitates the development of portable devices and ease of use, allowing use by non-specialized personnel in non-centralized laboratories (Sin *et al.*, 2014). The time-consuming preparative steps of gene probe evaluation make it hard to consider these as the basis of biosensors for *in situ* detection of pathogenic microorganisms. Although extensive efforts have been expended to develop biosensors, a relatively small number of analytics, particularly toxic materials, can be detected using commercially available devices (Alhamoud *et al.*, 2019). Although recent advancements in nanotechnology are facilitating the development of smaller, less-expensive, multiplex, easy-to-use and fully integrated biosensors for diagnostics and food and water safety (Bouguelia *et al.*, 2013; Verbarg *et al.*, 2013; Chung *et al.*, 2015), future research must also focus on simplifying their fabrication techniques, miniaturizing devices and developing the biocompatibility and environmental friendliness of the nanomaterials. Unfortunately, the preparation and manipulation of nanomaterials in a reproducible and economic fashion is still premature. More importantly, the development of point of care devices is the ultimate goal, particularly for human patients, which still faces a significant challenge. Consequently, techniques that employ and combine with chemistry, electrics, optics, material science and mechanical passivation approaches could speed up

Table 2. Summary of advantages and limitations of each nucleic acid-based method

Techniques	Advantages	Limitations	References
PCR	Relatively high specificity and sensitivity; can detect small amounts of target genes; lower turnaround time; relatively high throughput; high dynamic range of detection.	Relatively expensive instruments are required, risk of false negatives in pathogen detection.	(Diguta *et al*., 2010; Agarwal *et al*., 2014)
Multiplex PCR	High sensitivity and specificity; can detect multiple genes simultaneously with relatively high-throughput.	Primer design is critical; the primers may interfere with each other during amplification.	(Hajia *et al*., 2014; Vică et al., 2016)
ddPCR	High sensitivity and specificity; accurate and absolute quantification of the target without using reference materials with known target concentrations; low copy detection for target	Expensive instruments are required.	(Gutierrez-Aguirre *et al*., 2015)
LAMP	Rapid, highly efficient and cost-effective; denaturation step not required; more rapid than other conventional PCR-based methods; can directly visualize results; less equipment.	Primer design is critical for LAMP assay; risk of false-positive results.	(Tomita *et al*., 2008; Hsieh *et al*., 2014; Mori and Notomi, 2020)
RPA	Cost-effective, highly sensitive and specific, short amplification time. Takes place at the ambient temperature without the need for an initial denaturation step or the use of multiple primers.	Risk of false-positive outcomes.	(Daher *et al*., 2016; Shahin *et al*., 2018)
Biosensors	High sensitivity and reliability; fast detection; reduction in reagent consumption; detection of small molecules, proteins and nucleic acid.	Not commercially available for most pathogens, further research required for developing the system.	(Sin *et al*., 2014; Luz *et al*., 2016; Janissen *et al*., 2017)

the development of biosensors for commercial requirements. The advantages and limitations of each nucleic acid-based method are summarized in Table 2.

Conclusions

Traditional pathogen detection methods are usually time consuming, and associated with low sensitivity, selectivity or accuracy. Therefore, sensitive and specific methods for diagnosing parasitic diseases are urgently required. Recent advances in PCR, qPCR, ddPCR, LAMP and RPA are proving to be highly sensitive and specific diagnostic approaches. By identifying effective biomarkers during helminth infection, these approaches could be further improved by increasing the sensitivity of detection and diagnosis. Current biosensors have the potential to provide rapid, accurate and affordable diagnoses of parasite infection by offering significant advantages such as small fluid volume manipulation, high integration capability that facilitates the development of portable devices and ease of use. Therefore, the combination of these outcomes with advanced technologies such as nucleic acid-based biosensors may change the current scenario of clinical diagnosis.

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